Ubiquitylation of Nrf2 by the Keap1-Cullin3/RING box1 (Cul3-Rbx1) E3 ubiquitin ligase complex targets Nrf2 for proteasomal degradation in the cytoplasm and is an extensively studied mechanism for regulating the cellular level of Nrf2. Although mechanistic details are lacking, reports abound that Nrf2 can also be degraded in the nucleus. Here, we demonstrate that Nrf2 is a target for sumoylation by both SUMO-1 and SUMO-2. HepG2 cells treated with As$_2$O$_3$, which enhances that Nrf2 is a target for sumoylation by both SUMO-1 and SUMO-2, can undergo sumoylation, and can be degraded in PML-NBs.

# Results
Nrf2 localizes, in part, to promyelocytic leukemia-nuclear bodies (PML-NBs), can undergo sumoylation, and can be degraded in PML-NBs.

# Conclusion
Polysumoylated Nrf2 is polyubiquitylated by RING finger protein 4 (RNF4) and subsequently degraded by the proteasome in PML-NB domains.

# Significance
Nuclear degradation of Nrf2 involves PML-NBs.

Nrf2$^7$ is a basic leucine zipper transcription factor that is essential for cellular response to oxidative stress (1–3). In non-stressed cells, its abundance is restricted by Keap1, a redox-regulated inhibitory protein that functions as a substrate adaptor for ubiquitylation of Nrf2 by the Cullin3/RING box1 (Cul3-Rbx1) E3 ubiquitin ligase complex. This ubiquitylation targets Nrf2 for proteasome-dependent degradation in the cytoplasm (4–6). In stressed cells, stressors such as electrophiles, reactive oxygen species, or various polyphenolic phytochemicals that have anti-oxidant effects activate Nrf2 by inducing its separation from Keap1 (1, 2). Endogenously produced factors such as low density lipoproteins, prostaglandins, growth factors, nitric oxide, and shear stress also activate Nrf2 (7).

Since the discovery of Nrf2, the mechanism as well as the cellular locale for its degradation have attracted a great deal of attention, much of which focuses on Keap1-mediated Cul3/Rbx1-dependent ubiquitylation of Nrf2 and its subsequent proteasomal degradation in the cytoplasm (4, 6, 8–12). There are reports that Nrf2 can also be degraded in the nucleus (8, 10, 13), but mechanistic details are lacking. McMahon et al. (14) have postulated the existence of three spatially different modalities for the degradation of Nrf2 in cells, one dependent on Keap1 and the other two independent of it. They posited that the Keap1-dependent modality occurs only under homeostatic conditions for its degradation in the nucleus, independent of Keap1.

The abbreviations used are: Nrf2, nuclear factor erythroid 2-related factor 2; AKAP95, A-kinase-anchoring protein 95; ARE, antioxidant-response element; HO-1-ARE-luc, heme oxygenase 1-luciferase reporter gene construct; Keap1, Kelch-like ECH-associated protein 1; MEM, modified essential medium; MG-132, carboxbenzoxyl-L-leucyl-L-leucyl-L-leucinal; PML, promyelocytic leukemia protein; PML-NB, promyelocytic leukemia-nuclear body; RFP, red fluorescent protein; SENP, sentrin protease; SP100, 100-kDa speckled protein; SUMO, small ubiquitin-like modifier; Co-IP, co-immunoprecipitation; IP, immunoprecipitation; WB, Western blot; DS, detergent-soluble; DI, detergent-insoluble; BHQ, butylhydroquinone.

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**Background:** Nrf2 (nuclear factor erythroid 2-related factor 2) enables cells to mount a cytoprotective response to oxidative stress.

**Results:** Nrf2 localizes, in part, to promyelocytic leukemia-nuclear bodies (PML-NBs), can undergo sumoylation, and can be degraded in PML-NBs.

**Conclusion:** Polysumoylated Nrf2 is polyubiquitylated by RING finger protein 4 (RNF4) and subsequently degraded by the proteasome in PML-NB domains.

**Significance:** Nuclear degradation of Nrf2 involves PML-NBs.
Sumoylation and Nuclear Degradation of Nrf2

conditions, involves the cytoskeleton, and is directed by the DIDLID sequence (amino acid residues 17–32) within the Neh2 domain of Nrf2. The second modality was postulated to also occur in the cytoplasm and also to be directed by the DIDLID sequence, but away from the cytoskeleton, and in a Keap1-independent manner. The third modality was described as Keap1-independent, Neh6-mediated and to occur in the nucleus. Recently, the Neh6 domain was shown to contain two phosphodegrons that mediate degradation of Nrf2 by the E3 ubiquitin ligase β-TrCP in a Keap1-independent manner (15, 16). The authors remarked that there might also be other potential proteasome degradation motifs apart from those involving Keap1 and β-TrCP.

Here, we demonstrate that Nrf2 is a target for sumoylation (post-translational modification by small ubiquitin-like modifier (SUMO) proteins (17, 18)) and that it traffics, in part, to PML-NBs. We show that RNF4, a poly-SUMO-specific E3 ubiquitin ligase ubiquitylates polysumoylated Nrf2, leading to degradation of the modified Nrf2 in PML-NB domains. This work provides a novel mechanism for the degradation of Nrf2 in the nucleus.

EXPERIMENTAL PROCEDURES

Plasmids—Construction of the expression plasmid for the fusion protein GFP-Nrf2 has been described previously (19). Dr. Edward T. H. Yeh (University of Texas M.D. Anderson Cancer Center, Houston, TX) provided us with an expression plasmid for wild-type SENP1. Expression plasmid for FLAG-SENP1 mutant was purchased from Addgene (Cambridge, MA; catalog no. 17358). Expression plasmids for wild-type RNF4 (pBOS-RNF4wt-YFP) and mutant RNF4 (pBOS-RNF4-CSI-YFP), respectively, were obtained from Dr. Ronald T. Hay (University of Dundee, Dundee, Scotland, UK). The vector pBOS-H2BGFP was purchased from BD Biosciences. Expression plasmid for PML-I (pEGFP-C3-PML-I) was obtained from Dr. Peter Hemmerich (Leibniz Institute for Age Research, Fritz Lipman Institute, Jena, Germany). Plasmid pCMV6-RNF4-myc-DDK expressing RNF4 and the empty vector pCMV6-myc-DDK were obtained from OriGene Technologies. Expression plasmids for RFP-SUMO-1 and -2 and mutants thereof were obtained from Dr. Angela Chen (National Sun Yat-Sen University, Kaohsiung, Taiwan).

Cell Culture—HepG2 cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mm L-glutamine, 1 mm sodium pyruvate, 1× MEM nonessential amino acids (Invitrogen), and antibiotics (100 units of penicillin and 100 μg of streptomycin per ml) at 37 °C in 95% air, 5% CO2 atmosphere.

Localization of Nrf2 in HepG2 Cells by Fluorescence Microscopy—HepG2 cells (~5 × 105 cells per well) were seeded onto poly-d-lysine-coated coverslips in 6-well plates in 2 ml of medium and incubated overnight at 37 °C. The cells were then transfected with expression plasmid(s) indicated in the appropriate figures, using GeneJamer transfection reagent (Stratagene, La Jolla, CA) at a 4:1 ratio of GeneJamer (μl) to DNA (μg). Twenty four hours after transfection, some cells were incubated with 10 μM MG-132 for 4 h, then harvested by removing the medium, and rinsing once with 1 ml of 1× phosphate-buffered saline (PBS). The cells were fixed with 500 μl of 3.7% formaldehyde solution for 10 min at room temperature, then washed twice with 1 ml of 1× PBS followed by a 5-min wash with 1 ml of 0.1x glycine-Tris buffer (pH 7.4), and rinsed with 1 ml of 1× PBS. After fixation, the coverslips were mounted onto glass slides using a drop of Prolong Gold Antifade with DAPI (Invitrogen), dried, and kept overnight at 4 °C. Imaging was performed on a Nikon A1R confocal laser scanning microscope at excitation/emission wavelengths of 488/505–550 nm (for green fluorescence), 561/570–620 nm (red fluorescence), 640/662–737 nm (for PML, colored blue), and 405/425–475 nm (for DAPI, colored purple). When necessary, images of the fluorescence patterns were merged to visualize co-localization.

Immunocytochemistry to Detect PML Protein—HepG2 cells were grown and processed as for fluorescence imaging. After the fixation step, the cells were rinsed with PBS, followed by incubation in blocking solution (1% bovine serum albumin (BSA) in PBS containing 1% Triton X-100) for 30 min at room temperature. After removing the blocking solution, the cells were incubated with anti-PML antibody (sc-966, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in blocking solution for 1 h at room temperature. The primary antibody was removed by aspiration, and the coverslips were washed three times with PBS (5 min for each wash). Coverslips were then incubated with Alexa Fluor633 goat anti-mouse IgG (Invitrogen) diluted 1:2,500 in blocking solution for 1 h at room temperature in the dark and then washed and stained for nuclei as described above. The cells were then mounted in Aqua-Poly/Mount and dried overnight at 4 °C for visualization. PML protein was visualized at excitation/emission wavelengths of 633/650 nm LP filter, respectively, on a Nikon TE2000-U CI confocal microscope or a Nikon A1R confocal laser scanning microscope at excitation/emission wavelengths of 640/662–737 nm, respectively.

Assessment of Interaction of Nrf2 with SUMO Using Fluorescence Resonance Energy Transfer (FRET) Analysis—Cells prepared for cellular localization by fluorescence microscopy as described above were used for FRET analysis, using the acceptor photobleaching method. Individual dots containing PML, GFP-Nrf2, and RFP-SUMO were analyzed. Donor (GFP) and acceptor (RFP) images were acquired with a Nikon A1R laser scanning confocal microscope. Images were deliberately underexposed to avoid saturation of the donor-dequenched GFP signal. For each dot analyzed, three consecutive images were captured (with no delay between captures) for both pre- and post-bleach conditions, and fluorescence intensity for each condition was averaged. In some cases, only values for the last pre-bleach and first post-bleach intensities were used in the calculations for FRET efficiencies. This was deemed necessary to correct for general photobleaching when overall signal loss masked a clear increase in initial post-bleach intensity. The average pre- and post-bleach fluorescence intensities were then used for calculating FRET efficiency for the various treatment conditions studied. For each treatment, 10–20 cells were imaged. FRET efficiency was expressed as the percent increase of pre-bleach GFP fluorescence in the region of interest com-
pared with that observed after RFP photobleaching, according to the following equation: FRET efficiency (%) = (GFP\text{after} - GFP\text{before})/GFP\text{after} \times 100 (20).

Co-immunoprecipitation Assays—Whole cell lysates were immunoprecipitated with appropriate antibody, according to the experiment, using PureProteome Protein G magnetic beads (Millipore, Temecula, CA) or Dynabeads (Invitrogen) according to the manufacturer’s protocol. Immunoprecipitated proteins were eluted in 30 μl of 2× Laemmli’s sample buffer. The beads were collected using a magnetic rack, and the supernatant solution was resolved on 7% SDS-PAGE. Proteins were then analyzed by Western blotting using appropriate antibody as indicated in the legends to the various figures.

**Fractionation of Cell Lysate into Detergent-soluble and Detergent-insoluble Fractions**—HepG2 cells were grown to 80% confluency in T-75 flasks and then treated with either MG-132 (10 μM) for 4 h or As2O3 (2 μM) for up to 18 h. The cells were washed with 1× PBS and then scraped into ice-cold Triton X-100 lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1.0% Triton X-100, 1 mM PMSE, 10 mM dithiothreitol, 10 μl/ml protease inhibitor mixture (Sigma)) and kept on ice for 45 min, with occasional mixing, followed by centrifugation at 14,000 \( \times g \) for 15 min at 4 °C. The pellet (the detergent-insoluble fraction) was resuspended in 100 μl of buffer containing equal volumes of 62.5 mM Tris-HCl (pH 6.8) and 2× SDS sample buffer, then sonicated on ice, and boiled for 5 min. After quantification using a modified Bradford protein assay, the detergent-soluble and -insoluble fractions were used for Western blotting assays.

**In Vitro Sumoylation Assay**—Using glutathione-agarose resin, GST-Nrf2 was purified as described by Rachakonda et al. (21). The GST-Nrf2 fusion protein was then used as substrate in in vitro SUMO-1 and SUMO-2 conjugation assays, performed by using in vitro SUMOylation kits purchased from Active Motif (Carlsbad, CA), with p53 as a positive control.

**Reporter Gene Assay**—Nrf2-dependent gene transcription was assessed by reporter gene assay using ARE-driven minimal promoter HO-1-ARE-luc as described previously (19, 22).

**Preparation of PML-Nuclear Body-enriched Fraction**—Beginning with a partially purified nuclear pellet (19), a PML-NBs-enriched fraction was prepared according to the protocol described by He et al. (23) as modified by Vertegaal et al. (24).

**RESULTS**

**Nrf2 Traffics to PML-NBs**—We used enhanced green fluorescent protein-tagged Nrf2 (designated GFP-Nrf2) (19, 22) to monitor cellular localization of Nrf2. Under confocal microscopy (Fig. 1, column 2), we observed aggregates or dots in nuclei of HepG2 cells incubated with the proteasome inhibitor MG-132. The dots were observed in at least 90% of such cells. Because the nucleus of the eukaryotic cell contains a variety of nuclear bodies exhibiting different subnuclear locations and various functions (25–28), we wondered whether the nuclear dots contained components of PML-NBs, which can be visualized as punctate nuclear structures when cells are treated with proteasome inhibitors such as MG-132 (29). If these nuclear structures contained PML-NBs, we expected their presence to be disrupted by de-sumoylation, given that sumoylation is important in the formation of PML-NBs (30–34). Indeed, when pEGFP-Nrf2 was transfected into HepG2 cells along with expression plasmid for the SUMO-specific protease SENP1 (sentrin protease 1), the GFP fluorescence intensity of the nuclear dots was substantially diminished (Fig. 1, column 3). In contrast, mutant SENP1 that is catalytically inactive, and therefore cannot catalyze de-sumoylation, failed to abolish the nuclear dots (Fig. 1, column 4), suggesting that sumoylation was involved in the formation of these nuclear dots/aggregates. The data are quantified in the histogram. Western blotting (Fig. 1) showed that the expressed levels of SENP1 were higher in cells
transfected with wild-type SENP1 (lane 3) or mutant SENP1 (lane 4) than in cells displaying endogenous levels of SENP1 (lanes 1 and 2).

PML-NBs are nuclear domains that are initiated by sumoylated PML. The sumoylated PML then interacts noncovalently with other proteins containing SUMO interaction motifs (30–34). Characteristically, PML-NBs contain PML, SUMO, the nuclear antigen SP100, DAXX (also known as death-associated protein 6), and other proteins that dynamically localize to these nuclear domains (30–37).

To ascertain whether Nrf2 associates with PML-NBs, we used a cell fractionation technique to track localization of Nrf2 and key components of PML-NBs. To this end, we isolated a nuclear body-enriched fraction (designated NB in Fig. 2) from a partially purified nuclear pellet (19). As shown in Fig. 2A, the NB fraction prepared from As2O3-treated cells contained PML and SP100, two key components of PML-NBs. The unmodified 97-kDa isoform of PML protein (38) is shown in Fig. 2A; the slower migrating band (indicated by an asterisk) represents modified (sumoylated) PML inasmuch as treatment with As2O3 enhances formation of sumoylated PML (34, 38, 39). SP100 is known to be sumoylated (34, 35); therefore, the SP100 band (91 kDa) shown in Fig. 2A most likely represents sumoylated SP100. Remarkably, the NB fraction contained Nrf2 (Fig. 2A) as well as high content of SUMO-1- and SUMO-2/3-modified proteins (Fig. 2B). The NB fraction did not contain cytoplasmic contamination as evidenced by the absence of the cytoplasmic markers GAPDH and LDH (Fig. 2A). Given that AKAP95, a nuclear matrix protein (40), was abundant in the NB preparation (Fig. 2A), and that PML-NB domains are associated with the nuclear matrix (34), we infer that our NB preparation is rich in components of the nuclear matrix and therefore that Nrf2 traffics, in part, to PML-NB domains.

Next, we used confocal microscopy imaging to localize Nrf2. In cells transfected with plasmids expressing GFP-Nrf2 and either wild-type RFP-SUMO-1 or wild-type RFP-SUMO-2, and then immunostained for endogenous PML, we observed that GFP-Nrf2 co-localized in the nucleus not only with RFP-SUMO-1 and PML protein (Fig. 3A, panels 5–9) but also with RFP-SUMO-2 and PML protein (Fig. 3B, panels 5–9). Panels 7–9 in Fig. 3 (A and B) show additional co-localization results similar to that in panel 5 of each figure. Clearly, endogenous PML protein was present in some but not all of the SUMO-containing aggregates. In Fig. 3, yellow dots represent co-localization of GFP-Nrf2 with RFP-SUMO; white arrows point to white dots representing co-localization of GFP-Nrf2 with RFP-SUMO and endogenous PML. Scale bars, 10 μm.

FIGURE 2. Nuclear body-enriched preparation contains Nrf2 and components of PML-NBs. Whole cell lysate (W), prepared from HepG2 cell cultures treated with As2O3 (2 μM) for 4 h, was separated into cytoplasmic (C) and partially purified nuclear pellet (18). Beginning with the partially purified nuclear pellet, nuclear body-enriched fraction (NB) was prepared according to the protocol described by He et al. (23) as modified by Vertegaal et al. (24). The final pellet was suspended in 100 μl of buffer containing equal volumes of Tris-HCl solution (pH 6.8) and 2× SDS sample buffer. The cytoplasmic, whole cell lysate, and NB fractions were then blotted for the proteins indicated in A and B. *, modified (sumoylated) PML protein.

FIGURE 3. GFP-Nrf2 co-localizes with endogenous PML protein and wild-type RFP-SUMO. HepG2 cells grown in MEM on coverslips to 50–80% confluence were co-transfected with 2 μg of pEGFP-Nrf2 with 0.5 μg of expression plasmid for RFP-SUMO-1 or RFP-SUMO-2 followed by immunostaining for endogenous PML, as described under “Experimental Procedures.” A, RFP-SUMO-1; B, RFP-SUMO-2; inset in panel 5 is an enlargement of the boxed area within panel 5. Panels 7–9 are representative images of cells from parallel experiments showing co-localization as in panel 5. Yellow arrows point to yellow dots representing co-localization of GFP-Nrf2 with RFP-SUMO; white arrows point to white dots representing co-localization of GFP-Nrf2 with RFP-SUMO and endogenous PML. Scale bars, 10 μm.
SUMO-1 did not co-localize with PML. Displayed a diffuse pattern in which the nonconjugatable epithelial (Hep2) cells expressing nonconjugatable SUMO-1 resemble those of Bailey and O’Hare (29) who observed that SUMO molecules or with endogenous PML. These results imply that GFP-Nrf2 did not co-localize with the mutant yellow dots were evident (Fig. 4, A). When they were immunostained for endogenous PML, diffuse patterns were also observed, and neither white nor yellow dots were evident (Fig. 4, C and D). The diffuse patterns imply that GFP-Nrf2 did not co-localize with the mutant SUMO molecules or with endogenous PML. These results resemble those of Bailey and O’Hare (29) who observed that epithelial (Hep2) cells expressing nonconjugatable SUMO-1 displayed a diffuse pattern in which the nonconjugatable SUMO-1 did not co-localize with PML.

FRET Analysis Reveals the Presence of SUMO-modified Nrf2 in PML-NBs—Protein-protein interaction involving sumoylation can be studied by FRET analysis (39, 45, 46). FRET is based on the ability of a higher energy donor fluorophore to transfer energy to a lower energy acceptor fluorophore if the donor and acceptor molecules are within 50–100 Å of each other (47). Photobleaching (quenching) of the acceptor fluorophore, such that energy from the donor can no longer be transferred to the acceptor, causes an increase in fluorescence (enhanced fluorescence) of the donor molecule. We used this approach to provide independent evidence that Nrf2 interacts with SUMO in the intact cell.

When we measured FRET between GFP-Nrf2 (donor fluorophore) and RFP-SUMO-1 (acceptor fluorophore), the results revealed enhanced GFP fluorescence after photobleaching RFP (Fig. 5A, compare arrows in pre-bleach panel to arrows in the post-bleach panel), indicating that Nrf2 interacts with SUMO-1. Similar results were obtained with respect to RFP-SUMO-2 (Fig. 5B), indicating that Nrf2 also interacts with SUMO-2. However, use of RFP-SUMO-1ΔGG did not generate any detectable FRET signal (Fig. 5C). Similarly, little or no FRET signal was detected between GFP-Nrf2 and RFP-SUMO-2ΔGG (Fig. 5D). Quantitative summation of the FRET data is displayed in Fig. 5D. We interpret the lack of FRET signal between the nonconjugatable SUMO molecules and GFP-Nrf2 to mean that the FRET signal observed between wild-type SUMO and GFP-Nrf2 reflects conjugation of SUMO to Nrf2. The relatively small FRET signal observed with RFP-SUMO-2ΔGG (Fig. 5D) was not observed with RFP-SUMO-1ΔGG. This signal most likely resulted from noncovalent interaction between SUMO interaction motifs in Nrf2 and RFP-SUMO-2ΔGG, rather than covalent modification. A similar phenomenon was also observed by Percherancier et al. (48) who used bioluminescence resonance energy transfer to analyze interac-

FIGURE 4. Nonconjugatable SUMO does not co-localize with GFP-Nrf2 or with endogenous PML protein. HepG2 cells grown as in Fig. 3 were co-transfected with 2 μg of pE GFP-Nrf2 with 0.5 μg of expression plasmid for RFP-SUMO-1ΔGG (A and C) or RFP-SUMO-2ΔGG (B and D); the cells in C and D were immunostained for endogenous PML protein, as described under “Experimental Procedures.” Scale bars, 10 μm.
tion between PML protein and RNF4. Other investigators have also noted that nonconjugatable SUMO molecules fail to interact with SUMO-modifiable proteins (29, 49).

In Vitro Sumoylation of Nrf2

To apply a direct method to establish that Nrf2 is conjugatable to SUMO, we performed in vitro sumoylation assays, using purified Nrf2 (as GST-Nrf2) (Fig. 6, A) as substrate. To validate the assay, we used p53, a known sumoylatable protein, as a positive control (Fig. 6, B). As shown in this figure, the higher molecular weight band seen in assays containing SUMO-1 (Fig. 6B, lane 1) was absent in assays run with mutant SUMO-1 (i.e. nonconjugatable SUMO) (lane 2). When the membrane was blotted with anti-SUMO-1 antibody, only the high molecular weight band at ~73–75 kDa was detected (data not shown), indicating that this band represents sumoylated p53. Similar analyses were then performed with GST-Nrf2 as substrate (Fig. 6, C and D). Anti-Nrf2 antibody detected not only the GST-Nrf2 fusion protein but also higher molecular size species of this substrate in assays containing either SUMO-1 or SUMO-2 but not in assays containing non-conjugatable SUMO (Fig. 6, upper panels in C and D). We then confirmed that these higher molecular size species represented sumoylated species by blotting with anti-SUMO-1 and anti-SUMO-2/3 antibodies, respectively (Fig. 6, C and D, lower panels). The anti-SUMO antibodies did not detect any bands in

**FIGURE 5.** FRET analysis of SUMO-modified Nrf2 in PML-NBs. HepG2 cells were transfected with pEGFP-Nrf2 (1 μg) along with expression plasmid for RFP-SUMO (0.5 μg). The cells were harvested and fixed as described under “Experimental Procedures.” Individual spots containing PML, GFP-Nrf2, and RFP-SUMO were subjected to FRET analysis. Donor (GFP) and acceptor (RFP) images were acquired with a Nikon A1R laser-scanning confocal microscope, as described under “Experimental Procedures.” A and B, the arrows indicate locations of pre- and post-bleaching. The differential interference contrast (DIC) images are shown in panels 7 and 8. A, FRET analysis of interaction of GFP-Nrf2 with RFP-SUMO-1. B, FRET analysis of interaction of GFP-Nrf2 with RFP-SUMO-2. C, lack of FRET signals between GFP-Nrf2 and nonconjugatable SUMO-1 (RFP-SUMO-1 ΔGG). D, quantification of the FRET data. For each treatment, 10–20 cells were imaged. FRET efficiency (%) was calculated from the following equation: FRET efficiency (%) = (GFP after - GFP before) / GFP after × 100 (20). Values plotted are means ± S.E. for n = 17–52. Scale bars, 10 μm.

**FIGURE 6.** Sumoylation of Nrf2 in vitro. Using GST-Nrf2 fusion protein (purified as described under “Experimental Procedures”) as substrate (0.5 μg), the assays were performed with in vitro sumoylation assay kits (Active Motif), with p53 supplied in the kit as a positive control. The GST-Nrf2 fusion protein as well as products of the reaction were separated on 7% SDS-PAGE and analyzed by Western blotting using the indicated antibodies. A, representative Western blot of the purified GST-Nrf2 fusion protein. B, SUMO-1-modified p53. C and D, SUMO-1-conjugated (C) and SUMO-2-conjugated (D) Nrf2 detected with anti-Nrf2 antibody are shown in the upper panels. Lower panels show the same membranes blotted with anti-SUMO-1 and anti-SUMO-2/3 antibody, respectively.
assays run with mutant SUMO molecules (Fig. 6, C and D, lower panels, lane 3). These data provide direct evidence that Nrf2 is a SUMO-modifiable protein.

Use of the Desumoylase SENP1 Reveals Covalent Association of Nrf2 with SUMO in the Intact Cell—To establish that the association of GFP-Nrf2 and SUMO in the intact cell is covalent (i.e. caused by sumoylation), we repeated the co-localization experiments by co-transfecting HepG2 cells with expression plasmid for GFP-Nrf2, RFP-SUMO-1, and either wild-type SENP1 or mutant SENP1 that cannot catalyze de-sumoylation. We reasoned that if the association depended on sumoylation, then the number of yellow dots would be diminished by de-sumoylation imparted by SENP1. We observed fewer yellow dots/nucleus in cells that were transfected with wild-type SENP1 (Fig. 7B) than in control cells (Fig. 7A). Cells transfected with mutant SENP1 had a much higher number of yellow dots/nucleus than cells transfected with wild-type SENP1 (compare Fig. 7C with B). A quantitative summation of the data is shown in the histogram (Fig. 7D). When a similar experiment was performed with GFP-PML, a well established SUMO-modifiable protein (34, 39), as a positive control, the results (Fig. 7, E–H) resembled those obtained with GFP-Nrf2 (panels A–D), suggesting that the bonds formed between RFP-SUMO-1 and GFP-Nrf2 as well as RFP-SUMO-1 and GFP-PML were cleaved by the desumoylase SENP1. Thus, we infer that this enzymatic approach demonstrates that Nrf2 was covalently conjugated to SUMO-1 in the intact cell.

Endogenous SUMO Is Conjugated to Endogenous Nrf2—To establish that endogenous SUMO can be conjugated to endogenous Nrf2, we analyzed the interaction of SUMO-2/3 with Nrf2 in the absence of any transfection. We chose to analyze for SUMO-2, rather than SUMO-1, because SUMO-2 has been reported to be more abundant in cells than SUMO-1 (50). We incubated HepG2 cells with As2O3, a reagent that has been shown to induce oxidative stress (51–53), enhance SUMO-2/3 modification of proteins such as PML (54–56), and induce stabilization of stress response proteins such as Nrf2 (57–59). Using whole cell lysate, Co-IP assays (IP, Nrf2; WB, SUMO-2/3)
revealed that SUMO-2/3 interacted with Nrf2 (Fig. 8A). Fig. 8B represents input controls to show that the IP sample indeed contained Nrf2. Co-IP assay performed in reverse (IP, SUMO-2/3; WB, Nrf2) (Fig. 8C) also revealed that SUMO-2/3 interacted with Nrf2. Given that Nrf2 bands at ~95–110 kDa (60, 61), the higher molecular weight bands that were most prominent at 1–4 h after As2O3 treatment most likely represent As2O3-induced modification of Nrf2, i.e. SUMO-2/3-modified Nrf2. SUMO-2/3 is usually conjugated to proteins as SUMO chains (polysumoylation) (54–56). Therefore, the high molecular weight bands detected in Fig. 8 (A and C) most likely represent polysumoylated Nrf2. To validate this interpretation, we performed similar Co-IP assays in which we monitored sumoylation of endogenous PML as a positive control. Fig. 8D shows Western blots of nonmodified and modified PML; the input controls (Fig. 8E) show that the IP sample (generated with anti-PML antibody) indeed contained PML. Fig. 8F shows that the slower migrating high molecular weight species representing sumoylated PML were detected not only in cells that were not transfected with SUMO-2 but also in cells that were transfected with SUMO-2 (compare lanes 4–6 with lanes 1–3). These data, which are similar to those in Fig. 8, A–C, support our conclusion that the detected sumoylated Nrf2 (Fig. 8, A and C) represents endogenous Nrf2 conjugated to endogenous SUMO-2/3, especially because neither SUMO-2 nor Nrf2 was transfected in these experiments.

**Modified Nrf2 Is Degraded in PML-NBs**—Given that polysumoylation impacts the degradation of certain proteins in PML-NBs, specifically PML protein (39, 54), it was intriguing to investigate the fate of the Nrf2 that has trafficked to PML-NBs. Because mature PML-NBs contain the proteasome (29, 62, 63), one possibility is that turnover of Nrf2 might occur there. To begin to explore this idea, we used Triton X-100 to separate whole cell lysates into detergent-soluble (DS) and detergent-insoluble (DI) fractions, capitalizing on reports that detergent can be used to harvest PML-NBs into the DI fraction (64–67). To verify that our preparation of the DI fraction harvested PML-NBs, we blotted for SP100 and SUMO-1, which are prototypic residents of PML-NBs (34, 35). As expected, SP100 (Fig. 9A) and SUMO-1-modified proteins (Fig. 9B) were more abundant in our preparation of the DI fraction than the DS fraction. Next, we asked whether Nrf2 and/or Keap1 could be detected in the DI fraction, given that Keap1 is known to regulate cellular levels of Nrf2 (13, 68–70). To our surprise, biologically active Keap1 (~70 kDa) was not detectable in the DI fraction but was mostly restricted to the DS fraction (Fig. 9C). High molecular weight Keap1 (~150 kDa) was detected in the DI fraction, especially in cells treated with As2O3 (Fig. 9C). High molecular weight Keap1 has been described as representing multimeric
forms of Keap1 that are biologically inactive, i.e. unable to repress Nrf2 (21, 71, 72). Interestingly, the DI fraction was rich in modified Nrf2, which was very prominent in MG-132-treated cells (Fig. 9D). Most likely, these bands represent ubiquitylated Nrf2, but they could also include sumoylated Nrf2, especially because bands of similar size were also present in cells treated with As$_2$O$_3$, which is known to enhance polysumoylation of SUMO-2/3-modifiable proteins. This inference is consistent with the report (73) that inhibition of the proteasome in yeast and in human cells leads to accumulation of proteins simultaneously conjugated to both SUMO and ubiquitin.

Because the poly-SUMO-specific E3 ubiquitin ligase RNF4 has been shown to catalyze polyubiquitylation of polysumoylated PML in PML-NBs (74–77), we wondered whether polysumoylated Nrf2 could be a candidate substrate for ubiquitylation by RNF4. To explore this idea, we first checked for the presence of RNF4 in the detergent-separated fractions. We found that the DI fraction contained not only the monomeric form but also the dimeric form of RNF4 (Fig. 9E). Because dimerization is essential for the activity of RNF4 (78, 79), the presence of this form of RNF4 in the DI fraction, but not in the DS fraction (Fig. 9E), heightened our interest in whether RNF4 can ubiquitylate polysumoylated Nrf2.

To begin to address this question, we first assessed whether sumoylated Nrf2 was also ubiquitylated. Co-IP assays (IP, Nrf2; WB, ubiquitin) in whole cell lysates revealed endogenously ubiquitylated forms of Nrf2 (Fig. 10A). This was more evident when cells were treated with MG-132 (Fig. 10A, lane 3) than when they were treated with either As$_2$O$_3$ (lane 2) or As$_2$O$_3$ plus MG-132 (lane 4). When we blotted for SUMO-2/3, we noted that the slower migrating bands detected with the anti-SUMO-2/3 antibody (Fig. 10B) were similar in size to those detected with the anti-ubiquitin antibody shown in Fig. 10A, suggesting that polysumoylated Nrf2 was also ubiquitylated. Input controls are shown in Fig. 10C. Although treatment with As$_2$O$_3$ plus MG-132 resulted in higher levels of sumoylated Nrf2 than treatment with MG-132 alone (Fig. 10B, compare lane 4 with lane 3), the opposite was true with respect to ubiquitylated Nrf2 (Fig. 10A). This suggests that the same lysine residue(s) was/were sumoylated and/or ubiquitylated or that Nrf2 was simultaneously conjugated to both SUMO and ubiquitin.

To test whether RNF4 can ubiquitylate Nrf2, we transfected HepG2 cells with plasmid expressing wild-type RNF4-YFP and performed a Co-IP assay to establish that RNF4 can recognize endogenous Nrf2. Anti-GFP antibody was used to detect RNF4-YFP because this antibody recognizes YFP. Input controls demonstrate that the IP sample generated with anti-GFP antibody indeed detects RNF4-YFP (Fig. 10D). Next, we performed the Co-IP assay shown in Fig. 10E to measure interaction between RNF4-YFP and endogenous Nrf2. As evident in this figure, there was interaction between these two proteins; the interaction was more obvious when the cells were incubated with MG-132 (lane 2). Given that RNF4 recognizes only polysumoylated substrates, this result implies recognition of modified (i.e. polysumoylated) Nrf2 by RNF4.

To establish that the interaction between RNF4-YFP and Nrf2 yielded ubiquitylated Nrf2 that was also polysumoylated, we performed the Co-IP assay (IP, Nrf2; WB, ubiquitin) shown in Fig. 10F. With lysates of cells treated with As$_2$O$_3$ (Fig. 10F, lane 3) or MG-132 (lane 4), the band intensities of the ubiquitylated Nrf2 were higher than in control cells (lane 2). The intensities of these bands were also slightly higher (especially the band at ~150 kDa) than those of ubiquitylated Nrf2 in cells transfected with mutant RNF4 (Fig. 10F, compare lane 4 with lane 5). This is consistent with the fact that mutant RNF4, which lacks the RING domain, cannot mediate ubiquitylation...
Consistent with our idea that polysumoylated Nrf2 was also ubiquitylated, immunoprecipitating whole cell lysates with anti-Nrf2 antibody and blotting the IP sample with anti-SUMO-2/3 antibody (Fig. 10G) revealed that the slower migrating bands detected with anti-SUMO-2/3 antibody corresponded in size to the bands detected in Fig. 10F with anti-ubiquitin antibody. These results indicate that wild-type RNF4 can ubiquitylate polysumoylated Nrf2.

RNF4-mediated ubiquitylation of polysumoylated Nrf2 would make the polysumoylated and polyubiquitylated Nrf2 a substrate for degradation by the proteasome. As shown in Fig. 10H, wild-type RNF4, but not its mutant, enhances ubiquitylation of polysumoylated Nrf2. G, ubiquitylated Nrf2 is also sumoylated. H, steady-state levels of Nrf2 in cells transfected with plasmid encoding wild-type RNF4-YFP. Cells were treated with As2O3 (2 μM) for up to 8 h with or without MG-132 for 8.5 h, lysed, and then Western blotted for Nrf2. The plots were quantified densitometrically using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT). Values plotted are means for 2–3 experiments. The data are presented as percentage plots, taking the values for no treatment with As2O3 (controls) as 100%.

Next, we assessed the steady-state level of Nrf2 over time in NB fractions prepared from cells transfected with plasmid encoding RNF4-YFP. Much like in whole cell lysates, there was a clear decrease in the steady-state level of Nrf2 in these NB fractions (Fig. 11B, right panel). Given that the NB fraction contains key components of PML-NBs (Fig. 2A), these data are in agreement with the report that overexpressed RNF4 co-localizes with PML in PML-NBs (80).

To provide support that these events occurred in PML-NBs, we first analyzed the NB fraction for the presence of RNF4 and Keap1. Much like the detergent fractionation data in Fig. 9 (C and E), the NB fraction (Fig. 11A) contained RNF4-YFP but not biologically active Keap1. We detected slightly higher levels of RNF4-YFP in NB fractions prepared from cells that were treated with As2O3 than in cells that were not treated with As2O3 (Fig. 11B, right panel). Given that the NB fraction contains key components of PML-NBs (Fig. 2A), these data are in agreement with the report that overexpressed RNF4 co-localizes with PML in PML-NBs (80).

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We then assessed the degradation rate of Nrf2 by using the cycloheximide-chase approach. As shown in Fig. 11C, in NB fractions prepared from cells transfected with wild-type RNF4-YFP compared with transfection with vector only, the half-life ($t_{1/2}$) of the stabilized Nrf2 (cells were treated with As2O3) was substantially decreased. Taken together, the data in Figs. 10 and 11 strongly indicate
that RNF4 recognized polysumoylated Nrf2 and that its activity led to accelerated degradation of the modified Nrf2 in PML-NBs.

Impact of RNF4 on Nrf2-dependent Gene Transcription—To assess the functional significance of the RNF4-induced decrease in the steady-state level of Nrf2, we measured the ability of Nrf2 to transactivate an ARE-driven reporter gene in HepG2 cells transfected with plasmid expressing RNF4. Using the prototypic ARE-driven minimal promoter HO-1-ARE-luc (81) as the reporter gene construct, we found that the ectopic expression of RNF4 inhibited the transcriptional effect of Nrf2 by 30% (Fig. 12A). We have shown previously that tBHQ, which is known to antagonize Keap1-mediated inhibition of Nrf2 activity (82), potentiates the transcriptional effect of Nrf2 (83). As shown in Fig. 12B, RNF4 also inhibited Nrf2-dependent gene transcription in the presence of tBHQ (35%). These results suggest that the noted RNF4-induced degradation of Nrf2 impacts the biological function of Nrf2.

DISCUSSION

In this work, we uncovered three previously unrecognized aspects of the biology of Nrf2. First, we show that Nrf2 traffics, in part, to PML-NBs. Second, we demonstrate that Nrf2 is a target for conjugation with SUMO. Third, we show that polysumoylated Nrf2 can be ubiquitylated by RNF4 and then degraded in PML-NBs in a proteasome-dependent manner. Our work adds sumoylation to a short list of post-translational modifications that have been reported to impact the biology of Nrf2 after its separation from Keap1; the others are phosphorylation (12, 84–86) and acetylation-deacetylation (22, 87). Given that the consequences of sumoylating a protein vary (17, 18, 88–91) and are almost always context-dependent (92),

FIGURE 12. Nrf2-dependent gene transcription is abrogated by RNF4. HepG2 cells were co-transfected with 0.2 μg each of HO-1-ARE-luc reporter gene construct and heterologous Nrf2 (pCI-Nrf2) as inducer (19, 22), with or without plasmid pCMV6-RNF4-myc-DDK (OriGene Technologies) expressing RNF4. When needed, empty vector pCI-Neo (0.2 μg) or pCMV6-myc-DDK (0.2 μg) was also transfected. The total amount of DNA in each well was 0.6 μg. The electrophile tBHQ (20 μM) was added 24 h after transfection, and promoter activity was measured 8 h later, as described (19, 22). A, Nrf2-dependent gene promoter activity is inhibited by RNF4. B, tBHQ-enhanced Nrf2-dependent gene transcription is inhibited by RNF4. Values plotted are means ± S.E. for duplicate assays from 3 to 4 different experiments. *, statistically different (p < 0.05).
future investigation is warranted regarding other potential consequences of sumoylating Nrf2.

Our imaging data revealed two subsets of SUMO-containing populations to which Nrf2 co-localized within the nucleus. One subset contained PML protein, whereas the other did not. We identified the former as PML-NBs. We note that it is not unusual for a protein to localize to PML-NBs as well as to other bodies that do not contain PML. For example, HIPK2 localizes to PML-NBs (41) as well as to subnuclear speckles termed HIPK2 domains (also called nuclear deposition sites or HIPK-NUDES) that do not contain PML (42). Given the emerging interest in identifying what proteins traffic to the various nuclear neighborhoods (27) as well as in deciphering what physiological purpose(s) their localization there may serve, our study raises interesting questions about the potential biological significance of the trafficking of Nrf2 to PML-NBs. Arguably, this trafficking could be for temporary storage (36), or it could be for purposes of post-translational modification that eventually result in defined fate(s) of the transcription factor. Our observation that MG-132 increases the presence of ubiquitylated Nrf2 in these nuclear domains suggest that turnover of Nrf2 occurs there.

In recent years, degradation of certain proteins, specifically PML (39, 54), cAMP-response element-binding protein-binding protein (93), and the nucleolar protein N4BP1 (67), has been reported to occur within PML-NBs. In this context, degradation of PML protein, as SUMO-2/3-modified PML, was shown to require the poly-SUMO-targeted E3 ubiquitin ligase (STUbl) RNF4. RNF4 contains SUMO interaction motifs that enable it to recognize polysumoylated PML (39, 54), thereby targeting the modified PML for polyubiquitylation and subsequent degradation in PML-NBs. Given our demonstration that RNF4 recognizes polysumoylated Nrf2, the presence of polysumoylated Nrf2 in PML-NBs would position it for ubiquitylation by RNF4 and subsequent degradation in these nuclear domains.

Keap1-mediated degradation of Nrf2 (13, 14, 68, 69) has usually been considered to be a major means of regulating the cellular level of Nrf2 under homeostatic conditions. This well-documented modality involves the Keap1-Cullin3/RING box1 (Cul3-Rbx1) E3 ubiquitin ligase complex-dependent ubiquitylation of Nrf2 in the cytoplasm, leading to its degradation by the proteasome. However, although mechanistic details are lacking, reports abound that Nrf2 can also be degraded in the nucleus. Until now, no previous study has considered the involvement of either sumoylation or PML-NBs in the turnover of Nrf2. Our finding that polysumoylated Nrf2 is ubiquitylated by RNF4 in PML-NBs and subsequently degraded in these nuclear domains in a proteasome-dependent manner adds a novel dimension to our understanding of the mechanisms of the degradation of Nrf2 in the nucleus, and it provides an additional means of regulating the cellular level of Nrf2. At this time, mechanisms of the regulation of the cellular level of Nrf2 in stressed cells are not clearly delineated. That the PML-NB-dependent degradation of Nrf2 occurs in a Keap1-independent manner suggests it might be of major impact in oxidatively stressed cells. In such cells, Keap1 would be dysfunctional with respect to repressing Nrf2. Remarkably, overexpression of RNF4 resulted in decreased transcriptional activity of Nrf2, as measured by reporter gene assay (Fig. 12), indicating that the RNF4-induced degradation of Nrf2 impacts the biological function of Nrf2. PML-NB-mediated degradation of modified Nrf2 may also be important in cells in which the functionality of Keap1 has been impaired by somatic mutation(s) such as in certain cancers (94–96).

Taking into account the data presented here as well as recent studies (22, 87) on acetylation-deacetylation of Nrf2, we pro-
pose the model in Fig. 13, which highlights post-translational modifications of Nrf2 within the nucleus. In this model, we posit that RNF4 polyubiquitylates polysumoylated Nrf2 in PML-NBs, subsequently leading to its degradation in PML-NBs, in much the same way as PML (39, 54, 55). We do not yet know what proportion of total Nrf2 in the nucleus is degraded in PML-NBs. Future work using live cell imaging to monitor the kinetics of Nrf2 trafficking to PML-NBs may provide an answer to this question.

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Sumoylation and Nuclear Degradation of Nrf2

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