Reactive Oxygen Species Regulate Nucleostemin Oligomerization and Protein Degradation*[S]

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Nucleostemin (NS) is a nucleolar-nucleoplasmic shuttle protein that regulates cell proliferation, binds p53 and Mdm2, and is highly expressed in tumor cells. We have identified NS as a target of oxidative regulation in transformed hematopoietic cells. NS oligomerization occurs in HL-60 leukemic cells and Raji B lymphoblasts that express high levels of c-Myc and have high intrinsic levels of reactive oxygen species (ROS); reducing agents dissociate NS into monomers and dimers. Exposure of U2OS osteosarcoma cells with low levels of intrinsic ROS to hydrogen peroxide (H_2O_2) induces thiol-reversible disulfide bond-mediated oligomerization of NS. Increased exposure to H_2O_2 impairs NS degradation, immobilizes the protein within the nucleolus, and results in detergent-insoluble NS. The regulation of NS by ROS was validated in a murine lymphoma tumor model in which c-Myc is overexpressed and in CD34+ cells from patients with chronic myelogenous leukemia in blast crisis. In both instances, increased ROS levels were associated with markedly increased expression of NS protein and thiol-reversible oligomerization. Site-directed mutagenesis of critical cysteine-containing regions of nucleostemin altered both its intracellular localization and its stability. MG132, a potent proteasome inhibitor and activator of ROS, markedly decreased degradation and increased nucleolar retention of NS mutants, whereas N-acetyl-L-cysteine largely prevented the effects of MG132. These results indicate that NS is a highly redox-sensitive protein. Increased intracellular ROS levels, such as those that result from oncogenic transformation in hematopoietic malignancies, regulate the ability of NS to oligomerize, prevent its degradation, and may alter its ability to regulate cell proliferation.

Nucleostemin (NS) is a GTP-binding nucleolar protein that has been implicated in a variety of cellular processes, including cell cycle progression involving the G_1-S (1, 2) and G_2-M transitions (3), pre-rRNA processing (4), stress responses involving nucleoplasmic translocation (5, 6), cellular senescence (7), and inhibition of cell proliferation (8). NS shuttles from the nucleolus to the nucleoplasm in response to a variety of cellular stressors, including inhibition of RNA synthesis (5). We have recently demonstrated that reduction of intracellular GTP levels results not only in the nuclear translocation of NS, as had been observed previously (6), but also its rapid proteasomal degradation in an apparent Mdm2-dependent manner (9). A number of nuclear proteins interact with nucleostemin (10), including p53 (1, 8), Mdm2 (2, 3), ribosomal L1 domain-containing 1 (RSL1D1) (11), and telomeric repeat binding factor 1 (TRF-1) (7, 12). The regulation of these diverse proteins through their interactions with NS results in functional alterations in cell cycle control and telomere maintenance (10), although the precise mechanisms of regulation remain to be elucidated. Complete loss of NS, either through siRNA knockdown experiments (1, 2, 13, 14) or through its proteolytic degradation (9), leads to p53 up-regulation and cell cycle arrest or apoptosis, whereas heterozygous loss of NS leads to cellular senescence (7). Because NS is expressed at particularly high levels in tumor cells (8), understanding its regulation at the protein level has important ramifications for cancer cell biology and treatment.

Reactive oxygen species (ROS),[2] which include hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), hydroxyl radicals (OH·), nitric oxide (NO), and monoxide radical (NO·), play crucial roles in modulating many physiologic and pathologic processes. ROS act as mitogenic signals to promote cellular proliferation at lower concentrations and as inducers of apoptosis or necrotic cell death at higher concentrations (15–17). Reduced GSH is a major antioxidant and intracellular free radical scavenger of ROS, whereas N-acetyl-L-cysteine (NAC) is a cell-permeable precursor of GSH (18).

Increased levels of ROS also result from the expression of oncogenes (19, 20). The BCR-ABL translocation that results in chronic myelogenous leukemia (CML) has been shown to increase ROS levels in both BCR-ABL-transformed hematopoietic cell lines and primary CML cells (21–23). Mutations within the BCR-ABL tyrosine kinase domain that lead to resistance to imatinib as well as to other manifestations of DNA damage are accelerated by ROS generation and reduced by the concomitant administration of antioxidants (23). Similarly, increased

[2] The abbreviations used are: ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; CML, chronic myelogenous leukemia; PBMC, peripheral blood mononuclear cell; IMDM, Iscove's modified Dulbecco's medium; H2-DCFDA, 2′,7′-dichlorofluorescein diacetate; DHE, dihydroethidium; aa, amino acid(s); CML-BC, chronic myelogenous leukemia in blast crisis; EGFP, enhanced green fluorescent protein.

expression of the Myc oncogene because of amplification, mutation, increased transcription, or translocation results in increased ROS levels that appear to promote genomic instability in transformed cells (19, 24). Thus, ROS induction as a consequence of oncogenic transformation contributes to genomic instability and most likely to disease progression in tumors expressing these oncogenes. Further understanding of the oxidant-dependent signaling pathways induced during transformation should therefore be useful in identifying previously unrecognized redox-sensitive targets that may play a role in the progression of diseases such as CML or those resulting from increased c-Myc expression.

We have identified NS as a target of increased ROS levels. Cell lines with Myc amplification express an oligomerized form of NS that is reduced after incubation with NAC and other reducing agents. This observation led us to examine the role of intracellular ROS as a potentially important regulator of NS stability and function.

MATERIALS AND METHODS

Cell Culture and Reagents—Raji, HL-60, and U2OS cell lines were obtained from ATCC. The U2OS cell line stably expressing EGFP-tagged NS was described previously (9). Cells were grown in DMEM or RPMI 1640 medium supplemented with 10% FCS (HyClone, Perbio) and 100 units/ml penicillin and streptomycin at ambient oxygen concentration. The human c-Myc lymphoma cell line 6780 (25) was grown in RPMI 1640 medium with 10% FCS, 100 units/ml penicillin and streptomycin, 1% L-glutamine, and 50 μM 2-mercaptoethanol. The primary peripheral blood mononuclear cells (PBMC) and primary lymphoma cells from mice transgenic for both EμSR-tTA and Tet-o-Myc tumors (26) were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS (HyClone; Perbio) and 100 units/ml penicillin and streptomycin. GSH, NAC, H_2O_2, and FLAG peptides were obtained from Sigma. DAPI, 2′,7′-dichlorofluorescein diacetate (H2-DCFDA), and dihydroethidium (DHE) were purchased from Molecular Probes, Inc. (Eugene, OR). Rhodamine-conjugated goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Goat anti-nucleostemin polyclonal antibody was from R&D Systems, Inc. Full-length *Aequorea victoria* polyclonal EGFP antibody was from Clontech Laboratories. Anti-Mdm2 monoclonal antibody, anti-FLAG-BioM2 (F-9291), and anti-FLAG-M2-agarose (A2220) were from Sigma. Mouse monoclonal anti-nucleolin (MS-3), anti-p53 (DO-1), and anti-Mdm2 (sc-965) antibodies and protein A/G PLUS-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA).

Mutagenesis of NS, Construction of FLAG-tagged NS, and Transient Transfection Experiments—EGFP (C3)-NS was described previously (9). The human NS deletion construct EGFP (C3)-d111–160 amino acids (aa) was created using the QuikChange mutagenesis kit (Stratagene). Nine cysteines within NS were individually mutated to serines. Primer sequences are listed in supplemental Table 1. All constructs were confirmed by sequencing. The pcDNA3-FLAG-NS plasmid was constructed using EGFP-C3-NS as a template and PCR amplification with high-fidelity Platinum pfx DNA polymerase

(Invitrogen). The primer sequences for amplifying the FLAG-NS are shown in supplemental Table 1. The italicized bases are the restriction enzyme sites (BamH1 and Xho1) with four extra bases added at each end to promote efficient digestion. Amplified NS with an N-terminal FLAG tag was cloned into the BamH1and Xho1 sites of pcDNA3, and the resulting plasmids were sequenced. Transient transfections were performed using the Superfect transfection reagent (Qiagen) according to the manufacturer's instructions.

Analysis of c-Myc-induced Tumors—Conditional human c-Myc transgenic mice have been described previously (26). Transgenic mice from a cross between EμSR-tTA and Tet-o-Myc mice produce hematopoietic tumors with 100% frequency (26). In the absence of doxycycline treatment, mice transgenic for both EμSR-tTA and Tet-o-Myc die within 5 months of age with invasive hematopoietic tumors. In this study, 2- to 3-month-old mice transgenic for both EμSR-tTA and Tet-o-Myc and control FVB/N mice were sacrificed by CO_2 asphyxiation. Tumors were manifest by gross enlargement of the thymus, spleen, and mesenteric lymph nodes. The spleen, thymus, and lymph nodes were removed and collected in a sterile 70-μm cell strainer (BD Biosciences) over a Petri dish half filled with media, and single cell suspensions from these tissues were isolated by gently pressing the tissues through the cell strainer mesh with a plunger from a 15-ml syringe. Bone marrow cells were collected by flushing femurs and tibias with IMDM containing 10% FCS (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin with a 23- or 25-gauge needle. 5×10^5 cells/sample were incubated with the fluorochrome dyes H2-DCFDA and DHE for 30 min, and the level of intracellular H_2O_2 and superoxide was measured by flow cytometry. 5×10^6 cells/sample were centrifuged at 2,000 rpm for 5 min, washed with PBS twice, and then cell pellets were lysed for Western blot under non-reducing and reducing conditions.

Isolation of Human PBMC—PBMC from CML patients in blast crisis (CML-BC) were obtained from the Stanford Tissue Bank following Ficoll-Hypaque gradient separation and storage in 10% dimethyl sulfoxide in FCS. All analyzed CML-BC samples were obtained prior to treatment. After thawing, cells were washed three times with PBS, and 5×10^5 cells/sample were incubated in 20 ml IMDM supplemented with 10% FBS with or without 50 mM NAC/50 mM GSH for 4 h. The pH of the NAC- or GSH-containing medium was adjusted to 7.4. 5×10^6 PBMC/sample were harvested, washed, and sonicated as described above. 60 μg of the total lysate was then separated on SDS-PAGE and immunoblotted for NS, nucleoplasmin (NPM-1), and β-actin in the presence or absence of DTT with or without heat denaturation. To measure the intracellular level of H_2O_2, 5×10^5 cells/sample were incubated with H2-DCFDA for 30 min, and the level of intracellular H_2O_2 was determined by flow cytometry.

Cytospin and Immunostaining—Ficoll-purified PBMC from CML-BC patients were first stained with phycoerythrin-coupled anti-CD34 for 30 min on ice. After washing with PBS, the CD34-stained PBMC were cytospun onto coverslips at 500 rpm for 5 min, fixed with 4% paraformadehyde in PBS for 20 min at RT, and permeabilized with 0.1% Tritonin PBS for 15 min at RT. Following blocking with 3% BSA in PBS, slides were incu-
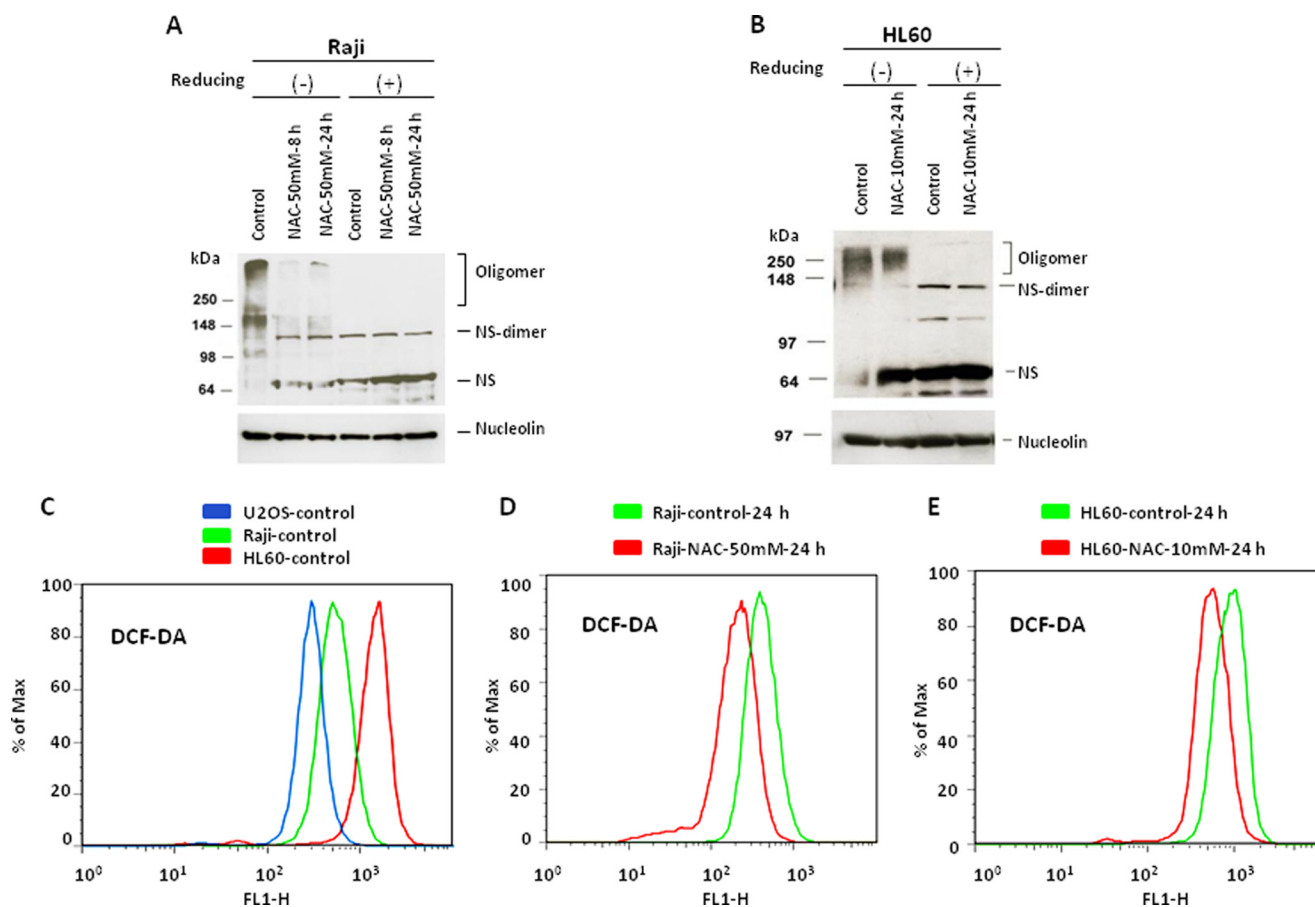
FIGURE 1. **NS oligomerization in Raji and HL-60 cells: effect of reducing agents.** Cultured Raji (*A*) or HL-60 (*B*) cells were untreated or exposed to 50 mM NAC for 8 or 24 h (*A*) or 10 mM for 24 h (*B*). Cell lysates (60 μg) were separated on SDS-PAGE under non-reducing or reducing conditions and immunoblotted with anti-NS or anti-nucleolin antibodies. *C*, ROS levels in U2OS, HL-60, and Raji cells measured by DCFDA fluorescence (*FL1-H*). *D* and *E*, effect of NAC on ROS levels in Raji and HL-60 cells as measured by DCFDA fluorescence.

bated with anti-NS antibody and stained with a FITC-conjugated secondary antibody (9). Immunostaining of NS, NPM-1, and nucleolin in U2OS cells was performed as described previously (9).

*Immunoprecipitation and Western Blotting Experiments—*U2OS cells were transfected with FLAG-tagged NS. 24 h after transfection, cells were treated with 10 mM H_2O_2 for 30 min. Cells were rinsed twice with PBS and lysed at 4 °C in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, and the protease inhibitors (150 μM Na_3VO_4, 0.25 mM PMSF, 5 μg/ml leupeptin, and 10 nM microcystin LR). The lysate was then sonicated and centrifuged at 12,000 × *g* for 10 min. The supernatants from the cell lysates were precleared with protein A/G PLUS-agarose at 4 °C for 1 h. 800 μg of total protein in 1 ml cell lysis buffer containing inhibitors from the precleared extracts were then incubated with 40 μl of anti-FLAG-agarose beads with gentle rocking at 4 °C for 2 h. Beads were washed five times with lysis buffer and twice with PBS. The immunoprecipitates were eluted from beads in 100 μl of TBS (50 mM Tris HCl/150 mM NaCl (pH 7.4)) elution buffer containing 500 μg/ml synthetic FLAG peptides. For immunoblot analysis, 30 μl of the elution fraction and 60 μg of total protein lysate were electrophoresed on 4–10% gradient SDS-PAGE gels and transferred onto nitrocellulose membranes (Immobilon-P, Millipore, Bedford, MA). Specific antigens were blotted with the corresponding primary antibody followed by

horseradish peroxidase-conjugated secondary antibody. Western blots were visualized using ECL (Chemiluminescence Reagent Plus, PerkinElmer Life Sciences, Boston, MA).

*Extraction of Triton-soluble and Triton-insoluble Protein—*Cells were rinsed twice with PBS and lysed at 4 °C in a lysis buffer containing protease inhibitors. The lysate was then sonicated while immersed in ice and centrifuged at 12,000 × *g* for 15 min. at 4 °C. The supernatant was transferred to fresh tubes, and the Triton-insoluble fractions were resuspended in the same volume of lysis buffer containing protease inhibitors and sonicated three times. An identical amount of Triton-insoluble material was separated on SDS-PAGE.

*Detection of Intracellular H_2O_2 and Superoxide—*Intracellular ROS levels were measured as described previously (27). Cells were washed with PBS and incubated in growth medium containing 10 μM H2-DCFDA or DHE at 37 °C for 30 min in the dark. H_2O_2 oxidizes the H2-DCFDA probe to a green fluorescent DCFDA, whereas superoxide (O_2^-) oxidizes the DHE probe to a red fluorescent hydroethidium. U2OS cells were harvested by trypsinization, washed with PBS, and analyzed by flow cytometric analysis (FACS Calibur™, BD Biosciences).

RESULTS

*ROS-dependent Oligomerization of NS—*Having first observed during purification from Raji cells that NS existed as a
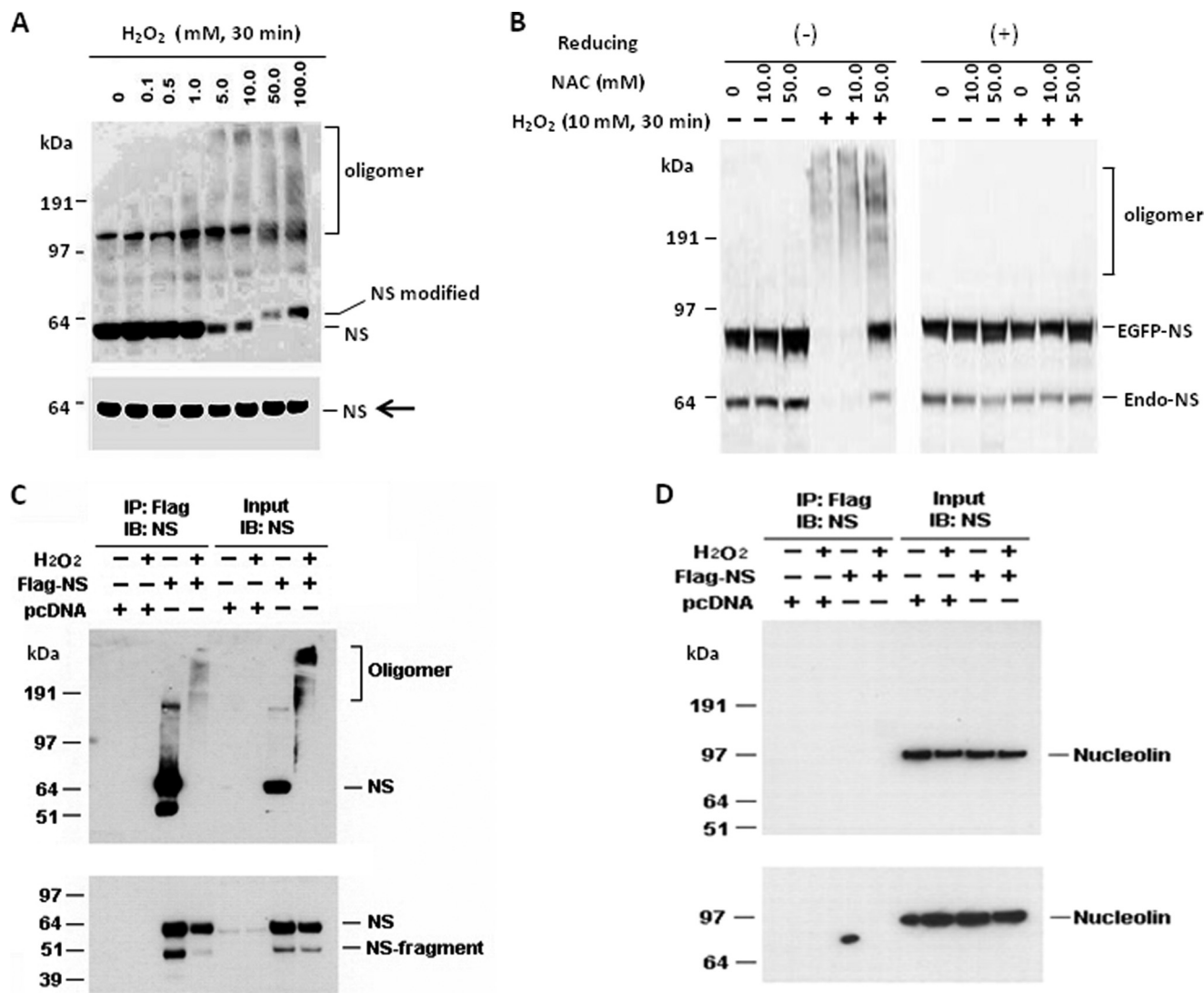
FIGURE 2. **Induction of NS oligomerization by H₂O₂ and reversal by reducing agents.** U2OS cells were exposed to increasing concentrations of H_2O_2 for 30 min. Cell lysates were separated using SDS-PAGE under non-reducing (*A, upper panel*) or reducing (*lower panel*) conditions. U2OS cells expressing EGFP-NS were preincubated with NAC (10 mM or 50 mM) for 16 h and exposed to 10 mM H_2O_2 for 30 min, followed by separation on SDS-PAGE and analysis for NS using goat antibody against full-length NS under non-reducing (*B, left panel*) or reducing conditions (*right panel*). *Endo-NS* represents endogenous NS. *C* and *D*, U2OS cells were transfected with pcDNA (*control*) or a FLAG-NS construct. After 24 h, cells were either untreated or exposed to 10 mM H_2O_2 for 30 min. FLAG-NS was immunoprecipitated (*IP*) from 800 μg of cell lysate, separated using SDS-PAGE under non-reducing (*upper panel*) or reducing (*lower panel*) conditions, and immunoblotted (*IB*) for NS (*C*) or nucleolin (*D*). Input (whole cell lysate) constituted one-twentieth of the total amount of immunoprecipitated protein.

high molecular weight multimer (data not shown), we proceeded to examine NS expression in cell lysates from Raji and HL-60 cells under non-reducing conditions by Western blot analysis. Separation by SDS-PAGE demonstrated a high molecular weight smear of NS in both Raji (Fig. 1*A*) and HL-60 cells (Fig. 1*B*). Incubation of intact cells with NAC diminished, but did not eliminate, the high molecular weight form, whereas boiling of cell extracts with DTT reduced NS to its monomeric and dimeric forms (Fig. 1, *A* and *B*). These observations suggested that NS, which contains nine cysteine residues, exists in these cell lines as higher molecular weight oligomers that are dissociable in the presence of reducing agents, both in cell lysates and in intact cells. DTT-resistant dimerization of NS suggests that mechanisms other than disulfide bond formation may be involved in dimer formation. Dimer formation may be related to the presence of a coiled-coil domain at residues

47–115 of NS (3, 8). Coiled-coil domains have been shown to play a role in the dimerization of other proteins (28, 29).

Raji and HL-60 cells both have amplification of the c-Myc protooncogene (30–32), which has been associated with increased intracellular levels of ROS (19, 32–34). We therefore analyzed the levels of intracellular hydrogen peroxide in both cell lines using the H2-DCFDA assay (27). As shown in Fig. 1*C*, both HL-60 and Raji cells have increased levels of DCFDA fluorescence as compared with U2OS osteosarcoma cells that do not express high levels of c-Myc. In both Raji and HL-60 cells, fluorescence was decreased by incubation of cells with N-acetyl-L-cysteine (Fig. 1, *D* and *E*) or glutathione (data not shown).

To determine whether the induction of ROS could promote the formation of high molecular weight complexes in cells with lower ROS levels, we added increasing concentrations of H_2O_2
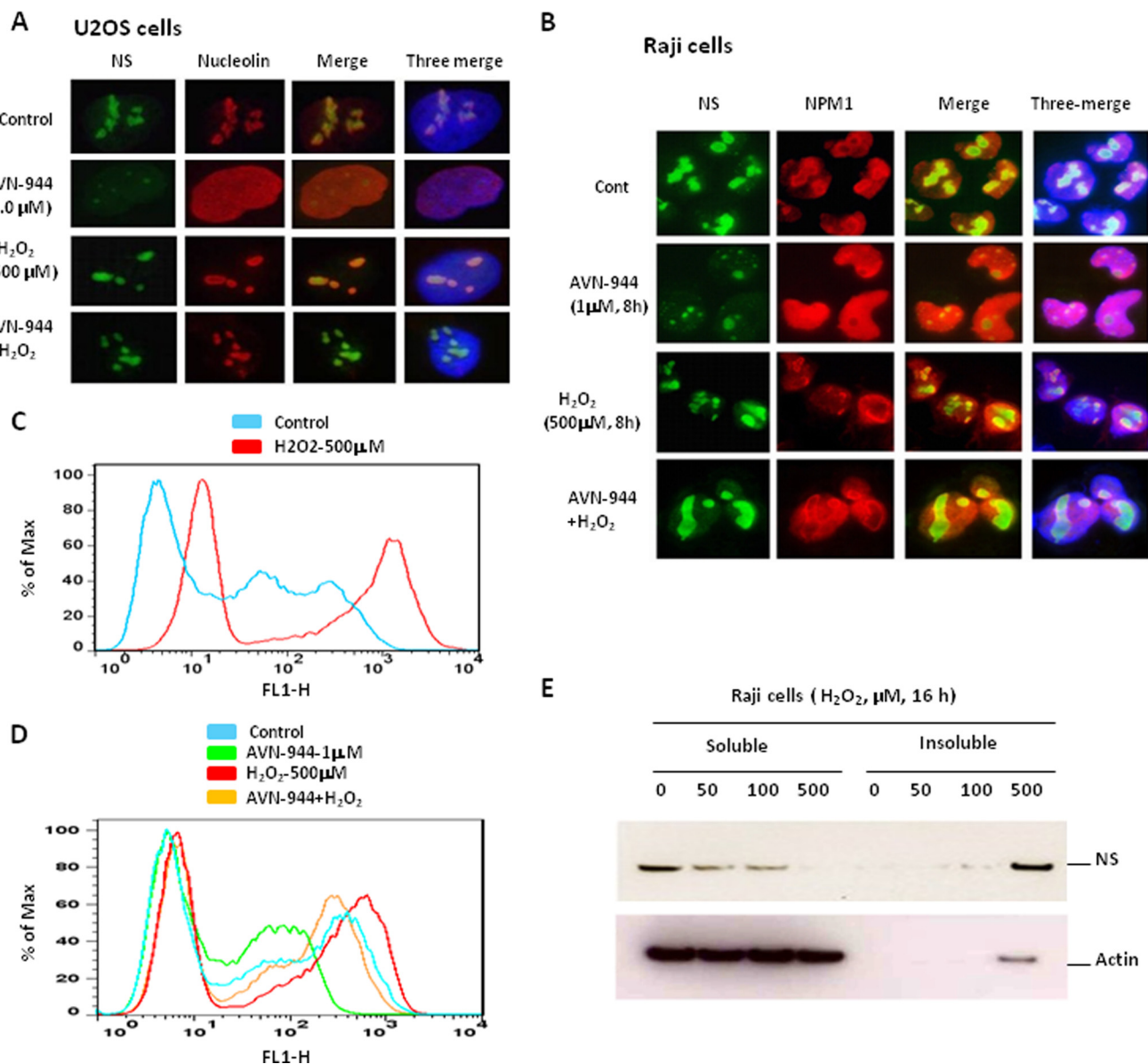
FIGURE 3. **Effect of hydrogen peroxide on NS protein levels, nucleoplasmic translocation, and degradation.** *A* and *B*, U2OS cells were pretreated with 500 μM H_2O_2 for 30 min, 1 μM AVN-944 for 8 h, or both, as indicated. Cells were then permeabilized and stained for nucleostemin and nucleolin. *C*, U2OS cells expressing EGFP-NS were treated with 500 μM H_2O_2 for 5 days and analyzed for EGFP-NS fluorescence by flow cytometric analysis (*FL1-H*). *D*, U2OS cells stably expressing EGFP-NS were untreated or preincubated with 500 μM H_2O_2 for 4 h and then left untreated or exposed to 1 μM AVN-944 for 20 h, followed by flow cytometric analysis of EGFP-NS fluorescence. *E*, Raji cells were exposed to H_2O_2 at concentrations indicated for 16 h. Triton-soluble and Triton-insoluble NS was extracted and blotted for NS.

to U2OS cells in which NS is present predominantly as a monomer. The addition of H_2O_2 resulted in a pronounced shift of NS to higher molecular weight forms and resulted in a new band that ran slightly above the monomeric form at the highest doses of H_2O_2 (Fig. 2*A*). This DTT-reversible band shift may represent one of several reversible oxidative modifications of NS, such as glutathionylation, sulfenic or nitroso modification of cysteines, or intramolecular disulfide bond formation within the molecule. The oligomerization of NS was both dose- and time-dependent. Boiling U2OS cell extracts in the presence of a reducing agent following H_2O_2 treatment reduced NS to the monomeric form (Fig. 2*A*, *lower panel*), and heat denaturation was not required (data not shown). Immunoblotting using goat anti-NS antibody developed against full-length NS (Fig. 2*A*) or

rabbit anti-NS antibody developed against the C-terminal peptide of NS (data not shown) demonstrated similar patterns of oligomerization in response to H_2O_2 exposure. Preincubation of cells with 50 mM NAC to increase intracellular GSH partially decreased complex formation induced by H_2O_2 (Fig. 2*B*).

Immunoprecipitation of FLAG-tagged Nucleostemin—To further confirm the ability of NS to oligomerize, a FLAG-tagged NS expression construct and control pcDNA3 vector were transfected into U2OS cells. NS protein immunoprecipitated using anti-FLAG-agarose beads was eluted with synthetic FLAG peptides and analyzed by Western blotting. In the absence of H_2O_2, NS was eluted predominantly as a monomer with some dimer, but exposure to 10 mM H_2O_2 for 30 min resulted in oligomer formation (Fig. 2*C*, *upper panel*), which
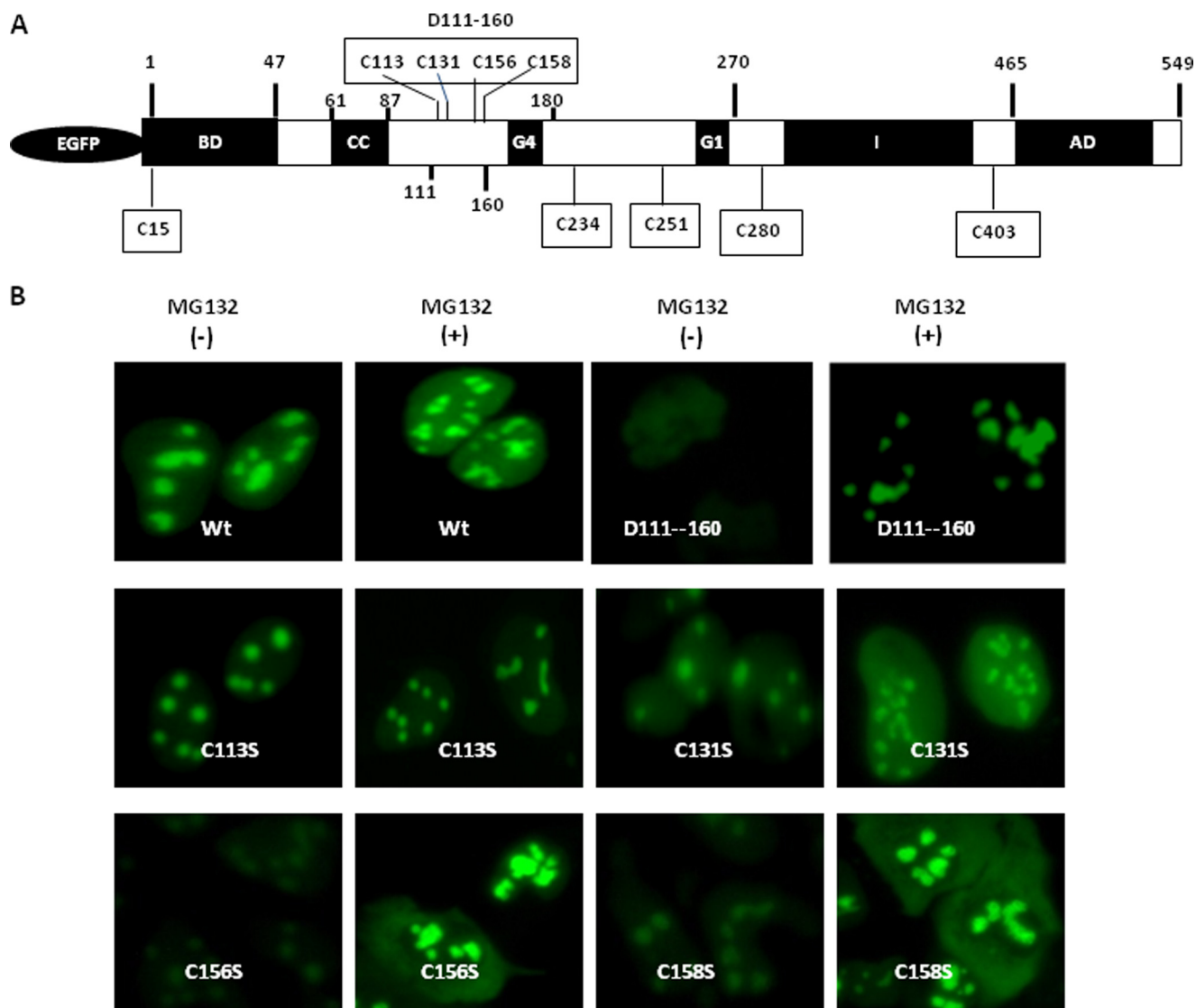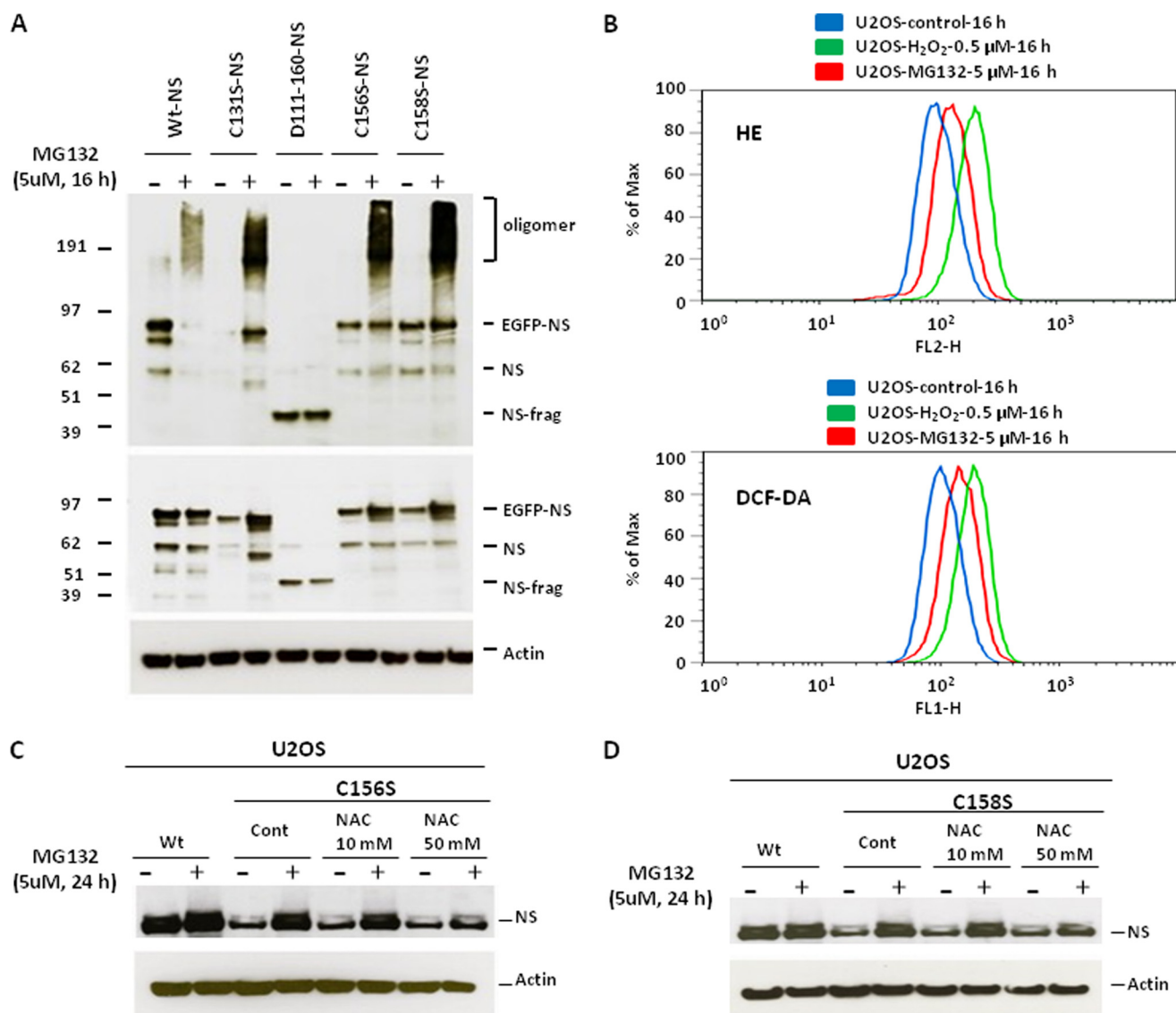
FIGURE 4. **Effects of deletion of a cysteine-rich region (aa 111–160) and individual cysteine mutations on the nucleolar localization and effects of MG132.** *A*, the locations of the nine cysteine residues are shown in *open boxes* and the structural domains of NS in *black boxes*. *BD*, basic domain; *CC*, coiled-coil domain; *G4*, GTP binding site 4; *G1*, GTP binding site 1; *I*, intermediate domain; *AD*, acidic domain. *B*, U2OS cells were transfected with EGFP-tagged wild-type NS or the cysteine or domain deletion mutants indicated. 24 h after transfection, cells were seeded onto coverslips for an additional 12 h and then treated with 5 μM MG132 for 16 h. Cells were washed, fixed, and observed using fluorescent microscopy at a magnification of 100$\times$.

was reduced by DTT (*lower panel*). In contrast, nucleolin, a nucleolar protein used for control, did not immunoprecipitate with NS and was present only as a monomer under non-reducing conditions (Fig. 2*D*).

Effect of H_2O_2 Treatment on NS Localization, Stability, and Solubility—U2OS cells stably expressing EGFP-tagged NS (9) were used to determine whether NS localization and stability were altered by H_2O_2 treatment. This cell line has the advantage of demonstrating excellent nucleolar morphology by immunostaining. Previous studies have shown that depletion of guanine nucleotide levels by the inosine monophosphate dehydrogenase inhibitor AVN-944 causes nucleolar stress and results in NS translocation from the nucleolus to the nucleus with subsequent degradation of the protein (9). As shown in Fig. 3*A*, H_2O_2 treatment prevented the nuclear translocation of NS, which remained within the nucleolus despite AVN-944 treatment. Similar results were obtained with low-dose actino-

mycin D, a well known inducer of nucleolar stress (data not shown). The retention of nucleolin within the nucleolus was concomitantly increased (Fig. 3*A*). We have used EFGP-NS to follow protein stability by flow cytometry in previous studies (9). Treatment of cells with 500 μM H_2O_2 for 5 days markedly impaired degradation of the protein (Fig. 3*C*) and prevented AVN-944-induced degradation (Fig. 3*D*). H_2O_2 treatment of Raji cells also stabilized NS within the nucleolus, and degradation induced by AVN-944 (Fig. 3*B*) and actinomycin D (data not shown) was prevented. Prolonged exposure of Raji cells to H_2O_2 also increased the Triton-insoluble fraction of NS in a dose-dependent manner (Fig. 3*E*), possibly because of irreversible oxidative modifications such as carbonylation. We conclude that, at high levels of ROS, NS forms an insoluble aggregate within the nucleolus and is no longer able to function as a shuttle protein in the face of nucleolar stress.

FIGURE 5. **Effects of deletion of aa 111–160 and of individual cysteine mutations on NS protein levels and oligomerization.** U2OS cells were transfected with EGFP-tagged wild-type NS or the various cysteine mutants shown in Fig. 4A. *A,* 24 h after transfection, cells were exposed to 5 μM MG132 for 16 h. Sixty micrograms of cell extracts were subjected to SDS-PAGE and immunoblotted for NS under non-reducing conditions without heat denaturation (*upper panel*) and NS (*center panel*) or β-actin (*lower panel*) under reducing conditions plus heat denaturation. *B,* U2OS cells were exposed to MG132 or H₂O₂ for 16 h, then incubated with DHE dye or H2-DCFDA dye followed by flow cytometric analysis of oxidized hydroethidium (*HE*) (*upper panel, FL2-H*) or DCFDA fluorescence (*lower panel, FL1-H*). U2OS cells were transfected with EGFP-tagged wild-type NS constructs and C156S-EGFP-NS or C158S-EGFP-NS. 24 h after transfection, cells were pretreated with 10 or 50 mM NAC for 2 h, after which 5 μM MG132 was added for 24 h. Sixty micrograms of cell extracts were separated using SDS-PAGE and immunoblotted for NS and β-actin under reducing conditions with heat denaturation (*C* and *D*).

Role of Individual Cysteines in the Regulation of NS Nucleolar Retention and Stability—Analysis of the amino acid sequence of NS reveals a cysteine-rich region at aa 111–160 that contains four cysteine residues (Cys-113, Cys-131, Cys-156, and Cys-158). To determine whether this region is involved in the regulation of the oxidative modification, nucleolar localization, and/or protein stability of NS, an EGFP-NS construct containing a deletion of aa 111–160 was made. In addition, each of the nine cysteines within EGFP-NS (Fig. 4A) was mutated to serine. Constructs were transfected into U2OS cells, seeded onto coverslips, and treated with 10 mM H₂O₂ for 10 min or 5 μM MG132, a potent proteasome inhibitor, for 16 h. Deletion of aa 111–160 resulted in reduced nucleolar retention of NS (Fig. 4B) and cleavage of NS into smaller fragments (Fig. 5A, *center panel*). MG132 partially increased the nucleolar retention of

EGFP-tagged NS del111–160 (Fig. 4B) but did not prevent its cleavage (Fig. 5A, *center panel*). A similar reduction in nucleolar localization (Fig. 4B) and protein stability (Fig. 5A, *center panel*) was also observed with three of the four individual cysteine mutations (C131S-NS, C156S-NS, and C158S-NS), each of which was also rescued by MG132 (Figs. 4B and 5A, *center panel*). In contrast, the amount of NS monomer and the level of β-actin were unaffected by MG132 treatment (Fig. 5A, *lower panel* and data not shown). Because MG132 also increases intracellular ROS levels (Fig. 5B), we then asked whether increased ROS resulting from this drug caused oligomerization and stabilization of the NS protein. As shown in Fig. 5A (*upper panel*), MG132 treatment increased NS oligomerization under non-reducing, but not reducing, conditions (Fig. 5A, *center panel*). However, NS oligomerization was largely diminished in
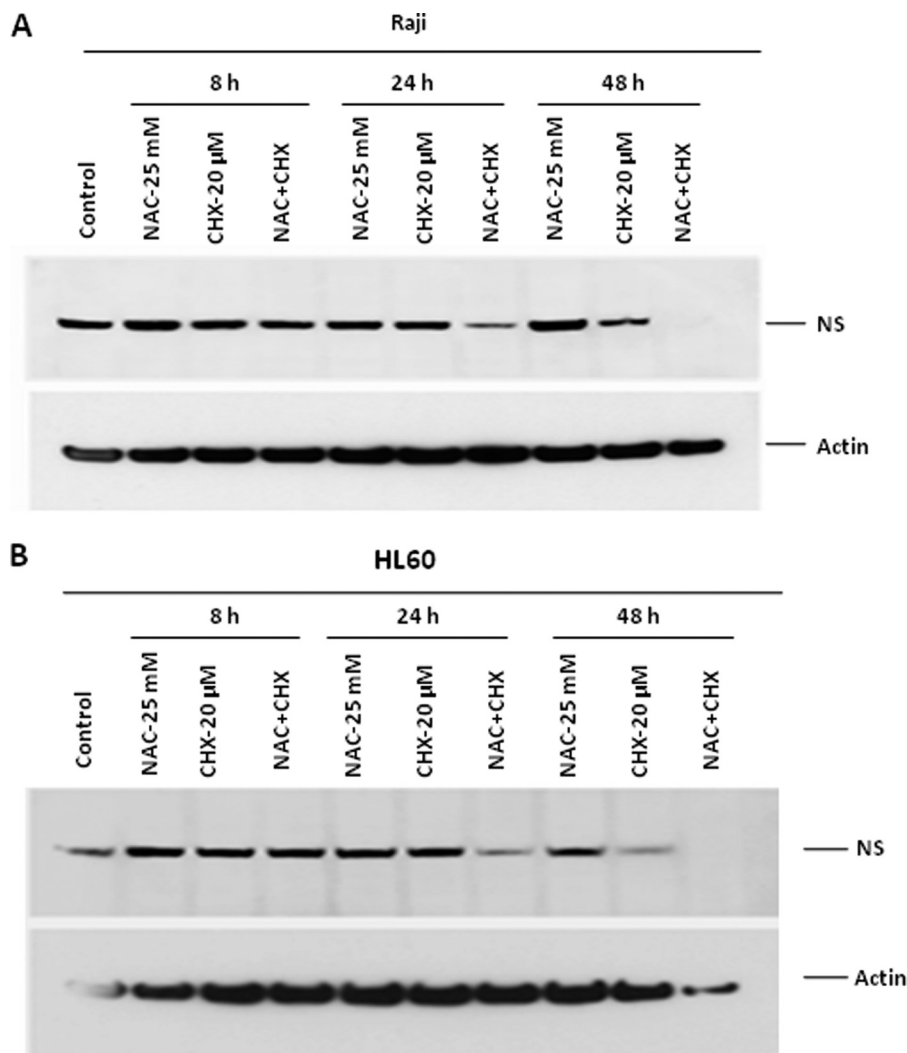
FIGURE 6. **Reversal of MG132 stabilization of NS mutants by NAC in U2OS cells and effect of NAC on endogenous NS levels after cycloheximide treatment in Raji and HL-60 cells.** Raji (*A*) and HL-60 (*B*) cells were untreated or preincubated with 25 mM NAC for 4 h and then treated with 20 μg/ml cycloheximide or vehicle control for 8, 24, and 48 h. Cell lysates were separated on SDS-PAGE and blotted for NS using goat antibody against full-length NS.

the del111–160 mutant. The response to H_2O_2-induced oligomerization and NS protein levels were not altered when any of the remaining six cysteines (Cys-113, Cys-15, Cys-234, Cys-251, Cys-280, and Cys-403) were mutated (Figs. 4*B* and 5*A* and data not shown). These results suggest that Cys-131, Cys-156, and Cys-158 are directly involved in the disulfide bond formation responsible for NS oligomerization. To further determine the role of ROS as generated by proteasome inhibition, U2OS cells were transfected with constructs encoding wild-type or mutated NS and exposed to MG132 in the presence or absence of NAC for 24 h. The results demonstrate that NAC treatment reduced the stabilizing effects of MG132 on NS mutants at cysteines 156 and 158 (Fig. 5, *C* and *D*). Collectively, our data suggest that the stabilizing effect of MG132 on NS cysteine mutants may occur at least partially through the generation of increased levels of ROS.

Effects of NAC on Endogenous NS Protein Turnover in Raji and HL-60 Cells—To further examine the effects of reducing agents on endogenous NS protein turnover, we measured the effect of NAC on protein levels following cycloheximide treatment. As shown in Fig. 6, *A* and *B*, treatment with cyclohexi-

mide alone reduced endogenous NS levels to less than 50% of the control level in both Raji and HL-60 cell lines, suggesting that the NS half-life is between 24 h and 48 h in these cell lines. The addition of NAC to cycloheximide markedly decreased NS levels at both 24 and 48 h, further underscoring the role of ROS in increasing the level of protein expression.

Effects of Oncogene-induced Tumorigenesis on ROS Levels and NS—The conditional expression of human c-Myc in transgenic mice invariably results in hematopoietic tumors with a predominance of T-cell lymphomas (26). We therefore used this animal model to examine the effects of ROS elevation on NS oligomerization and protein stability in murine tumors that result from c-Myc overexpression. Consistent with what was observed in Myc-expressing HL-60 and Raji cell lines, oligomerization of NS was present in tumors isolated from the spleen, thymus, lymph nodes, and bone marrow of these mice (Fig. 7*A*, *upper panel*). NS was reduced to monomeric form after incubation of tumor lysates with DTT and heat denaturation (Fig. 7*A*, *center panel*). Strikingly, a very dramatic increase in NS protein expression was observed in all tumor tissues over that seen in control tissues from non-transgenic mice (Fig. 7*A*,
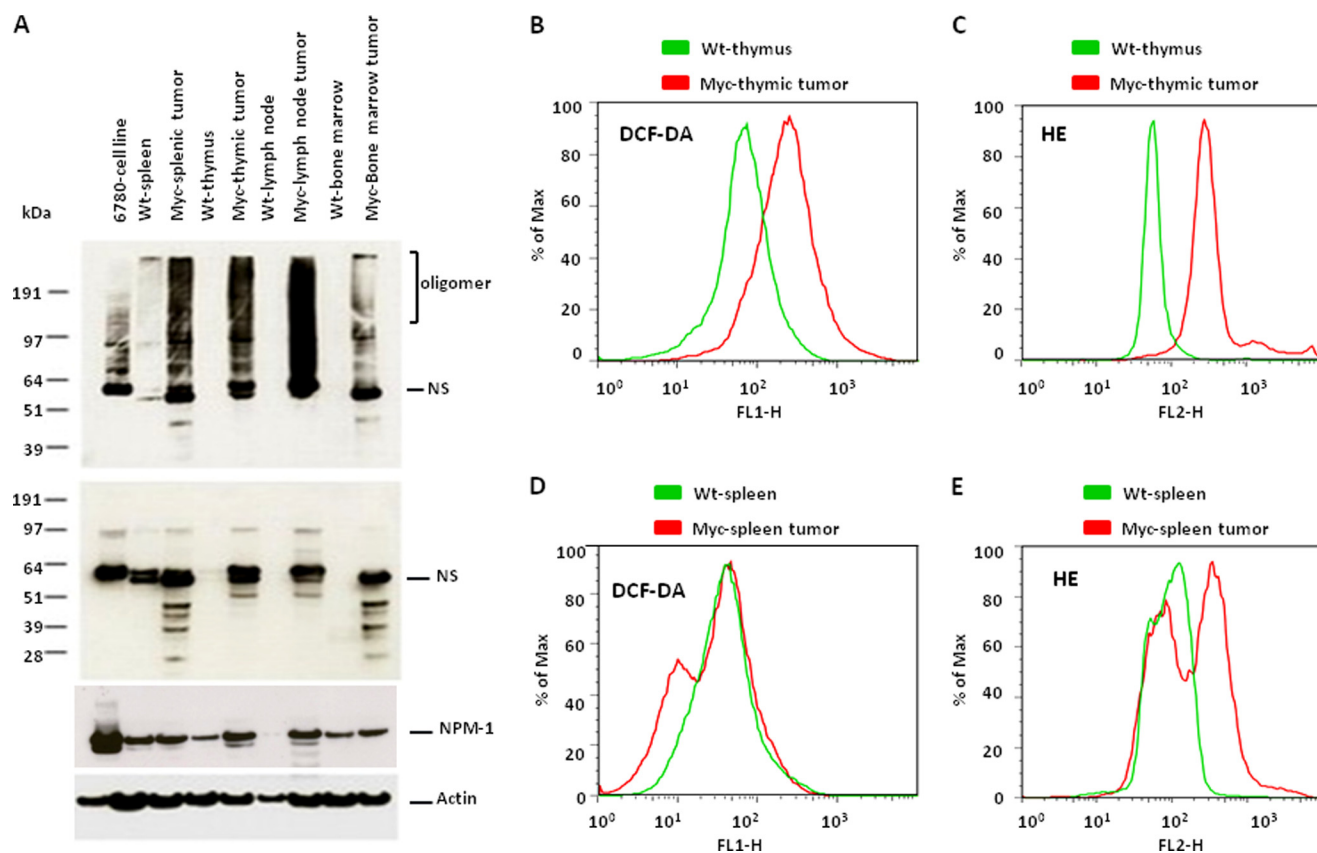
FIGURE 7. **Effect of c-Myc-induced tumorigenesis on NS expression, oligomerization, and intracellular ROS levels in murine cells.** *A*, cell lysates from the c-Myc-expressing lymphoma cell line 6780 and from the spleen, thymus, lymph node, and bone marrow obtained from wild-type FBV/N or c-Myc over-expressing mice were separated by SDS-PAGE under non-reducing (*upper panel*) or reducing (*center panel*) conditions and immunoblotted with anti-NS, anti-NPM-1, or anti-actin antibodies (*lower panels*). *B–E*, cells isolated from thymus and spleen from these mice were exposed to H2-DCFDA (*B and D*) or DHE (*C and E*) dyes followed by flow cytometric analysis (*FL1-H*).

upper and *center panels*). Of note, NS expression in the spleen of the control mice was relatively higher than that in other normal tissues, such as lymph node, thymus, bone marrow (Fig. 7*A*), and liver (data not shown). The increased expression of NS was associated with a number of proteolytic degradation products in the splenic and marrow tumors, despite the use of a mixture of proteolytic inhibitors during extraction. Levels of H_2O_2 and superoxide were determined in tumor and normal cell suspensions using the intracellular dyes H2-DCFDA and DHE, respectively. As shown in Fig. 7, *B* and *C*, cells from the thymic tumor exhibited an increased level of both H_2O_2 (DCFDA fluorescence) and superoxide (hydroethidium fluorescence) when compared with control thymus. Similarly, splenic tumors from the c-Myc-overexpressing mice exhibited a higher level of intracellular superoxide than did a normal spleen, despite a comparable level of H_2O_2 between the two cell types (Fig. 7, *D* and *E*). The dual peaks in the tumor sample most likely reflect an admixture of untransformed and transformed cell types.

Effect of ROS Levels on NS in CD34+ PMBC of Patients with CML-BC—Because increased ROS levels are also associated with BCR-ABL transformation, especially in CML-BC (23), we hypothesized that NS would be present as an oligomer in the CD34+ cells of these patients. Ficoll-purified PBMC from a CML-BC patient were co-stained for CD34 and NS. Markedly enhanced NS staining was evident only in CD34+ CML-BC

and not in the CD34− cells (Fig. 8*A*). We then examined the effect of *in vitro* incubation of PBMC cells from this patient with either NAC or GSH. Incubation of cells with either NAC or GSH for 4 h reduced intracellular H_2O_2 levels in the leukemic cells as measured by DCFDA fluorescence (Fig. 8*B*) and also reduced, but did not eliminate, the oligomerization of NS (*C*). In contrast, neither NAC nor GSH affected NPM-1 pentamers (35) (Fig. 8*D*), confirming a previous report that NPM-1 pentamers are not dependent on disulfide bridge formation (36). NS protein levels were also somewhat reduced after incubation with NAC or GSH (Fig. 6*C*), whereas the expression of NPM-1 and actin was unaffected (Fig. 8, *D* and *E*). Dicoumarol, an activator of 20 S proteasomal degradation (37), did not alter NS levels under these conditions (Fig. 8*C*).

DISCUSSION

Oxidative stress results from an imbalance between excessive ROS production and limited antioxidant defenses (17). Moderate increases in ROS may act to promote cell proliferation, signal transduction, and possibly genomic instability (38, 39), whereas higher levels of ROS can induce extensive oxidation of essential cellular proteins, leading to the accumulation of unfolded or misfolded aggregates and protein inactivation (18, 40, 41). Accumulation of such aggregated proteins is known to play an important role in oxidative damage-mediated cell death (41).
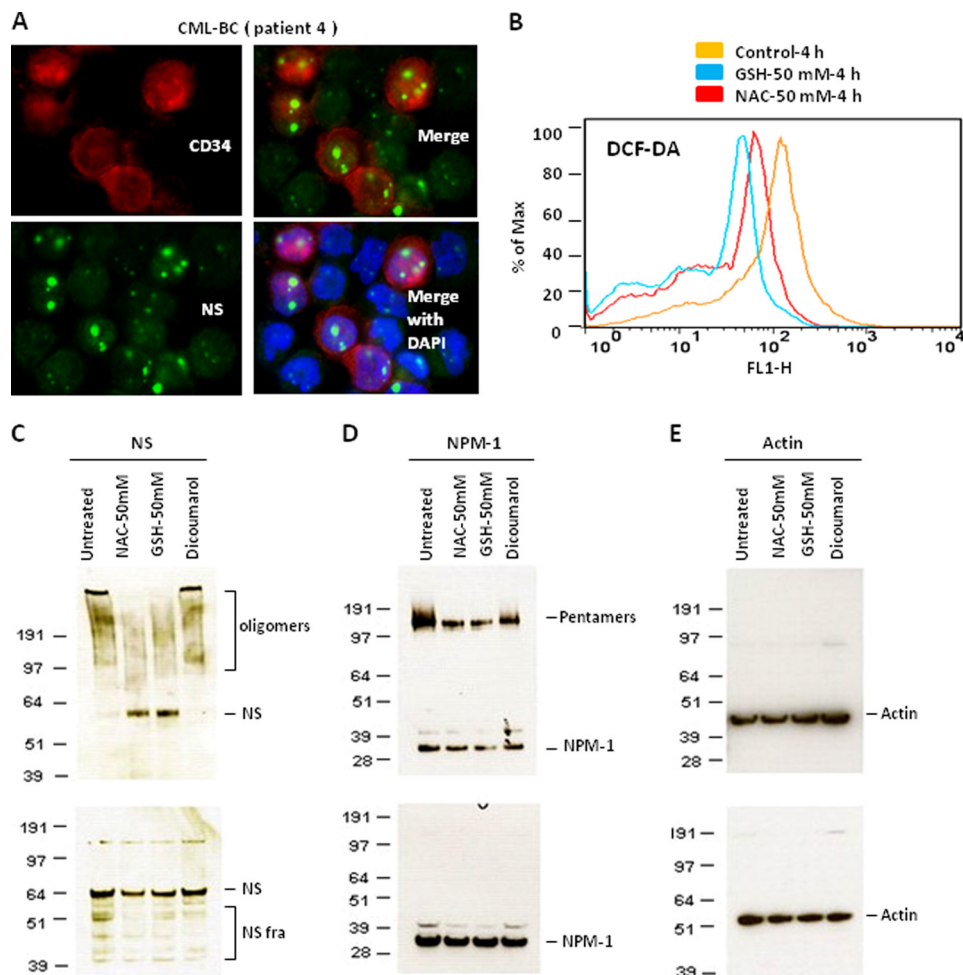
FIGURE 8. **Expression of NS in CD34+ CML-BC cells and reversal of ROS-mediated oligomerization of NS by reducing agents.** 5×10^5 PBMC from one CML-BC patient were co-stained for CD34 and NS (*A*). Cells (5×10^5 PBMC) of CML-BC were incubated in the absence or presence of NAC or GSH for 4 h, as indicated. After washing, cells were then incubated with H2-DCFDA for 30 min and analyzed by flow cytometry (*FL1-H*) (*B*). Cells (5×10^6 PBMC/sample) from the same patient were incubated with NAC, GSH, or 400 μM dicoumarol for 4 h, harvested, and sonicated. Cell lysate was separated by SDS-PAGE under non-reducing (*C*, *upper panel*) or reducing (*lower panel*) conditions and immunoblotted for NS. The blots were reprobed for either NPM-1 or actin (*D* and *E*).

We have demonstrated that NS, which contains nine cysteine residues, forms oligomers in response to oxidative stress. Oxidative modifications of cysteines have been shown to result in changes in protein conformation, activity, localization, stability, or interactions (15, 18, 20, 42–44). Our data have demonstrated that ROS, generated endogenously in transformed cells or induced exogenously by oxidizing agents, result in NS oligomerization, impaired protein degradation and, under more pronounced oxidative conditions, reduced solubility and immobilization of NS in the nucleolus. We also show that inhibition of endogenous ROS by antioxidants such as GSH and NAC abolishes NS oligomerization, indicating that disulfide bond formation is essential to oligomerization.

Elevated levels of NS are present in tumor cells compared with their untransformed counterparts (8). Whereas the transcriptional regulation of NS may contribute to this elevation, in particular as the NS gene is directly regulated by c-Myc (45, 46), the lack of NS degradation now appears to be a major contributing factor. Not only do elevated ROS levels stabilize the protein against degradation induced by GTP depletion, they also enhance basal levels of NS protein expression, as demonstrated by the shorter half-life of NS resulting from NAC exposure. The

precise mechanisms by which ROS modulate NS protein levels remain to be fully explored. We postulate that one potential mechanism involves the regulation of degradation of NS by the ROS-mediated formation of high molecular weight NS oligomers that decrease its proteolysis. Alternatively, ROS levels may directly alter the activity of proteases that are responsible for ongoing NS degradation (47).

The expression of many oncoproteins, including c-Myc, Bcr-Abl, activated Ras, and Flt3, have been shown to result in increased levels of ROS in tumor cells (19–21, 32–34, 48, 49). Thus, oxidative modifications of NS should be prevalent in the setting of oncogenic transformation. To further examine the link between oncogene-generated ROS and NS oligomerization, we turned to a murine lymphoma model resulting from c-Myc overexpression. In this model, Myc-induced lymphomas are associated with a dramatic increase in NS expression and oligomerization compared with that present in normal lymphoid tissues. High levels of expression are also associated with a minor degree of proteolytic degradation. To determine whether similar results are present in primary human tumor cells, we turned to cells from patients with CML-BC. ROS levels were markedly and selectively elevated in CD34+ cells isolated

from a patient with CML-BC and were associated with oligomerization of NS that was reversible with exposure to either NAC or GSH. Overall, these data support the conclusion that NS levels are directly influenced by the levels of endogenous ROS and that transformation by either c-Myc or Bcr-Abl is associated with both high ROS levels and with major increases in NS protein levels.

The cellular localization of NS is another major factor in regulating its function. As a shuttle protein, it is capable of binding both Mdm2 and p53 when released from the nucleolus into the nucleus. With increased oxidation induced by more prolonged exposure to H_2O_2, NS undergoes irreversible oxidation that is associated with immobilization of the protein within the nucleolus and conversion into a detergent-insoluble fraction. Therefore, the extent of NS oxidation and its potential reversibility in any individual cell type is most likely determined by the relative intracellular levels of ROS in relationship to the expression and activity of ROS-scavenging enzymes. One form of irreversible protein modification that results from high levels of oxidative stress is carbonylation. Carbonylated proteins are more susceptible to proteolytic degradation and aggregation than are their non-carbonylated counterparts (50, 51), and significantly higher levels of carbonylated proteins have been found in the plasma of CML-BC patients as compared with plasma from patients with chronic-phase CML (52). Thus, it is conceivable that the progressive increase in ROS levels that occurs in CML with progression to blast crisis may irreparably alter NS function.

Because identification of the specific redox-sensitive cysteine residues within NS could provide insights into the functional consequences of oligomerization, we constructed serial domain deletion mutants that lack one or more cysteine residues. Neither the deletion mutant (del111–160-EGFP-NS) nor the nine individual cysteine mutations completely abrogated the ability of NS to form oligomers in response to H_2O_2, suggesting that more than one cysteine residue is involved. NS contains two known nucleolar-targeting regions, the N-terminal basic domain and the GTP-binding domain (11). We found that deletion of the N-terminal basic domain (aa 1–46) and the GTP binding domain (aa 116–270) led to predominantly nucleoplasmic accumulation of NS (data not shown), consistent with previous reports (5). Deletion of a region containing four cysteines (aa 111–160) decreased both nucleolar localization and protein levels. Within this region, the three cysteine residues located at Cys-131, Cys-156, and Cys-158 appear to be involved in regulating both nucleolar localization and protein expression. We conclude that these cysteine residues are essential for the correct folding of the NS protein. The appearance of a large degradation fragment from the combined deletion of Cys-131 with either Cys-156 or Cys-158 indicates that this cysteine mutant is important in preventing proteolytic degradation (data not shown). In addition, the aa 111–160 region is in proximity to the two major GTP binding sites, G4 (aa 178–182) and G1 (aa 261–268) (53). Thus, it is possible that this cysteine-rich domain may affect the binding of GTP to NS, which is essential for both nucleolar localization and protein stability (5, 9, 11, 53). The nucleolar localization and stability of NS may also be affected by the formation of intermolecular disulfide bonds that

could enhance its ability to interact with a variety of other proteins. These hypotheses will have to be tested by obtaining a crystal structure of NS to further elucidate the positioning of these cysteine residues within the molecule.

Proteasome inhibitors are commonly used as a first approximation to determine whether proteins are subject to ubiquitination and proteolytic degradation by the 26 S proteasome. We previously argued that NS could be a substrate for Mdm2 based on its enhanced stability upon GTP depletion in the presence of MG132 (9), although we had no direct evidence that the protein was ubiquitinated. The reversal of MG132 effects by NAC leads us now to implicate ROS-dependent oxidative modifications of NS as at least a partial explanation for its increased expression and nucleolar retention in the presence of a proteasome inhibitor.

In summary, this study demonstrated that NS is highly responsive to changes in ROS levels that are found in oncogene-transformed cells and that the elevated levels of NS found in these cells result in part from increased protein stability resulting from oxidative modification. Deletion of a cysteine-rich domain and mutation of three cysteine residues within this domain reduced nucleolar retention and protein stability. Further identification of sites or types of oxidative modifications that regulate NS function will provide insights into the structural basis of these changes.

REFERENCES

1. Ma, H., and Pederson, T. (2007) *Mol. Biol. Cell* **18,** 2630–2635
2. Dai, M. S., Sun, X. X., and Lu, H. (2008) *Mol. Cell. Biol.* **28,** 4365–4376
3. Meng, L., Lin, T., and Tsai, R. Y. (2008) *J. Cell Sci.* **121,** 4037–4046
4. Romanova, L., Grand, A., Zhang, L., Rayner, S., Katoku-Kikyo, N., Kellner, S., and Kikyo, N. (2009) *J. Biol. Chem.* **284,** 4968–4977
5. Tsai, R. Y., and McKay, R. D. (2005) *J. Cell Biol.* **168,** 179–184
6. Huang, M., Ji, Y., Itahana, K., Zhang, Y., and Mitchell, B. (2008) *Leuk. Res.* **32,** 131–141
7. Zhu, Q., Yasumoto, H., and Tsai, R. Y. (2006) *Mol. Cell. Biol.* **26,** 9279–9290
8. Tsai, R. Y., and McKay, R. D. (2002) *Genes Dev.* **16,** 2991–3003
9. Huang, M., Itahana, K., Zhang, Y., and Mitchell, B. S. (2009) *Cancer Res.* **69,** 3004–3012
10. Tsai, R. Y., and Meng, L. (2009) *Int. J. Biochem. Cell Biol.* **41,** 2122–2124
11. Meng, L., Yasumoto, H., and Tsai, R. Y. (2006) *J. Cell Sci.* **119,** 5124–5136
12. Romanova, L., Kellner, S., Katoku-Kikyo, N., and Kikyo, N. (2009) *J. Biol. Chem.* **284,** 26685–26694
13. Sijin, L., Ziwei, C., Yajun, L., Meiyu, D., Hongwei, Z., Guofa, H., Siguo, L., Hong, G., Zhihong, Z., Xiaolei, L., Yingyun, W., Yan, X., and Weide, L. (2004) *J. Exp. Clin. Cancer Res.* **23,** 529–538
14. Nikpour, P., Mowla, S. J., Jafarnejad, S. M., Fischer, U., and Schulz, W. A. (2009) *Cell Prolif.* **42,** 762–769
15. Chiarugi, P., and Cirri, P. (2003) *Trends Biochem. Sci.* **28,** 509–514
16. Chandra, J. (2009) *Antioxid. Redox Signal.* **11,** 1123–1137
17. Uttara, B., Singh, A. V., Zamboni, P., and Mahajan, R. T. (2009) *Curr. Neuropharmacol.* **7,** 65–74
18. Gopalakrishna, R., Gundimeda, U., Schiffman, J. E., and McNeill, T. H. (2008) *J. Biol. Chem.* **283,** 14430–14444
19. Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T. K., Hampton, G. M., and Wahl, G. M. (2002) *Mol. Cell* **9,** 1031–1044
20. Rodrigues, M. S., Reddy, M. M., and Sattler, M. (2008) *Antioxid. Redox Signal.* **10,** 1813–1848
21. Sattler, M., Verma, S., Shrikhande, G., Byrne, C. H., Pride, Y. B., Winkler, T., Greenfield, E. A., Salgia, R., and Griffin, J. D. (2000) *J. Biol. Chem.* **275,** 24273–24278
22. Naughton, R., Quiney, C., Turner, S. D., and Cotter, T. G. (2009) *Leukemia*

23, 1432–1440

23. Koptyra, M., Falinski, R., Nowicki, M. O., Stoklosa, T., Majsterek, I., Nieborowska-Skorska, M., Blasiak, J., and Skorski, T. (2006) *Blood* **108,** 319–327

24. Egler, R. A., Fernandes, E., Rothermund, K., Sereika, S., de Souza-Pinto, N., Jaruga, P., Dizdaroglu, M., and Prochownik, E. V. (2005) *Oncogene* **24,** 8038–8050

25. Wu, C. H., van Riggelen, J., Yetil, A., Fan, A. C., Bachireddy, P., and Felsher, D. W. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 13028–13033

26. Felsher, D. W., and Bishop, J. M. (1999) *Mol. Cell* **4,** 199–207

27. van Reyk, D. M., King, N. J., Dinauer, M. C., and Hunt, N. H. (2001) *Free Radic. Biol. Med.* **30,** 82–88

28. Burkhard, P., Stetefeld, J., and Strelkov, S. V. (2001) *Trends Cell Biol.* **11,** 82–88

29. Ciani, B., Bjelic, S., Honnappa, S., Jawhari, H., Jaussi, R., Payapilly, A., Jowitt, T., Steinmetz, M. O., and Kammerer, R. A. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107,** 19850–19855

30. Von Hoff, D. D., Needham-VanDevanter, D. R., Yucel, J., Windle, B. E., and Wahl, G. M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85,** 4804–4808

31. Ben-Yosef, T., Yanuka, O., Halle, D., and Benvenisty, N. (1998) *Oncogene* **17,** 165–171

32. Cerimele, F., Battle, T., Lynch, R., Frank, D. A., Murad, E., Cohen, C., Macaron, N., Sixbey, J., Smith, K., Watnick, R. S., Eliopoulos, A., Shehata, B., and Arbiser, J. L. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 175–179

33. Dang, C. V., Li, F., and Lee, L. A. (2005) *Cell Cycle* **4,** 1465–1466

34. Graves, J. A., Metukuri, M., Scott, D., Rothermund, K., and Prochownik, E. V. (2009) *J. Biol. Chem.* **284,** 6520–6529

35. Okuwaki, M. (2008) *J. Biochem.* **143,** 441–448

36. Herrera, J. E., Correia, J. J., Jones, A. E., and Olson, M. O. (1996) *Biochemistry* **35,** 2668–2673

37. Asher, G., Reuven, N., and Shaul, Y. (2006) *BioEssays* **28,** 844–849

38. Hussain, S. P., Hofseth, L. J., and Harris, C. C. (2003) *Nat. Rev. Cancer* **3,** 276–285

39. Storz, P. (2005) *Front. Biosci.* **10,** 1881–1896

40. Calabrese, V., Guagliano, E., Sapienza, M., Panebianco, M., Calafato, S., Puleo, E., Pennisi, G., Mancuso, C., Butterfield, D. A., and Stella, A. G. (2007) *Neurochem. Res.* **32,** 757–773

41. Esteves, A. R., Arduíno, D. M., Swerdlow, R. H., Oliveira, C. R., and Cardoso, S. M. (2009) *Antioxid. Redox Signal.* **11,** 439–448

42. Savitsky, P. A., and Finkel, T. (2002) *J. Biol. Chem.* **277,** 20535–20540

43. Finkel, T. (2006) *Antioxid. Redox Signal.* **8,** 1857–1863

44. Herrmann, J. M., and Jakob, U. (2008) *Biochim. Biophys. Acta* **1783,** 519

45. Dave, S. S., Fu, K., Wright, G. W., Lam, L. T., Kluin, P., Boerma, E. J., Greiner, T. C., Weisenburger, D. D., Rosenwald, A., Ott, G., Müller-Hermelink, H. K., Gascoyne, R. D., Delabie, J., Rimsza, L. M., Braziel, R. M., Grogan, T. M., Campo, E., Jaffe, E. S., Dave, B. J., Sanger, W., Bast, M., Vose, J. M., Armitage, J. O., Connors, J. M., Smeland, E. B., Kvaloy, S., Holte, H., Fisher, R. I., Miller, T. P., Montserrat, E., Wilson, W. H., Bahl, M., Zhao, H., Yang, L., Powell, J., Simon, R., Chan, W. C., and Staudt, L. M. (2006) *N. Engl. J. Med.* **354,** 2431–2442

46. Wu, C. H., Sahoo, D., Arvanitis, C., Bradon, N., Dill, D. L., and Felsher, D. W. (2008) *PLoS Genet.* **4,** e1000090

47. Xu, Z., Lam, L. S., Lam, L. H., Chau, S. F., Ng, T. B., and Au, S. W. (2008) *FASEB J.* **22,** 127–137

48. Kopnin, P. B., Agapova, L. S., Kopnin, B. P., and Chumakov, P. M. (2007) *Cancer Res.* **67,** 4671–4678

49. Sallmyr, A., Fan, J., Datta, K., Kim, K. T., Grosu, D., Shapiro, P., Small, D., and Rassool, F. (2008) *Blood* **111,** 3173–3182

50. Nyström, T. (2005) *EMBO J.* **24,** 1311–1317

51. Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R., and Milzani, A. (2006) *J. Cell. Mol. Med.* **10,** 389–406

52. Singh, R. K., Tripathi, A. K., Tripathi, P., Singh, S., Singh, R., and Ahmad, R. (2009) *Hematol. Oncol. Stem Cell Ther.* **2,** 285–288

53. Meng, L., Zhu, Q., and Tsai, R. Y. (2007) *Mol. Cell. Biol.* **27,** 8670–8682