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Identification of NCF2/p67phox as a novel p53 target gene

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Abbreviations: Nox, NADPH oxidase; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; PI, propidium iodide; ChIP, chromatin immunoprecipitation

Analysis of microarrays performed in p53-, TAp63α- and ΔNp63α-inducible SaOs-2 cell lines allowed the identification of NCF2 mRNA upregulation in response to p53 induction. NCF2 gene encodes for p67phox, the cytosolic subunit of the NADPH oxidase enzyme complex. The recruitment of p67phox to the cell membrane causes the activation of the NADPH oxidase complex followed by the generation of NADP+ and superoxide from molecular oxygen. The presence of three putative p53 binding sites on the NCF2 promoter was predicted, and the subsequent luciferase and chromatin immunoprecipitation assays showed the activation of NCF2 promoter by p53 and its direct binding in vivo to at least one of the sites, thus confirming the hypothesis. NCF2 upregulation was also confirmed by real-time PCR in several cell lines after p53 activation. NCF2 knockdown by siRNA results in a significant reduction of ROS production and stimulates cell death, suggesting a protective function of Nox2-generated ROS in cells against apoptosis. These results provide insight into the redox-sensitive signaling mechanism that mediates cell survival involving p53 and its novel target NCF2/p67phox.

Introduction

The family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was originally detected in neutrophils as a multicomponent enzyme that catalyzes monoelectronic reduction of external oxygen using NADPH or NADH as an internal electron donor, thus producing superoxide anions and NADP+ or NAD+.[1] Currently, the family is formed by seven members, Nox1–5 and Duox1/2, which are now recognized to have specific cellular and subcellular localizations, thus serving a variety of biological functions, including signal transduction, host defense, development, angiogenesis, blood pressure regulation and biosynthetic processes.[2-6] The most extensively studied member is Nox2; this enzyme is composed of two membrane-bound subunits (p22phox and gp91phox), three cytosolic subunits (p67phox, p47phox and p40phox) and a small G-protein Rac (Rac1 and Rac2). Nox2 becomes activated during phagocytosis of invading microbes as a result of membrane translocation of a ternary regulatory complex, formed by p47phox, p67phox and p40phox subunits, as well as of the small GTPase Rac.[7] The p47phox organizer protein contains two SH3 domains, which cooperatively interact with the proline-rich-domain of p22phox, thus promoting membrane translocation and oxidase activation.[8] In normal conditions, the two SH3 domains are masked by intramolecular interaction with an auto-inhibitory region. Upon stimulation, serine residues are unmasked by phosphorylation. The p67phox, encoded by the NCF2 gene, is the "activator" of the Nox2 complex. p67phox contains four conserved domains: a C-terminal SH3 domain, which mediates membrane translocation through binding to the proline-rich region present in the p47phox tail; four tetratricopeptide repeat motifs at the N-terminal domain interacting with the Rac; an activation domain able to act on gp91phox and a domain interacting with p40phox (PB1).[9,10] The PB1 domain is dispensable for oxidase activation, but is necessary for oxidase assembly, as it enhances p67phox recruitment to the phagosomal membrane.

Nox2 signaling has been extensively studied, given its physiological relevance in immune defense.[6] Along with its well-established role in immune function, Nox2 has also been shown to be activated in endothelial cells by several stimuli, including vascular endothelial growth factor and thrombin, thus implicating Nox2 in new blood vessel formation.[11-13] Recently, Nox2 and other Nox isoforms have been shown to be involved in tumor cell proliferation.[14-18] The upregulation of Nox is critical to support the elevated glycolysis by providing additional NAD+, and it has been consistently observed in cancer cells and in primary pancreatic cancer tissues with compromised mitochondria.[9,19] In addition, in acute leukemic cell lines, Nox2- and/or Nox4-derived ROS are crucially involved in the modulation of glucose transport (mediated by Glut1), which is frequently upregulated

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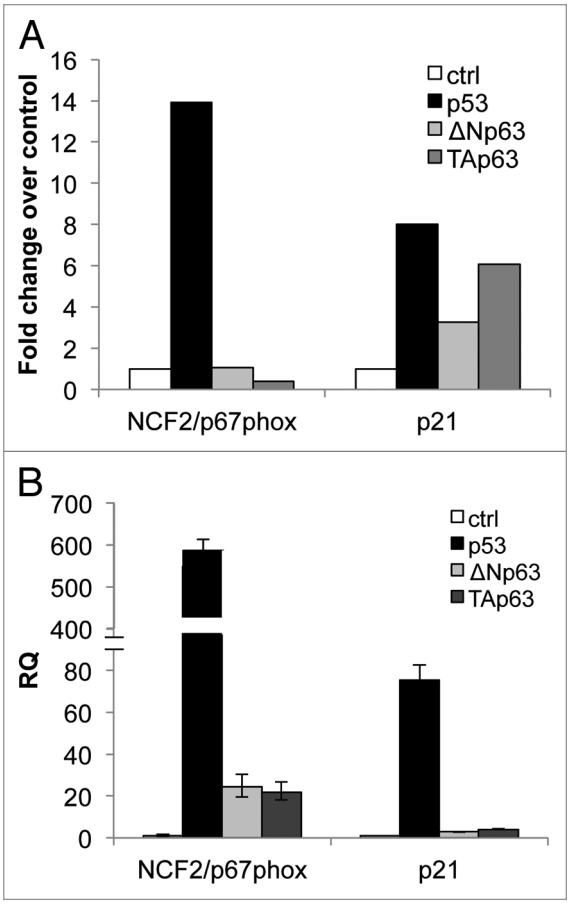

Figure 1. mRNA expression of NCF2/p67phox in SaOs-2 and in H1299 by p53 family members. (**A**) Microarray in SaOs-2 inducible clones for each transcription factor. Expression of NCF2/p67phox was measured 24 h after induction with doxycycline. p21 is used as a positive control. (**B**) Real-time PCR analysis of NCF2/p67phox expression following transfection of p53, ΔN or Tap63 in non-small cell lung carcinoma H1299 cells. p21 is used as a positive control.

in cancer cells, suggesting that both Glut1 and Nox homologs may be considered new potential targets in the treatment of leukemia.[16] In parallel with these studies, other results support the finding that ROS-generating Nox family enzymes are involved in neoplastic formation. For instance, Nox1 downregulation suppresses oncogenic Ras transformation,[20] and Nox4/Nox5 appear to control cell survival of malignant melanoma, pancreatic carcinoma and prostate cancer cells.[21-25] These data suggest that the modulation of survival signaling by ROS is also critical for some types of cancer development, although the genotoxic effect has been mainly emphasized for the role of ROS in tumor formations.[26-30] In pancreatic cancer, instead, Nox4-generated ROS have a protective function against apoptosis through the inhibition of AKT-ASK1 phosphorylation signaling.[22,23,28]

ROS are even generated downstream of p53 and p53 family members, p63 and p73, most likely by the transcriptional modulation of genes that regulate the cellular redox state and that directly contribute to p53/p63/p73-mediated cell death.[31-36] To maintain the cellular redox state, ROS levels need to be tightly controlled, a task which is performed by two interconnected systems: thioredoxin and glutathione system.[37-41] Interestingly, p53 and its family members positively regulate expression of genes whose products are directly involved in both systems; therefore, p53 clearly acts in an anti-apoptotic manner.[42-46] The list of p53 target genes includes the glutathione peroxidase (GPX), manganese superoxide dismutase (MnSOD, SOD2), aldehyde dehydrogenase 4 (ALDH4), p53-induced glycolysis and apoptosis regulator (TIGAR), as well as PA26 and Hi95, which encode two proteins of the sestrin family, namely sestrin1 (SESN1) and sestrin 2 (SESN2), respectively.[47-49]

In the present study, we identified NCF2, the gene encoding for p67phox, the cytosolic subunit of the NADPH oxidase enzyme complex, as direct p53 target gene. We demonstrated that NCF2 downregulation by siRNA implicates a significant reduction in cellular ROS production that stimulates apoptosis, suggesting a protective function of Nox2-generated ROS in cells against apoptosis. Here, we provide insight into the redox-sensitive signaling mechanism that mediates cell survival involving p53 and its novel target NCF2/p67phox.

Results

NCF2 expression and p53 family members. To characterize how downstream functions of the p53 family members are mediated, we performed a microarray analysis to identify genes that are differentially regulated by the TAp63α and ΔNp63α isoforms of p63 compared with p53.[50] We focused our attention on the NCF2 gene encoding for p67phox protein. Following upregulation of p53 expression by doxicycline addiction in a Tet-On-inducible SaOs2 cell line, the gene NCF2 was found upregulated (14-fold over control), while no upregulation was observed upon TAp63α and ΔNp63α induction (**Fig. 1A**). As positive control, the data related to p21 are also shown (**Fig. 1A**). In order to verify the data obtained by the array, we transiently transfected H1299 cell line with expression vectors bearing the p53, TAp63α and ΔNp63αHA-tagged cDNAs. Twenty-four hours post-transfection, NCF2 levels were greatly upregulated, as evaluated by real-time PCR, in p53-expressing cells as compared with TAp63α- and ΔNp63α-expressing cells (**Fig. 1B**). p21 expression levels are also shown as positive controls (**Fig. 1B**). These data indicate that p53 possibly regulates NCF2 expression in two different cellular systems.

NCF2 is a direct transcriptional target of p53. Based on the observations described above, we investigated the possibility that p53 directly transactivates the NCF2 gene. By using bioinformatics tools (MatInspector Professional software[51]), we searched the promoter region of human NCF2 for putative p53-responsive elements (REs). We identified three p53 consensus motifs (RE1, RE2 and RE3), which contained the core sequence CWWG (**Fig. 2A**). A luciferase reporter vector containing the promoter region,[52] with the three REs (RE1, RE2 and RE3) to control the expression of a luciferase cDNA, was used in co-transfection with expression vectors for different p53/p63/p73 isoforms (**Fig. 2B**). Twenty-four hours after co-transfection of HEK293 cells, luciferase assays were performed. p53 overexpression significantly

increased luciferase activity (10.2-fold activation over control), TAp73α also produced an enhancement of NCF2 promoter activity although to a minor extent (4.0-fold activation over control); the other members of p53 family did not exert a positive control on NCF2 promoter (**Fig. 2B**). As control, using the same cell extracts of the luciferase assay, we performed a western blot to show that the absence of luciferase upregulation is not due to evident differences in the expression level of the different transcription factors (**Fig. 2C**). This strongly argues in favor of the conclusion that the NCF2 gene is a direct p53 transcriptional target. Direct evidence has been found by chromatin immunoprecipitation (ChIP) experiments followed by PCR amplification of REs genomic fragments. **Figure 2D** clearly shows the ability of p53 protein to bind directly only the p53 RE2 of the NCF2 promoter sequence.

To further demonstrate a direct link between p53 activation and NCF2/p67phox expression, we treated HCT116 p53+/+ or HCT116 p53-/- cell lines with doxorubicin (1 μM, for 24 h). We observed an increase in NCF2 expression (13-fold over control), evaluated by real-time PCR, only in HCT116 p53+/+ cells (**Fig. 3A**). As a positive control, we evaluated using the same experimental conditions p21 expression (**Fig. 3B**).

NCF2/p67phox-mediated ROS production in HCT116 and in HaCat cell lines. Data previously published point out that the activity of the Nox enzyme families is critical for survival in cancer cell lines.[22,23] To investigate the role of NCF2/p67phox in cell signaling and survival, we took advantage of the loss-of-function analysis using siRNA. By transfection of specific NCF2 siRNA in HCT116 cells, we strongly suppressed its expression as evaluated at mRNA and at protein levels (**Fig. 4A**). Intracellular superoxide production was evaluated by using flow cytometry in cells loaded with oxidation-sensitive 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). In normal growing conditions, HCT116 spontaneously generated a certain level of ROS; upon siNCF2, ROS production was significantly reduced (50% less), as compared with scramble control transfected cells (**Fig. 4B**). This decrease indicates that NCF2/p67phox, and therefore Nox2, at least in part, is responsible for the intracellular ROS generation occurring in proliferating cells, and that other mechanisms also contributed to ROS synthesis. To demonstrate that this result is not cell line-specific, we performed the same experiment in HaCat cell line. HaCat cells were transiently transfected to obtain NCF2 silencing. As indicated in **Figure 5A**, we totally abrogated NCF2 expression as evaluated at mRNA and at protein levels. In this experimental condition, we evaluated ROS levels by DCFH-DA

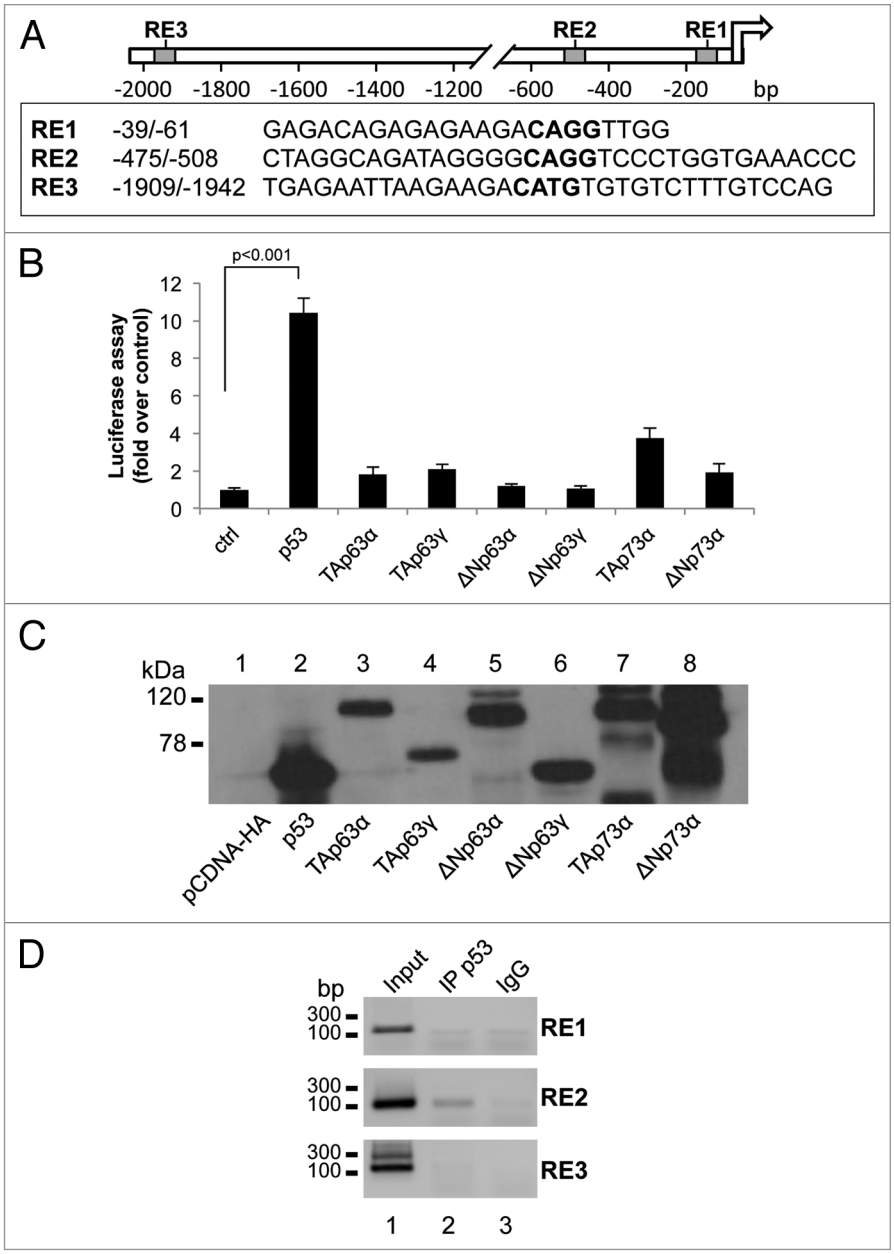

Figure 2. p53 transcriptionally activates NCF2/p67phox. (**A**) Map of the human NCF2/p67phox promoter. The promoter possesses three putative p53-responsive elements as indicated by the boxes. (**B**) p53 induces NCF2/p67phox promoter activity. HEK293 cells were transfected with pGL3-p67phox and the transactivators cloned in pcDNA vectors to evaluate the promoter activity by luciferase assay. Results are shown as the mean of three independent experiments. (**C**) Following transfection, HEK293 cells were lysed, and a western blot was performed using an anti-HA antibody to verify the transcription factors expression. The figure shows a representative experiment. (**D**) p53 binds to NCF2/p67phox promoter on RE2. Chromatin immunoprecipitation was performed in p53-inducible SaOs-2 cells using an anti-p53 antibody (IP p53) or a non-specific serum IgG. Non-immunoprecipitated chromatin was loaded as a positive control (input). The figure shows one representative experiment of three.

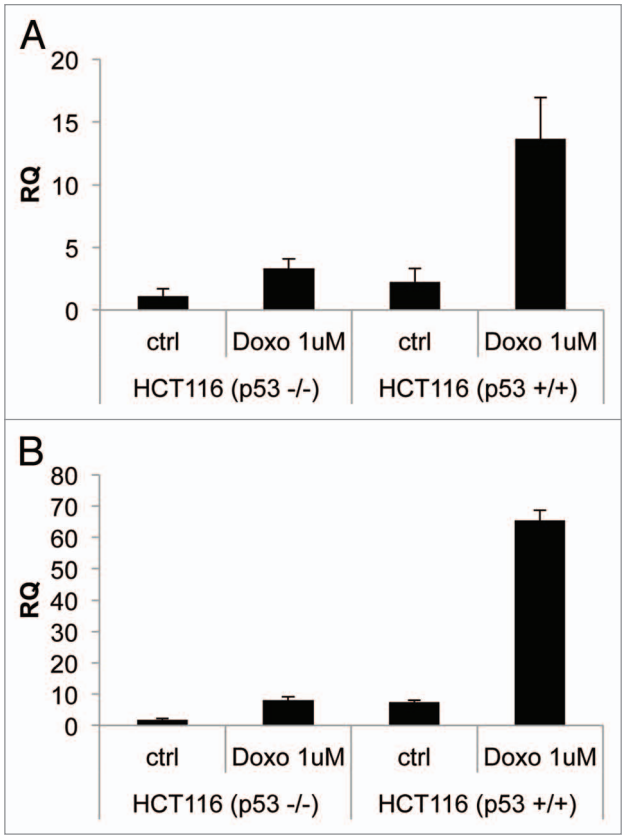

Figure 3. Endogenous-activated p53 induces NCF2/p67phox expression. (**A**) Colorectal carcinoma HCT116 cells, available in both p53 negative or positive clones, were treated with doxorubicin 1 μM to induce activation of p53. Cells were collected 24 h after treatment and real-time PCR was performed. (**B**) p21 is used as a positive control. Results are shown as the mean of three independent experiments.

cell loading followed by flow cytometry. We found that ROS production was significantly reduced (80% less) as compared with scramble control transfected cells (**Fig. 5B**), indicating that NCF2/p67phox is responsible for the majority of intracellular ROS generation in HaCat cells as well, suggesting that our observation is not cell line-specific.

Suppression of ROS generation by siNCF2 induces apoptosis in HCT-116 and in HaCat cell lines. We next examined whether suppression of NCF2/p67phox by siNCF2 affects cell viability in HCT116 and in HaCat cell lines. Apoptosis in NCF2-silenced cells was evaluated as sub-G_1 events by propidium iodide (PI) staining, followed by flow cytometry analysis and PARP enzyme cleavage. The data indicate that NCF2 knockdown in HCT116 induces an increasing cell number undergoing to apoptosis (from 15–44%), whereas scrambled control had no effect (**Fig. 4C**), suggesting that depletion of ROS, obtained by impairing Nox2 activity, is sufficient for apoptosis induction. Similar results were obtained in HaCat cells (**Fig. 5C**), suggesting that depletion of Nox2-generated ROS induces apoptosis (sub-G_1 events increase from 12–55% and PARP enzyme is cleaved, **Fig. 5A**). Taken together, the data suggested that Nox2, and its subunit NCF2/p67phox, exerts an anti-apoptotic activity in HCT116 and in HaCat cells, and that suppression of this activity, lowering the intracellular ROS, leads to cell death.

Discussion

ROS are not only generated as a cellular response to exogenous stress stimuli, but also as products of normal aerobic metabolism[53-59] or as second messengers in various signal transduction pathways.[27,60-64] ROS production may have deleterious effects on cells, and this event has been traditionally related to cancer development, aging and neurodegeneration.[65-69] However, it is now accepted that ROS may have an important role in regulating signal transduction pathways, gene expression and differentiation, although the molecular mechanisms upstream and downstream ROS generation are not fully understood.[70-75] The main non-mitochondrial sources of ROS are the NADPH oxidases, which are membrane-associated multi-protein complexes, of which NFC2/p67phox is an essential and crucial component, which produce superoxide. In this study, we identified NCF2 gene as a novel p53 target.[76-79] Expression of NCF2/p67phox, by activating the Nox2 complex,[4] generates ROS that have protective functions against apoptosis in HCT116 and HaCat cell lines, confirming previous results obtained for Nox4 in pancreatic cancer cells.[22,23] The pro-apoptotic activity of p53 is well documented and important for its multiple tumor suppressor functions. However, over the past years, it became clear that there is a second, less known, aspect of p53 function, implicating p53 also as an active mediator of pro-survival pathways[45,80-84] as demonstrated by a long list of pro-survival p53 targets.[47,83-86] The data presented here suggest that the novel p53 target gene, NCF2/p67phox, should be part of the above mentioned list. Furthermore, our results provide a molecular connection between the redox-sensitive signaling mechanism that mediates cell survival and p53 activity with its targets NFC2/p67phox.

Materials and Methods

Cell culture and transfection. HCT116, TAp63, ΔNp63 inducible SaOs-2 cells were cultured as described in Gressner et al., 2005.

RNA extraction, quantitative real-time RT-PCR and array. RNA was extracted from cells by using the RNAeasy Mini Kit (Qiagen). A total of 1,000 ng of RNA was used for reverse transcription using the GoScript Kit (Promega), and 2 ul of the reaction were used for real-time PCR. Normalization was performed amplifying human β-actin housekeeping mRNA using the following primers: forward 5'-GTT GCT ATC CAG GCT GTG CTA-3' and reverse 5'-AAT GTC ACG CAC GAT TTC CCG C-3' (237 bp product). For detection of human p21 isoform expression, the primers used were: forward 5'-TGA GCG ATG GAA CTT CGA C-3' and reverse 5'-ACA AGA CAG TGA CAG GTC C-3' (214 bp product); while for human NCF2 expression, the primers were: forward 5'-ATC AGC CTC TGG AAT GAA GGG G-3' and reverse 5'-GCA GCC AAT GTT GAA GCA AAT CC-3' (117 bp product). Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. All the

details related to the p53/TAp63/ΔNp63 dox-inducible SaOs-2 array have already been described previously.[50]

Luc assay and constructs. HEK293 cells were cultured in 12-well dishes and transfected at 50–80% confluency. Transfections were performed with Effectene (Qiagen) according to the manufacturer's recommendations. We used a 1:3 ratio between the reporter plasmid (containing the firefly luciferase gene under the control of the NCF2 promoter) and the given expression vectors encoding for all p53 family members. When needed, empty vector was added to keep the total amount of DNA (400 ng) used in each transfection constant. In all cases, 10 ng of Renilla Luciferase Vector (pRL-CMV; Promega) was co-transfected as a control of transfection efficiency. Twenty-four hours after transfection, luciferase activities in cellular extracts were measured by using a Dual Luciferase Reporter Assay System (Promega); light emission was measured over 10 sec using a Lumat LB9507 luminometer. pGL3-p67phox promoter construct was kindly provided by Professor Mark T. Quinn of Montana State University.

Western blotting. Cells were resuspended in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS) plus protease inhibitor cocktail (Sigma-Aldrich). After homogenization through QIA shredder columns (Qiagen) and protein determination, 50 μg of total proteins were loaded onto 10%SDS-PAGE and transferred to PVDF membranes (GE Healthcare). The blots were blocked with TBST (Tris-buffered saline and Tween 20)/10% nonfat dry milk (Biorad) and then incubated with the specific primary antibody: anti-NCF2/p67phox (610913, BD Biosciences), anti-HA-7 monoclonal mouse antibody (Sigma) and anti-PARP (SA-250, Enzo Life Sciences). Actin (A5441 mouse monoclonal antibody, Sigma-Aldrich) was used as a loading control. After washings and incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Biorad), signal detection was performed with the Western Lightning Plus ECL (Perkin Elmer).

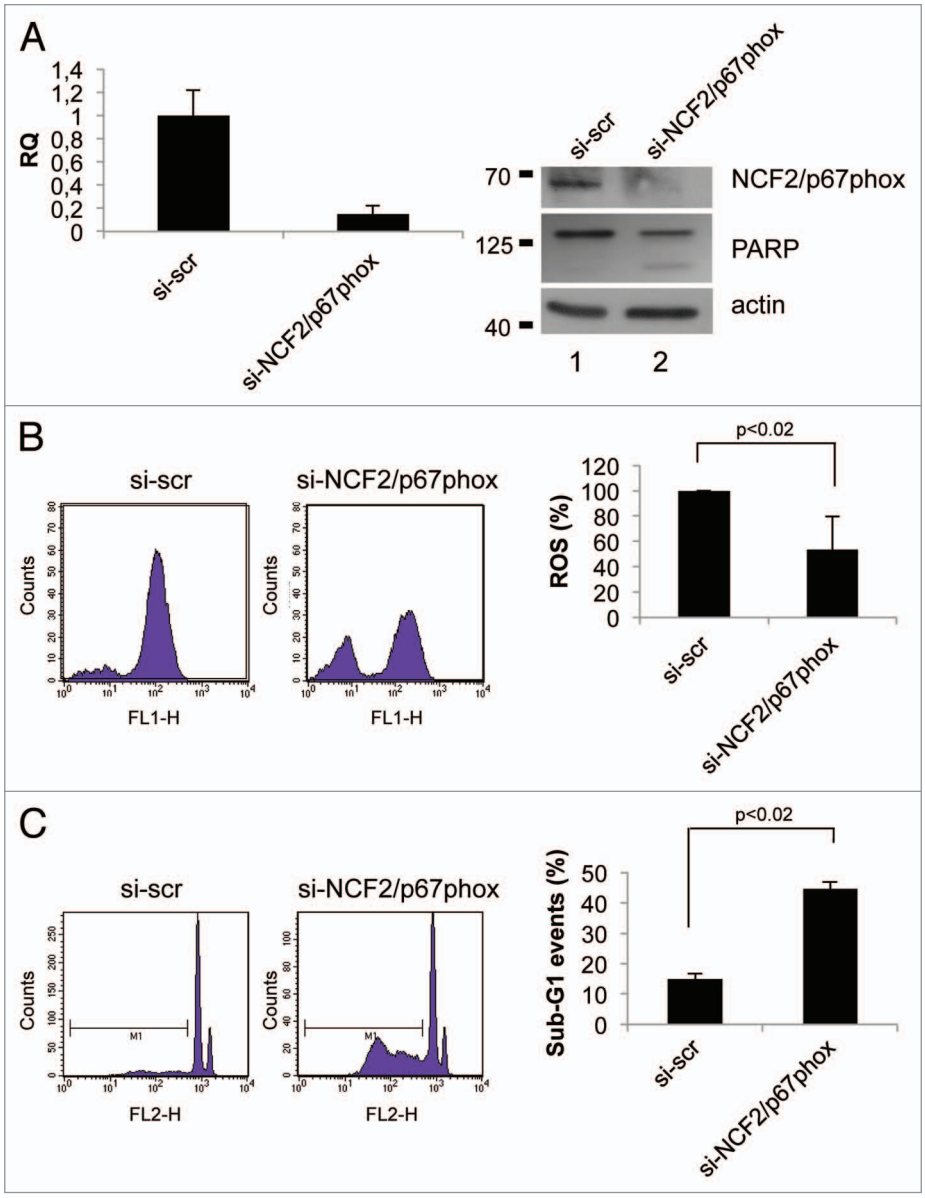

Figure 4. Inhibition of NCF2/p67phox in HCT116 cells decreases ROS and induces apoptosis. (**A**) HCT116 cells were transfected with either a control siRNA (si-scr) or specific NCF2/p67phox-targeting siRNA (si-NCF2/p67phox) and collected after 48 h. NCF2/p67phox protein and transcript levels were examined by western blot and real-time PCR, respectively. The western blot shown is one representative experiment. Real-time PCR shows the mean of three independent experiments. Actin is shown as a loading control. (**B**) ROS levels were assayed using a DCFDA staining and FACS analysis. Left panel shows one representative experiment of four independent ones. Right panel shows the mean of four independent experiments as a percentage respect to the control (presented as 100%). (**C**) Apoptosis levels were assayed by propidium iodide staining and FACS analysis. Percentage of sub-G_1 events (M1) is shown. Left panel shows one representative experiment of three. Right panel shows the mean of three independent experiments.

Chromatin immunoprecipitation. ChIP was performed using MAGnify Chromatin Immunoprecipitation System (Life Technologies) according to manufacturer's protocol. Cells (1.5×10^6) were incubated with anti-p53 antibody DO-1 (sc-126, Santa Cruz Biotechnology). DNA samples were analyzed with 33 cycles of PCR to amplify NCF2 promoter sequences (94°C for 25 sec, 59°C for 25 sec, 72°C for 25 sec). We used three primer pairs amplifying the RE1, RE2 and RE3 p53 responsive element in the NCF2 promoter (RE1: forward 5'-CTG CCA GGA GAC AGA GAG AAG-3' and reverse 5'-GCC CAG AAA GTG AAC ACC TTG-3'; RE2: forward 5'-GGT GAT AAT GAC AGG

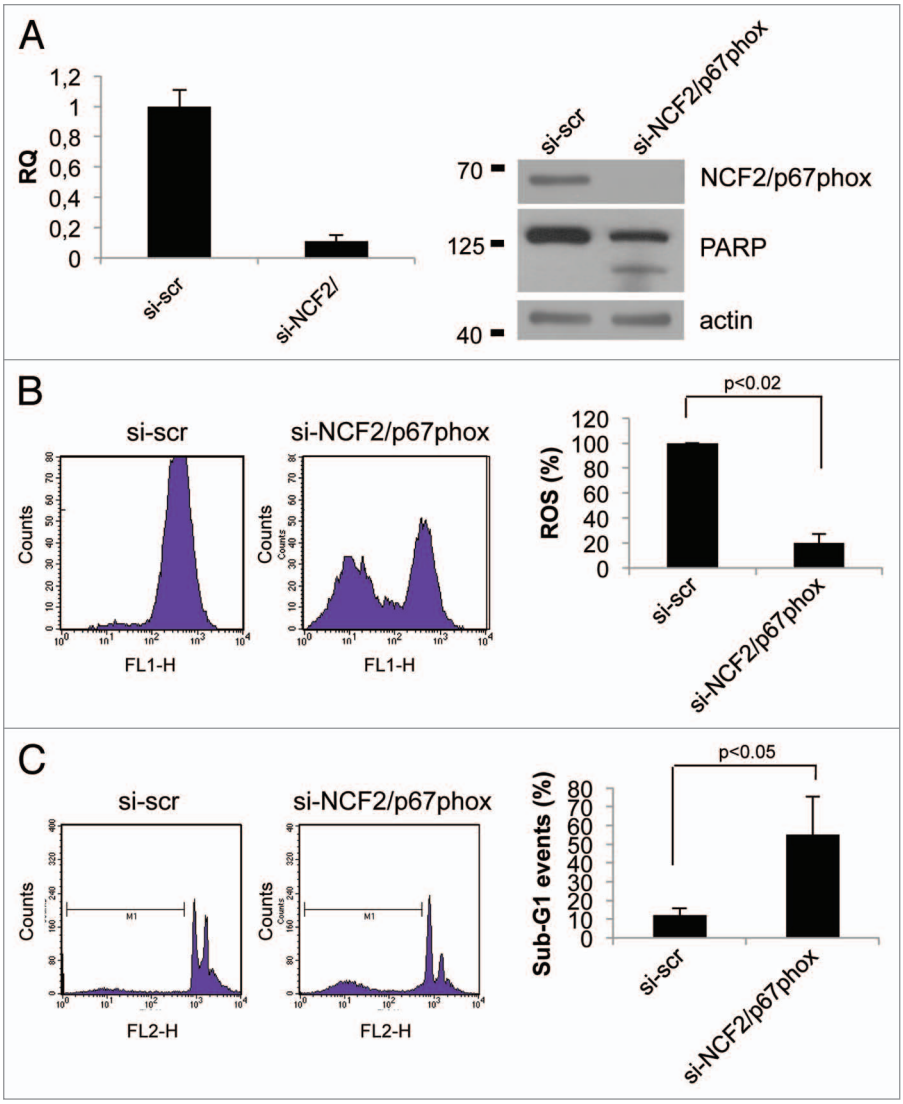

Figure 5. Inhibition of NCF2/p67phox in HaCat cells decreases ROS and induces apoptosis. (**A**) HaCat cells were transfected with either a control siRNA (si-scr) or specific NCF2/p67phox-targeting siRNA (si-NCF2/p67phox) and collected after 48 h. NCF2/p67phox protein and transcript levels were examined by western blot and real-time PCR, respectively. Western blot for both whole and cleaved PARP was performed to assay apoptosis. The western blot is one representative experiment. Real-time PCR shows the mean of three independent experiments. Actin is shown as a loading control. (**B**) ROS levels were assayed using a DCFDA staining and FACS analysis. The mean of three independent experiments as a percentage respect to the control (presented as 100%) is shown. (**C**) Apoptosis levels were assayed by PI staining and FACS analysis. Percentage of sub-G_1 events (M1) is shown for one of three experiments. The mean of three independent experiments is shown on the right panel.

AGG CAG C-3' and reverse 5'-TGG CTT GAA GGT GGG GTT TC-3'; RE3: forward 5'-TGC TTG TAG GAT TGA AGG TGT GAG-3' and reverse 5'-GAA AGA TGA GTA GGA GTT TGC CAG G-3').

A valuation of apoptosis and ROS by flow cytometry. HaCat and HCT-116 cells were collected 48 h after transfection, washed twice in PBS, stained with 10 μM DCFDA (dissolved in DMSO to a concentration of 1 mM then further diluted in PBS) (Life Technologies) for 30 min at 37°C. Twenty thousand events were collected by FacsCalibur flow cytometer (BD Biosciences) and analyzed with CellQuest software. For subsequent apoptosis analysis, cells were fixed with Methanol:Acetone 4:1, stored at 4°C overnight, then pelleted, treated with RNase and propidium iodide and analyzed by flow cytometry as described.

Bioinformatics. NCF2/p67phox promoter sequence was analyzed in search of p53-like responsive elements using GenomatixMatInspector software (www.genomatix.de).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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