NAD(P)H:quinone Oxidoreductase 1 (NQO1) Competes with 20S Proteasome for Binding with C/EBPα Leading to Its Stabilization and Protection against Radiation-induced Myeloproliferative Disease

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Background: NQO1 protects against myeloproliferative diseases.

Results: Radiation-induced NQO1 competes with 20S proteasome for binding with myeloid differentiation factor C/EBPα leading to stabilization of C/EBPα and protection against myeloproliferative diseases.

Conclusion: NQO1 is an endogenous factor in stabilization of C/EBPα and protection against myeloproliferative diseases during radiation and chemical stress.

Significance: NQO1 might serve as a therapeutic target for prevention of hematological diseases.

This article has been retracted by the publisher. An investigation at the University of Maryland, Baltimore determined that the data shown in Fig. 2A are unreliable and do not support the hypothesis of this work.

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a flavoprotein that protects cells against oxidative stress. Disruption of NQO1 increased susceptibility to radiation stress, respectively (3–4). C/EBPα is a leucine zipper factor that protects cells against radiation and chemical-induced oxidative stress. Disruption of the NQO1 gene in mice leads to increased susceptibility to radiation and chemical stress. Disruption of NQO1 gene in mice leads to increased susceptibility to radiation and chemical stress. Disruption of NQO1 gene in mice leads to increased susceptibility to radiation and chemical stress.

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NQO1 is an endogenous factor in stabilization of C/EBPα and protection against myeloproliferative diseases during radiation and chemical stress. This protein that protects cells against radiation and chemical-induced oxidative stress. Disruption of NQO1 gene in mice leads to increased susceptibility to radiation and chemical stress.

The NAD(P)H:quinone oxidoreductase 1 (NQO1) gene is located on chromosome 16q22. It encodes a flavoprotein that is involved in the detoxification of electrophilic and oxidizing metabolites. It is also involved in the defense against oxidative stress. The NQO1 gene is expressed in various cell types, including hematopoietic cells, where it plays a role in protecting against radiation and chemical-induced oxidative stress.

Mutations in the NQO1 gene have been associated with an increased risk of hematological diseases, including myeloproliferative disorders and acute myeloid leukemia. A more recent study reported that NQO1 deletion in murine hematopoietic cells led to increased susceptibility to radiation-induced myeloproliferative diseases.

A cytokine to thymidine (C→T) polymorphism in exon 6 of human NQO1 gene on chromosome 16q22 produces a proline to serine (P187S) substitution that destabilizes the enzyme which is degraded by proteasomes (15–16). This mutation is also associated with increased susceptibility to radiation-induced myeloproliferative diseases.

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to γ-radiation resulted in myeloproliferative disease in NQO1−/− mice (26). NQO1−/− mice exposed to γ-radiation demonstrated bone marrow hypercellularity and enlarged lymph nodes and spleen. The spleen showed disrupted follicular structure, loss of red pulp, and granulocyte and megakaryocyte invasion. The above studies demonstrated a role of NQO1 in protection against myeloproliferative diseases including leukemia and raised questions regarding the mechanism of this protection.

In this report, we demonstrate that NQO1 protects myeloid differentiation factor C/EBPs against 20S proteasomal degradation. This leads to stabilization of C/EBPs and protection against myeloproliferative diseases including leukemia. NQO1 achieves this function by competing with 20S proteasomes for binding with C/EBPs. Further studies identified the C/EBPs and NQO1-interacting domains and determined the mechanism of protection.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified 20S, Flag, and mouse NQO1 antibodies, Easyview Red Anti-Flag M2 affinity gel, and MG132 were obtained from Sigma (St. Louis, MO). Human NQO1 and C/EBPα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PU.1 antibody was from cell signaling technology (Santa Cruz, CA). PU.1 antibody was from cell signaling technology (Santa Cruz, CA). The specificities of antibodies are reported on the company web sites. In addition, the specificities of antibodies used in the present report have also been used in our previous publications (25–29). Nucleofector II and reagents were from Lonza (Cologne, Germany).

**Mice, Cells, and Cell Culture**—C57BL/6 WT and NQO1−/− mice were generated as reported previously (27). Human myelogenous leukemia HL-60 and human CD34-positive KG-1 cells purchased from ATCC (Manassas, VA), were cultured in RPMI/10% FBS/1% antibiotics. Bone marrow cells were collected from the mice femurs and suspended in RPMI/10% FBS.

**Construction of Plasmids**—All truncated constructs of mouse NQO1 cDNA were engineered by PCR followed by digestion with XbaI and Xhol and subsequently cloning into pCMX vector containing double Flag-tag. Site directed mutations of mouse C/EBPα cDNA were generated by first performing two PCRs to amplify the 5′-fragment of mouse C/EBPα cDNA (carrying EcoRI site) and the rest 3′-fragment of mouse C/EBPα cDNA (with site mutations and carrying Xhol site), after blunt-end ligation, the fragments with size of about 1080 base pairs were digested with EcoRI and Xhol and subsequently cloned into pCMX vector. This strategy was applied since C/EBPα is highly GC rich (72%) and full-length PCR was unsuccessful to amplify the DNA.

Flag-C/EBPα (1–162) and Flag-C/EBPα (1–279) were produced by PCR using the same forward primer 5′-TCTTTCTTGAAATGGATCTGGCGACTTCTC-3′ and reverse primers 5′-ACCTGGGATCCGATTGTGATCAACAGACC-3′ for construct 1–162 and 5′-ACCTGGGATCCGATTGTGATCAACAGACC-3′ for construct 1–279, respectively. Flag-C/EBPα (157–359), Flag-C/EBPα (189–359), Flag-C/EBPα (208–359), Flag-C/EBPα (235–359), Flag-C/EBPα (268–359), and Flag-C/EBPα(279–359) were produced by PCR all using the same reverse primer 5′-CTTACTCGAGTCAGCAGCATGTCGCTGCT-3′ and forward primers 5′-ACCTGGGATCCGATTGTGATCAACAGACC-3′ for construct 157–359; 5′-ATTATTGAATTCGACGAGTCTCCTCCGGC-3′ for construct 189–359; 5′-ATTATTGAATTCGACGAGTCTCCTCCGGC-3′ for construct 208–359; 5′-ATTATTGAATTCGACGAGTCTCCTCCGGC-3′ for construct 235–359; 5′-ATTATTGAATTCGACGAGTCTCCTCCGGC-3′ for construct 268–359; and 5′-ACCTGGGATCCGACGGCTGCGTGGCCG-3′ for construct 279–359, respectively.

Truncated constructs of mouse NQO1 cDNA were engineered by PCR followed by digestion. Mouse NQO1(1–140)–V5 was produced by PCR using the forward primer 5′-GCCAC- TGATCGCAGAAATATATCTCTATTGAAT-3′ and the reverse primer 5′-GCCAC-TGATCGCAGAAATATATCTCTATTGAAT-3′; mNQO1(141–279)–V5 was produced by PCR using the forward primer 5′-GCCAC-TGATCGCAGAAATATATCTCTATTGAAT-3′ and the reverse primer 5′-GCCAC-TGATCGCAGAAATATATCTCTATTGAAT-3′ and the reverse primer 5′-GCCAC-TGATCGCAGAAATATATCTCTATTGAAT-3′. NQO1-Y127/129A-V5, NQO1P187S-V5, and other V5-tagged proteins were generated in our laboratory as reported previously (28).

**Cell Lysate Preparations**—Cells at 90% confluence or from bone marrow were lysed in ice-cold lysis buffer (150 mmol/liter NaCl, 25 mmol/liter Tris (pH7.4), 1 mmol/liter EDTA, 2 mmol/liter phenylmethylsulfonyl fluoride, and 1% perfluorooctanoic acid). After 30 min incubation at 4 °C followed by brief sonication, the lysates were cleared by centrifugation at 16,000 × g for 30 min.

**γ-Radiation Treatment**—Mice and cells were either non-irradiated or irradiated with 1, 3, or 6 Grays of γ-radiation, waited for 12, 24, and 48 h and analyzed. However, 3 Grays and 48 h gave the best results are described. Mice were non-irradiated or irradiated with 3 Grays γ-radiation (Mark I Irradiator, cesium-137, J.L. Shepherd and Associates). Forty-eight hours later, femurs were removed and bone marrow flushed out with cold PBS. The cells were centrifuged and lysed with cold buffer. For ex vivo analysis, mice were sacrificed, and their bone marrow cells were obtained in RPMI/10% FBS. Cells were irradiated with 3 Grays γ-radiation and cultured in 12-well plates for 48 h for further analysis.

**Transfection**—Transfection of HL-60 cells with plasmid DNA and U937 or KG-1 cells with siRNA were performed using Amaxa transfection device, Lonza Cell Line Optimization Nucleofector Kit and manufacturer’s instructions.

**Immunoprecipitation**—Cells were lysed with buffer (150 mmol/liter NaCl, 25 mmol/liter Tris, pH 7.4, 1 mmol/liter EDTA, and 1% perfluorooctanoic acid) with protease inhibitor mixture (Roche). 1 mg lysates were precleared with washed protein A/G-agarose beads for 1 h at 4 °C, followed by bead
removal and immunoprecipitation overnight with 20S core subunit antibody (1:100), V5 antibody (1:1000), or antibodies against NQO1, C/EBPα, Pu.1 and unrelated protein Nrf2. The next day, fresh A/G-agarose beads were added and rotated for 4 h to bind antibodies, then, the samples were washed twice in lysis buffer, boiled for 5 min in 2× SDS loading buffer, and analyzed by SDS-PAGE. Immunoprecipitation of Flag-tagged proteins was done by Easyview Red anti-Flag M2 affinity gel according to manufacturer’s protocol.

Ubiquitination Assay—HL-60 cells were co-transfected with 0.5 μg of HA-Ub and 1 μg of wild type C/EBPα-V5 or mutant C/EBPαK276A-V5 or C/EBPαK277A-V5 or ubiquitination inactive Bcl2K17A-V5. The cells were lysed 24 h later, and 1 mg of cell lysates were immunoprecipitated with anti-V5 antibody. After SDS-PAGE and Western blotting, the PVDF membrane was probed with anti-HA antibody.

In Vitro Translation and 20S Proteasomal Degradation of Translated Protein—1 μg V5-tagged NQO1 or NQO1 mutants or C/EBPα plasmid DNAs were translated and 2 μl from each product were incubated together with purified 20S proteasome in degradation buffer (100 mmol/liter Tris-Cl (pH 7.5), containing 150 mmol/liter NaCl, 5 mmol/liter MgCl₂, and 2 mmol/liter DTT) for various time points and quenched via freezing at −80 °C. Samples were separated on 12% SDS-PAGE and immunoblotted. In similar experiments, in vitro translated NQO1 without or with NADH and dicoumarol were included and degradation of C/EBPα experiments repeated. In related experiments, in vitro translated C/EBPα was incubated with increasing concentration of in vitro translated purified 20S proteasome in varying amount of purified 20S proteasome that directly competes with 20S for the degradation of C/EBPα.

Real-time PCR—RNA was isolated from bone marrow cells with or without γ-radiation, as described with increasing concentration of C/EBPα-V5 plasmid or NQO1 siRNA using RNeasy Mini kit (Qiagen). RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. cDNA was used with Taqman Master Mix and NQO1 (ID: Mm01253561-m1), C/EBPα (ID: Mm00514283-S1), PU.1 (ID: Mm00488428-m1), or control GusB (ID: Mm00446953-m1) Primer and Probe. Final mixture was run on 7500 Real Time System (Applied Biosystems) using relative quantitation according to the manufacturer’s instructions.

Western Blot Analysis—Cell lysates were clarified by centrifugation at 10,000 × g. SDS-PAGE analysis was performed as previously described (25–29).

Statistical Analysis—The statistical analyses (student t test and ANOVA) were done by Microsoft Excel and Graphpad Prism. Differences were considered significant at p < 0.05.

RESULTS

NQO1 Protects C/EBPα against 20S Proteasomal Degradation—Wild type (WT) and NQO1−/− mice were non-irradiated or irradiated with 3 Grays γ-radiation, bone marrow collected, and immunoblotted (Fig. 1A). Bone marrow cells from non-irradiated NQO1−/− mice showed higher levels of C/EBPα (1.5 times) and PU.1 (2 times) as compared with WT mice. This suggested that increased C/EBPα and PU.1 were responsible for myeloid hyperplasia in NQO1−/− mice as reported earlier (25). Interestingly, exposure to 3 Grays of γ-radiation increased NQO1, C/EBPα and PU.1 to varying levels in WT mice but failed to do so in NQO1−/− mice bone marrow. These results indicated that NQO1−/− mice were incapable of inducing myeloid differentiation factors C/EBPα and PU.1 upon exposure to γ-radiation, as observed with WT mice.

It is noteworthy that non-irradiated NQO1−/− mice expressed higher C/EBPα and Pu.1, as compared with WT mice. Real time PCR analysis showed that this is due to increased transcription of C/EBPα in NQO1−/− mice, as compared with wild type mice (supplemental Fig. S1). This might have been to compensate the loss of C/EBPα in NQO1−/− mice.

Next, we performed ex vivo bone marrow studies to investigate the role of protein degradation and/or transcription in lack of induction of C/EBPα and PU.1 in response to γ-radiation. Bone marrow cells from WT and NQO1−/− mice were exposed to 3 Grays γ-radiation in absence and presence of proteasome inhibitor MG132 or epoxomycin and analyzed for p53, C/EBPα, Pu.1, NQO1, and actin. p53 was included as control of MG132-mediated down-regulation of proteasomes as reported earlier (Ref. 29 and supplemental materials). MG132 treatment protected C/EBPα from proteasomal degradation (Fig. 1B, right panels). This was evident from increased C/EBPα in bone marrow cells from WT and NQO1−/− mice after γ-radiation. Similar results were observed with proteasome inhibitor epoxomycin (Fig. 1B, left panels). The results together suggested that NQO1 is responsible for the stabilization and induction of C/EBPα in response to γ-radiation. Pu.1 showed similar alterations as C/EBPα because C/EBPα is known to regulate Pu.1 transcription (14). In related experiments, RNA was analyzed to determine the contribution of transcription in down-regulation of Pu.1. The results revealed that exposure to γ-radiation significantly down-regulated Pu.1 gene transcripts in NQO1−/− mice (supplemental Fig. S1). This was expected since C/EBPα is known to regulate Pu.1 transcription (14). In the same experiment, bone marrow cells from WT mice showed up-regulation of C/EBPα and Pu.1 in response to radiation exposure. Interestingly, the C/EBPα RNA was also down regulated in response to radiation exposure in bone marrow cells from NQO1−/− mice. This was also expected since C/EBPα is known to auto-regulate its own transcription (30–31). These results indicated that radiation-mediated degradation of C/EBPα in NQO1−/− mice led to decreased transcription of C/EBPα that contributed to down-regulation of C/EBPα protein. In related experiments, MG132 was used to determine if it has an effect on C/EBPα and Pu.1 transcription. The results showed that MG132 had no effect on C/EBPα and Pu.1 transcript (supplemental Fig. S2). In conclusion, the above results together reveal that radiation-induced instability/degradation of C/EBPα in the absence of NQO1 is a major mechanism leading to down-regulation of C/EBPα in NQO1−/− mice bone marrow.

Next, HL-60 and U937 cells were immunoblotted to compare NQO1 levels (Fig. 1C). The results showed that U937 cells express 2-fold higher levels of NQO1 as compared with HL-60 cells. HL-60 cells transfected with NQO1-V5 (Fig. 1D) and
U937 cells transfected with NQO1 siRNA (Fig. 1E) were analyzed for alterations in NQO1 and its effect on C/EBPα/H9251 and PU.1. The results revealed that overexpression of NQO1 in HL-60 cells led to up-regulation of both C/EBPα/H9251 and PU.1 proteins (Fig. 1D). In the same experiment, the over-expression of V5-His-tagged random peptide control failed to affect levels of C/EBPα and PU.1 indicating that NQO1 effect on C/EBPα/H9251 and PU.1 was specific (Fig. 1D). In addition, siRNA-mediated inhibition of NQO1 in U937 cells led to down-regulation of both C/EBPα/H9251 and PU.1 proteins (Fig. 1E). The HL60 cells overexpressing NQO1 in Fig. 1D and U937 cells expressing inhibited NQO1 were also analyzed for NQO1, C/EBPα, and PU.1 RNA to determine the contribution of transcription (supplemental Fig. S3, left and right panels). The results demonstrated NQO1 plasmid dose-dependent increase in NQO1 RNA, no change in C/EBPα RNA (C/EBPα increase in Fig. 1D is due to stabilization of C/EBPα), and dose-dependent increase in PU.1 (because of C/EBPα stabilization and transcriptional activation of Pu.1).

Similarly, the results with NQO1 siRNA demonstrated dose-dependent decrease in NQO1 RNA, no change in C/EBPα RNA (C/EBPα decrease in Fig. 1E is due to degradation of C/EBPα), and dose-dependent decrease in PU.1 (because of C/EBPα degradation and decreased transcription of Pu.1). Therefore, the results together demonstrate that increase in C/EBPα in NQO1-overexpressing HL60 cells in Fig. 1D is due to NQO1 stabilization of C/EBPα protein. Similarly, decrease in C/EBPα
**NQO1 Competition with 20S Proteasome for C/EBPα Binding**

in U937 cells expressing siRNA-mediated inhibition of NQO1 is due to degradation of C/EBPα protein.

NQO1 is known to stabilize p53 against 20S proteasome-mediated ubiquitination-independent degradation (5, 29). Therefore, we performed *in vitro* assays to investigate the role of 20S proteasome in C/EBPα degradation and NQO1 protection against 20S degradation of C/EBPα. Incubation of *in vitro* translated C/EBPα with purified 20S showed significant degradation of C/EBPα within 1 h (Fig. 1F). Inclusion of *in vitro* translated NQO1 partially protected C/EBPα. Interestingly, NQO1 in combination with its cofactor NADH in the same experiment provided complete protection of C/EBPα against 20S proteasomal degradation (Fig. 1F). The results collectively indicated that NQO1 stabilizes C/EBPα against 20S proteasomal degradation. We also used *in vitro* translated NQO1-V5 and Flag-C/EBPα proteins and purified 20S proteasome to investigate if NQO1 competed with 20S for C/EBPα leading to stabilization of C/EBPα against 20S degradation. The results are shown in [supplemental Fig. S4](#). The results reveal that NQO1 directly competed with 20S proteasome for C/EBPα protein. Increase in NQO1 led to NQO1 concentration-dependent increase in NQO1:C/EBPα interaction and decrease in 20S:C/EBPα interaction (supplemental Fig. S4).

The studies were extended to CD34 positive human myeloid KG-1 cells that upon transfection with NQO1 siRNA demonstrated siRNA concentration dependent decrease in NQO1, C/EBPα and PU.1 (Fig. 1G, *left panels*). In related experiments, NQO1 siRNA-mediated inhibition of NQO1 upon exposure to 3 Grays of γ-radiation also resulted in significant decrease in C/EBPα and PU.1 (Fig. 1G, *right panels*). The results revealed that NQO1 also protected C/EBPα downstream PU.1 in human myeloid KG-1 cells.

WT and NQO1−/− mice were exposed to 3 Grays of γ-radiation and NQO1−/− bone marrow granulocytes were isolated (supplemental Fig. S6). Bone marrow granulocytes were also isolated from Gr-1-positive mature granulocytes of bone marrow granulocytes in bone marrow (supplemental Fig. S6). Bone marrow granulocytes revealed radiation-mediated 7-fold decrease in granulocytes in NQO1−/− mice as compared with only a 2.2-fold decrease in WT mice (supplemental Fig. S5). In the same experiment, apoptosis in granulocytes was increased by only 1.6-fold in NQO1−/− compared with 2-fold in WT mice (supplemental Fig. S5). These results clearly suggested that differentiation of granulocytes in NQO1−/− mice was significantly decreased as compared with WT mice upon exposure to γ-radiation. Blood CBCs analysis also showed only 3.5-fold increase in neutrophils in NQO1−/− mice exposed to radiation as compared with 6.5-fold increase in WT mice, thus supporting the above observations (supplemental Fig. S6). Collectively, these results demonstrated that absence of NQO1 upon exposure to γ-radiation led to loss of induction of C/EBPα and Pu.1 and decreased granulocyte differentiation.

**NQO1 and 20S Both Interact with C/EBPα but Not with PU.1** — Immunoprecipitation assays were performed to investigate the role of NQO1 and 20S interaction with C/EBPα in inhibition of 20S degradation of C/EBPα. Bone marrow from WT and NQO1−/− mice, HL-60 cells and HL-60 cells transected with NQO1-V5, Flag-C/EBPα and an unrelated Nrf2 plasmids were analyzed for interactions among C/EBPα, 20S, and NQO1 (Fig. 2A). The transected HL-60 cells were also immunoprecipitated with anti-Nrf2 antibody as negative control of immunoprecipitation experiments. The results showed that all three (C/EBPα, 20S, and NQO1) proteins interacted with each other in WT mice (*left panels*), HL-60 (*middle panels*), and transected HL-60 cells (*right panels*). The results further showed that C/EBPα interacted with 20S in absence of NQO1 in NQO1−/− mice bone marrow. It is noteworthy that unrelated Nrf2 protein as expected failed to interact with NQO1, C/EBPα or 20S (Fig. 2A, *right panels*). This indicated that interactions among C/EBPα, NQO1, and 20S were specific. To further define the interactions, we used *in vitro* translated Flag-C/EBPα and NQO1-V5 and purified 20S proteins and performed immunoprecipitation assays to investigate the mutual interaction between two of the three proteins in the absence of the third one. Results indicated that any two of the three proteins interacted with each other independent of the third one (Fig. 2B).

These results collectively demonstrated that NQO1 and 20S both interact with C/EBPα besides NQO1 interaction with 20S. The results also showed that interactions of two of the three proteins are independent of the third one. In similar experiments, we investigated C/EBPα, immunoprecipitation assays failed to show PU.1 interaction with either NQO1 or 20S (Fig. 2B). Immunoprecipitation assays confirmed that NQO1 controls C/EBPα and 20S proteasome mediated transcription of PU.1.

**Interaction between Amino Acids S268 and S279 of C/EBPα** — *In vitro* competition experiments with C/EBPα A–D (Fig. 3A) showed that amino acids 268 and 279 are involved in interaction with both NQO1 and 20S (supplemental Fig. S7). The results also showed that interactions of two of the three proteins are independent of the third one. In similar experiments, we investigated C/EBPα, immunoprecipitation assays failed to show PU.1 interaction with either NQO1 or 20S (Fig. 2B). Immunoprecipitation assays confirmed that NQO1 controls C/EBPα and 20S proteasome mediated transcription of PU.1.

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NQO1 Competition with 20S Proteasome for C/EBPα Binding

**FIGURE 2. NQO1 and 20S both interact with C/EBPα but not PU.1.** A, NQO1 interacts with both NQO1 and 20S, which were pulled down with wild-type C/EBPα. Co-immunoprecipitation of Flag-C/EBPα and 20S were successfully immunoprecipitated (Fig. 3A, middle panel). All three peptides competed with C/EBPα (Fig. 3B, right panel). Based on these results, we generated double mutant C/EBPα K276A/K277A that also failed to interact with NQO1 (Fig. 4B, left and middle panels). These results suggested that K276/K277 of C/EBPα is required for its interaction with NQO1. Interestingly, all four C/EBPα mutants K274A, K276A, K277A, and double mutant K276A/K277A interacted with 20S in a similar capacity as normal C/EBPα (Fig. 4B, right panels). We also generated G273A and S278A that showed no effect on C/EBPα interaction with NQO1 or 20S (Fig. 4B). These results indicated that mutations in individual amino acid between S268 and V279 had no effect on C/EBPα interaction with 20S, suggesting the requirement of all of the amino acids in 268–279 domain for interaction of C/EBPα with 20S.

Histidine pull-down assays showed the same results as observed in immunoprecipitation assays (Fig. 4C). Both NQO1 and 20S were pulled down with full-length C/EBPα-V5-His6 (Fig. 4C, right panels). However, C/EBPαΔ268–279-V5-His6 deletion mutant failed to pull down either NQO1 or 20S (Fig. 4C, right panels). In related pull-down assays, 20S but not NQO1 was pulled down with both mutants C/EBPαK276A-V5-His6 and C/EBPαK277A-V5-His6 (Fig. 4C, right panels). Ubiquitination assays showed that C/EBPα full-length and

Protein containing internal deletion of amino acids S268–V279 failed to pull down NQO1 (Fig. 3D, right panels). These results together revealed that NQO1 and 20S proteasomes interact with the same domain in C/EBPα protein between amino acid S268–V279. To confirm this, three C/EBPα fragment peptides including S268–V279 (full-length), S268–G273 (N-terminal half), and K274–V279 (C-terminal half) were synthesized and used in *in vitro* assays to determine their effect on C/EBPα interaction with 20S (Fig. 3E). All three peptides competed with C/EBPα for binding with 20S and degradation. Interestingly, C-terminal half peptide containing basic lysine residues were significantly more efficient in competing C/EBPα against 20S degradation, as compared with N-terminal half containing small/neutral amino acids.

**Lysetine 276 and Lysine 277 of C/EBPα Play Critical Roles in Its Interaction with NQO1—Alignment of mouse, human, and rat C/EBPα domains between amino acid 268–279 showed conserved residues K274, K276, and K277; G269, G271, and 273; A272 and A275; S278; and V279 (Fig. 4A). Among these, basic lysine residues appeared most likely to interact with NQO1 and 20S. Site-directed mutagenesis was used to individually mutate K274, K276, and K277 to generate Flag-tagged C/EBPs K274A, K276A, and K277A mutant plasmids. Transfection and immunoprecipitation assays demonstrated that all C/EBPα mutants, NQO1-V5 and 20S were successfully immunoprecipitated (Fig. 4B, left, middle, and right panels). Transfection and forward/reverse immunoprecipitation experiments also showed that mutation of K276 and K277 to alanine resulted in the loss of interaction of C/EBPα with NQO1 (Fig. 4B, left and middle panels). On contrary, C/EBPα K274A mutant still interacted with NQO1 (Fig. 4B, left and middle panels). Based on these results, we generated double mutant C/EBPα K274A/K277A that also failed to interact with NQO1 (Fig. 4B, left and middle panels). These results suggested that K276/K277 of C/EBPα is required for its interaction with NQO1. Interestingly, all four C/EBPα mutants K274A, K276A, K277A, and double mutant K276A/K277A interacted with 20S in a similar capacity as normal C/EBPα (Fig. 4B, right panels). We also generated G273A and S278A that showed no effect on C/EBPα interaction with NQO1 or 20S (Fig. 4B). These results indicated that mutations in individual amino acid between S268 and V279 had no effect on C/EBPα interaction with 20S, suggesting the requirement of all of the amino acids in 268–279 domain for interaction of C/EBPα with 20S.

Histidine pull-down assays showed the same results as observed in immunoprecipitation assays (Fig. 4C). Both NQO1 and 20S were pulled down with full-length C/EBPα-V5-His6 (Fig. 4C, right panels). However, C/EBPαΔ268–279-V5-His6 deletion mutant failed to pull down either NQO1 or 20S (Fig. 4C, right panels). In related pull-down assays, 20S but not NQO1 was pulled down with both mutants C/EBPαK276A-V5-His6 and C/EBPαK277A-V5-His6 (Fig. 4C, right panels). Ubiquitination assays showed that C/EBPα full-length and
mutants all were ubiquitinated to similar extent (Fig. 4D). In the same experiment ubiquitination mutant Bcl2K17A-V5 showed absence of ubiquitination. These results together suggested that C/EBPα K276A and K277A ubiquitination were specific and not due to V5 since Bcl2K17A-V5 failed to show ubiquitination. The results together indicated that neither K276 nor K277 is the ubiquitination site of C/EBPα.

**NQO1 C- and N-terminal Deletions Result in Loss of Interaction with C/EBPα.**  NQO1 protein contains 8 α-helices and 9 β-sheets (32–33). V5-tagged C- and N-terminal deletions were generated to identify the protein domain(s) required for interaction with C/EBPα and 20S. Transfection of these mutants in HL-60 cells produced expected size of truncated NQO1-V5 proteins (Fig. 5A). Immunoprecipitation assays revealed that all the deletions of NQO1 failed to interact with C/EBPα or 20S proteasome (Fig. 5B). This suggested that full-length NQO1 protein is required for interaction with C/EBPα and 20S.

**Disruption of NADH Binding to NQO1 Abolished the Interaction between NQO1 and C/EBPα.**—Dicoumarol and NQO1Y127A/Y129A double mutant were used in in vivo and in vitro studies to disrupt NQO1 binding with NADH to determine its effect on NQO1 interaction with C/EBPα and 20S (in vivo studies) and protection of C/EBPα against 20S degradation (in vitro studies). Dicoumarol is a competitive inhibitor of NADH binding to NQO1 and NQO1Y127/Y129 is required for NADH binding (32–33). The results showed that both dicoumarol and double mutant NQO1Y127A/Y129A disruption of NADH binding to NQO1 led to the loss of NQO1 binding with C/EBPα but not with 20S (Fig. 6, A and B). In vitro translation/degradation assays showed that dicoumarol and double mutant NQO1Y127A/Y129A both inhibited NQO1 from protection of C/EBPα against 20S degradation (Fig. 6C). These results revealed that NADH binding to NQO1 is essential for NQO1 protection of C/EBPα against 20S degradation. In addition, the NADH binding to NQO1 is not required for NQO1 interaction with 20S.

**NQO1P187S Mutation Leads to Degradation of NQO1 and Upon Stabilization Protects C/EBPα against 20S Degradation.**—NQO1P187S mutant is an unstable protein and is degraded by proteasomes (15–16). HL-60 cells transfected with mutant plasmid expressed low level of NQO1P187S mutant protein that was significantly stabilized in presence of proteasome inhibitor MG132 (Fig. 6, left panels). Interestingly, addition of V5-tag at C terminus of mutant NQO1P187S protein stabilized the protein even in the absence of MG132 presumably due to conformational changes (Fig. 6, right panels). In
vitro translation and degradation assays revealed that NQO1P187S-V5 protein protected C/EBP against 20S degradation (Fig. 6). These results indicated that it is instability/loss not P187S mutation of NQO1 protein that associates with adverse effects.

\[\text{\textit{NQO1 Competition with 20S Proteasome for C/EBP}}\alpha\text{ Binding} \]

\[\text{\textit{FIGURE 4. Lysine 276 and lysine 277 of C/EBP}}\alpha\text{ play critical roles in interaction with NQO1. A, schematic showing of S268-V279 domain from human, mouse, and rat. B, mutation of K276 and K277 to alanine in C/EBP causes the loss of interaction with NQO1. C, HL-60 cells were co-transfected with mutant Flag-C/EBP and NQO1-V5, lysed, and immunoprecipitated and immunoblotted with anti-V5 and anti-Flag antibodies.} \]

\[\text{\textit{FIGURE 5. NQO1 with terminal deletions fails to interact with C/EBP}}\alpha\text{. A, schematic showing C- and N-terminal deletions of NQO1 (left panel). V5-tagged NQO1 and deletion mutants were transfected and immunoblotted to demonstrate input of expressed proteins (right panel). B, V5- tagged NQO1 deletion mutants were co-transfected with Flag-C/EBP. The transfected cells were lysed and immunoprecipitated with IgG (control), anti-Flag and anti-V5. Immunoprecipitated proteins were separated on SDS-PAGE and immunoblotted. The immunoblots were probed with anti-V5 to detect NQO1 deletions, anti-Flag to detect Flag-C/EBP and anti-20S 5 to detect 20S. All input were 10% of the proteins used in the experiments.} \]

\[\text{\textit{\gamma-Radiation Increases NQO1 Interaction with C/EBP}}\alpha\text{ and Decreases Interaction between 20S and C/EBP}—\text{HL-60 cells were non-irradiated or irradiated with \gamma-radiation and analyzed for C/EBP, NQO1, and 20S interactions by immunoprecipitation and immunoblotting (Fig. 7A, left panels). The exper-} \]
**DISCUSSION**

NQO1 protects against myeloproliferative diseases including leukemia (19–26). We demonstrate here that NQO1 controls myeloid differentiation factor C/EBPα and PU.1 to provide this protection. Disruption of C/EBPα and PU.1 both are known to be associated with development of myeloid leukemia (11–12, 34). WT mice upon exposure to γ-radiation showed significant induction of C/EBPα and PU.1 and myeloid differentiation (current report) and absence of myeloproliferative disease (26). On the other hand, NQO1−/− mice exposed to γ-radiation showed lack of induction of both C/EBPα and PU.1 and myeloid differentiation (current report) and development of myeloproliferative disease (26). Therefore, it is reasonable to suggest that the lack of induction of C/EBPα and PU.1 contributed to significantly decreased differentiation of myeloid cells and myeloproliferative diseases in NQO1−/− mice. Lack of induction of C/EBPα was due to rapid degradation by 20S proteasomes in the absence of NQO1. NQO1 directly interacted with C/EBPα and prevented its degradation by 20S proteasomes. This led to the stabilization of C/EBPα, normal differentiation of myeloid progenitor cells and protection against myeloproliferation. NQO1 indirectly regulates PU.1 since it does not interact with NQO1 or 20S proteasomes. NADH was required for NQO1 mediated stabilization or 20S degradation of PU.1 transcription, proteasomes interacted and competed for C/EBPα domain 2665GAGAGKAKKVS279. Internal deletion of this domain from C/EBPα resulted in the loss of interaction with both NQO1 and 20S. The NQO1 interaction with C/EBPα was further narrowed down to two lysine residues at amino acid position 276 and 277. Mutation of one or both of these lysine residues led to the complete loss of C/EBPα interaction with NQO1 but not with 20S. Interestingly, single amino acid mutations in the 20S interacting domain had no effect on CEBPα interaction with 20S. This indicated that complete domain was required for C/EBPα and 20S interaction. The NQO1 and 20S interacting domain in mouse C/EBPα characterized by us was found highly conserved in human and rat. Deletions in NQO1 protein had deleterious effect on NQO1 interaction with C/EBPα and 20S. This indicated that secondary/tertiary/folding structures are required for NQO1 interaction with C/EBPα and 20S. NADH was required for NQO1 binding with C/EBPα but not for that with 20S. This was clearly evident from the following. Dicoumarol that competes with NADH for binding with NQO1 abolished NQO1 interaction with C/EBPα. In addition, mutant NQO1Y127A/Y129A deficient in binding with NADH failed to interact with C/EBPα. Therefore, NADH binding with NQO1 regulates NQO1 control of stabilization of C/EBPα. It is possible that radiation/chemical stress-induced intracellular redox changes leads to higher levels of NADH which binds with NQO1 that in turn interacts with C/EBPα leading to stabilization of C/EBPα. NQO1 belongs to a battery of cytoprotective genes that coordinate activate in response to radiation and chemical stress (Ref. 29, present studies). This induction is critical in protection...
against myeloproliferative disease exposed to γ-radiation. The induction of NQO1 enhances its interaction with C/EBPα and reduces 20S interaction with C/EBPα, leading to the stabilization of C/EBPα.

NQO1P187S mutation is less robust for C/EBPα stabilization because of its degradation by proteasome. It is interesting to observe that addition of V5-tag at the C-terminal end of the protein led to stabilization of NQO1Y187S-V5 mutant protein. This mutant interacted with 20S and 20S in similar capacity as normal NQO1. This led to conclusion that higher susceptibility of human individuals carrying NQO1P187S mutation to develop myeloproliferative diseases including leukemia is due to loss of NQO1 protein and not due to lack of interaction with C/EBPα and 20S. Studies have shown that homozygous and heterozygous population for NQO1P187S mutation (17, 21, 23) might be at risk for development of myeloproliferative diseases.

A model of NQO1 protection of C/EBPα against 20S degradation is shown (supplemental Fig. S8). Under normal conditions, both 20S and NQO1 interact with C/EBPα and control physiological level of C/EBPα, PU.1 and myeloid differentiation. Exposure to γ-radiation stress led to increased NQO1 and increased NQO1 interaction with C/EBPα. Exposure to γ-radiation also reduced 20S:C/EBPα interaction. Increased NQO1 interaction with C/EBPα and decreased interaction of C/EBPα with 20S led to stabilization of C/EBPα, increased expression of PU.1 and differentiation of myeloid cells and protection against γ-radiation. On contrary, loss of NQO1 leads to increased 20S interaction with C/EBPα, degradation of C/EBPα, down-regulation of PU.1, lack of myeloid cell differentiation, and myeloproliferative diseases including leukemia.

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NQO1 competes with 20S proteasome for binding with C/EBPα leading to its stabilization and protection against radiation-induced myeloproliferative disease
Junkang Xu and Anil K. Jaiswal

Supplement Figure S1. Real Time PCR analysis of mRNA levels of C/EBPα and PU.1 in wild type (WT) and NQO1-null (NQO1-/-) mice.
NQO1 competes with 20S proteasome for binding with C/EBPα leading to its stabilization and protection against radiation-induced myeloproliferative disease
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Supplement Figure S2. Effect of MG132 on mRNA levels of C/EBPα and PU1. Mouse bone marrow cells were treated with DMSO or 20 μM MG132 for 10 hours, RNA was extracted and converted to cDNA. Equal amounts of cDNA were used with Taqman Master Mix and C/EBPα, PU.1, or GusB Primer and Probe. Final mixture was run on Real Time System (Applied Biosystems) using relative quantitation according to the manufacturer's instructions.
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**Supplement Figure S3.** Effect of NQO1 on mRNA levels of C/EBPα and PU1. HL-60 cells were transfected with 0.375 or 0.75 μg NQO1-V5 plasmid, and U937 cells were transfected with 100 or 200 nM NQO1 siRNA, 48 hours later, RNA was extracted and converted to cDNA. Equal amounts of cDNA were run on Real Time System (Applied Biosystems) using relative quantitation according to the manufacturer's instructions. * indicates significant difference.
Supplement Figure S4. NQO1 competes with 20S for binding with C/EBPα leading to its stabilization and protection against radiation-induced myeloproliferative disease
Junkang Xu and Anil K. Jaiswal

NQO1 competes with 20S proteasome for binding with C/EBPα leading to its stabilization and protection against radiation-induced myeloproliferative disease. Junkang Xu and Anil K. Jaiswal

Supplement Figure S4. NQO1 competes with 20S for C/EBPα binding. 1 µg flag C/EBPα and 1 µg NQO1-V5 plasmids were used for in vitro translation for 1.5 hours. 5 µl final products of flag-C/EBPα were mixed with 1 µl purified 20S and different amount of NQO1-V5 with NADH (5 mM). The mixture were then used for IP using anti-flag, precipitated proteins were probed with antibodies as indicated. All input were 10% of the proteins used in the experiments.
Supplement Figure S5. Bone marrow analysis. Seven to nine weeks old wild type and NQO1-null mice were either non-irradiated (0) or irradiated with 3 Grays γ-radiation, waited for 48 hours and analyzed for granulocytes and apoptotic death of granulocytes.
**Eosinophils**:  
Wild Type  
Non-irradiated 1.8±0.4  
Irradiated 1.9±0.5  
NQO1-/-  
Non-irradiated 2.4±0.9  
Irradiated 2.2±0.4  

**Basophils**:  
Wild Type  
Non-irradiated 0.5±0.1  
Irradiated 0.4±0.1  
NQO1-/-  
Non-irradiated 2.2±0.4  
Irradiated 2.1±0.2  

**Monocytes**:  
Wild Type  
Non-irradiated 1.9±0.4  
Irradiated 3.2±0.7  
NQO1-/-  
Non-irradiated 2.0±0.8  
Irradiated 3.5±0.4  

*Percent of WBC

Supplement Figure S6. CBC analysis of non-irradiated and 3 Grays γ-radiation irradiated wild type and NQO1-null mice. CBC analysis was done 48 hrs post-exposure to radiation. Seven to nine weeks old mice were used.
NQO1 competes with 20S proteasome for binding with C/EBPα leading to its stabilization and protection against radiation-induced myeloproliferative disease
Junkang Xu and Anil K. Jaiswal

Supplement Figure S7. Co-IP of Flag-C/EBPα (162-267) with NQO1-V5 or 20S. All input were 10% of the proteins used in the experiments.
NQO1 competes with 20S proteasome for binding with C/EBPα leading to its stabilization and protection against radiation-induced myeloproliferative disease

Junkang Xu and Anil K. Jaiswal

**Supplement Figure S8.** Model to demonstrate NQO1 protection against stress-induced myeloproliferative diseases.
NAD(P)H:quinone Oxidoreductase 1 (NQO1) Competes with 20S Proteasome for Binding with C/EBP α Leading to Its Stabilization and Protection against Radiation-induced Myeloproliferative Disease
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J. Biol. Chem. 2012, 287:41608-41618.
doi: 10.1074/jbc.M112.387738 originally published online October 18, 2012

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