Phosphorylated TP63 Induces Transcription of RPN13, Leading to NOS2 Protein Degradation

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Head and neck squamous cell carcinoma cells exposed to cisplatin display ATM-dependent phosphorylation of the most predominant TP63 isoform (ΔNp63α), leading to its activation as a transcription factor. Here, we found that the phospho-ΔNp63α protein binds to the genomic promoter of RPN13 through the TP63-responsive element. We further found that the phospho-ΔNp63α protein associates with other transcription factors (DDIT3 also known as CHOP, NF-Y, and NF-κB), activating RPN13 gene transcription. Furthermore, cisplatin-induced and phospho-ΔNp63α-dependent RPN13 gene transcription leads to NOS2 degradation. Finally, we show that RPN13 knockdown by siRNA essentially rescues NOS2 from cisplatin-dependent inactivation. These data provide a novel mechanism for the phospho-ΔNp63α-dependent regulation of NOS2 function in cells upon cisplatin treatment, contributing to the cell death pathway of tumor cells.

Misfolding and aggregation of proteins may play an important part in the pathogenesis of cancer because cells utilize a physiologic aggresome pathway targeting various proteins into proteasome-dependent degradation in response to stress (1–4). There is emerging evidence that inhibiting the aggresome pathway leads to accumulation of misfolded proteins and to cell death (apoptosis or autophagy) in tumor cells (1–4). One of these misfolded proteins, inducible NOS2, was shown to be targeted into an aggresome/proteasome pathway, which leads to termination of NO production by NOS2 (5–8). NOS2 plays a critical role in massive NO production in a variety of cell types under physiologic and pathophysiologic conditions (9–13). Understanding the molecular and cellular processes responsible for controlling NO production by NOS2 is critical for devising therapeutic strategies for many pathologic conditions, including cancer (14–20).

We previously found that the NAP110 (NOS2-associated protein of 110 kDa; also known as ADRM1 (adhesion regulatory molecule 1)) or RPN13 (regulatory particle non-ATPase subunit 13) forms protein-protein complexes with NOS2. This interaction modulates NOS2 activity by blocking its homodimerization (21, 22) and likely targeting NOS2 into a membrane fraction of cells, as reported by others (23). RPN13 is predominantly expressed as a non-glycosylated 42-kDa protein and is the ortholog of the yeast proteasome subunit Rpn13 (24–27). Furthermore, RPN13 functions as a novel 19 S proteasome cap-associated protein, acting as a receptor for ubiquitin, and recruits the deubiquitinating enzyme UCH37 to the 26 S proteasome (24–27).

Although RPN13 gene expression can be potentially induced by IFN-γ in gastric cancer cells, its transcriptional regulatory machinery is largely unknown (28). We thus undertook the study of transcription factors (TFs) implicated in RPN13 gene regulation in head and neck squamous cell carcinoma (HNSCC) cells exposed to cisplatin, the most used agent in chemotherapy for human cancers (29–32). Among a few of the TFs controlling RPN13 gene transcription, we found TP63 (tumor protein 63).

The TP53 homolog TP63 is a novel TF implicated in the regulation of genes involved in DNA damage response and chemotherapeutic stress in tumor cells (33). Because of the two independent promoters, the TP63 gene encodes two types of protein isoforms, one with a long transactivation domain and one with a short transactivation domain (34). The latter is designated ΔNp63. Because of several alternative splicing events, TP63 produces three isoforms with various lengths of the C terminus (α, β, and γ). ΔNp63α is the longest TP63 protein among the ΔNp63 isoforms and is the most predominant isoform expressed in HNSCC cells (35–37). We previously showed the importance of the ATM (ataxia telangiectasia mutated)-dependent phosphorylation of TP63 for its transcriptional activity in HNSCC cells upon cisplatin exposure (36, 37). Here, we define a novel molecular mechanism underlying the effect of the cisplatin-induced and phospho-ΔNp63α-dependent up-regulation of RPN13 gene expression on NOS2 proteasome-dependent degradation.

EXPERIMENTAL PROCEDURES

Cells and Reagents—We used HNSCC stable cell lines expressing wild-type ΔNp63α or ΔNp63α-S385G (with an altered ability to be phosphorylated by ATM kinase) as described previously (33, 35–37). Cells were maintained in RPMI 1640 medium and 10% fetal bovine serum. Cells were incubated with 10 μg/ml cis-diaminedichloroplatinum (cisplatin, Sigma), 25 μM lactacystin β-lactone (Calbiochem), or

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2 The abbreviations used are: TF, transcription factor; HNSCC, head and neck squamous cell carcinoma; qPCR, quantitative PCR; kbp, kilobase pair(s); TSS, transcription start site; 5′-RACE, 5′-rapid amplification of cDNA ends.
Isolation of Nuclear and Cytoplasmic Fractions—1–2 × 10^6 cells were resuspended in hypotonic lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA) with protease inhibitors (Sigma). After resuspension, Triton X-100 (final concentration of 0.6%) was added, and the nuclei were pelleted by centrifugation at 2500–3000 g for 10 min at 4 °C. Supernatants served as cytoplasmic fractions. Nuclear pellets were resuspended in extract buffer (20 mM HEPES (pH 7.9), 50% glycerol, 0.4 mM NaCl, 0.1 mM EDTA, and 0.1 mM EGTA) and rocked for 15 min at 4 °C, and nuclear lysates (supernatants) were recovered by centrifugation at 10,000 × g for 5 min at 4 °C (38).

Antibodies—We used a rabbit anti-DNp63 polyclonal antibody (Ab-1, EMD Chemicals); a rabbit anti-TP63 (clone Y289, NB110-57309) and a rabbit anti-Bcl-2 (Ab-1, EMD Chemicals); and rabbit polyclonal antibodies against DNA topoisomerase II α (sense, 5'-GTTACACAACACTGGCG-3'; antisense, 5'-GGCGGGTCCAAGTAATCCGCCCGC-3'); and rabbit polyclonal antibodies against DNA topoisomerase II β (sense, 5'-TGACGTCAAAAGCTGGTGC-3'; antisense, 5'-TGGTGCCA-GGCTCTCGGGG-3' (antisense); 5'-GAGTGCAATGTGC-CAAT-3' (sense) and 5'-TTGGTCCAGCCGCTTCGGGG-3' (antisense); 5'-CAAGTAAATCGCCGGC-3' (sense) and 5'-TGGTGGACGCGCTTCGGGG-3' (antisense); 5'-GAGCCA-CCGCCGCGCTAG-3' (sense) and 5'-TGGTGCCACGGCTTCGGGG-3' (antisense); 5'-GTTACAAACACTGGGC-3' (sense) and 5'-TGGTGGACGCGCTTCGGGG-3' (antisense); 5'-CCCGGACCCCAACCTTCTG-3' (sense) and 5'-TGGTGCCACGGCTTCGGGG-3' (antisense); and 5'-GAGCCA-CCGCCGCGCTAG-3' (sense) and 5'-TGGTGCCACGGCTTCGGGG-3' (antisense). The resulting PCR fragments were cloned into the MluI and XhoI sites or HindIII site of the promoterless pGL3-Basic luciferase reporter expression vector (Promega) as described previously (34). A PCR SuperMix High Fidelity kit (Invitrogen) was used for amplification (34). All constructs were sequenced from the 5'- and 3'-ends. 5 × 10^4 cells were plated per well in a 24-well plate. pGL3 luciferase reporter constructs (100 ng; Promega) and Renilla luciferase plasmid pRL-SV40 (1 ng; Promega) were introduced into cells using FuGENE 6 (Roche Applied Science) as described previously (34). 48 h after transfection, luciferase assays were performed using the Dual-Luciferase reporter assay kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well (34). For each experimental trial, wells were transfected in triplicate, and each well was assayed in triplicate. Luciferase activity was normalized to the activity produced from the empty vector. Cell lysates were cleared by centrifugation, 10 μl was added to 50 μl of firefly luciferase substrate, and light units were measured in a luminometer. Renilla luciferase activities were measured in the same tube after the addition of 50 μl of Stop & Glo reagent. Values for the firefly luciferase activity were divided by the Renilla luciferase activity to normalize for differences caused by unequal transfection efficiency (34).

RT-PCR—a first strand cDNA synthesis kit (Invitrogen) was used for reverse transcription. RT-PCR was performed with Taq DNA polymerase (Invitrogen) for 24–30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 30 s as described previously (34, 37). As a control, we amplified the GAPDH mRNA with primers 5'-CTACATGTTTTAATGTTG-3' (sense, +121) and 5'-GCCTCAAGCAACTG-3' (antisense, +920), yielding the 800-bp PCR product. For RN13 amplification, we used primers 5'-TCATCCTTCCCTGAC-GACTG-3' (sense, +301) and 5'-GCCCTGCTGGGAAACTAT-GTA-3' (antisense, +570), yielding the 270-bp PCR product. A quantitative PCR (qPCR) assay was performed using the StepOnePlus real-time PCR system kit (Applied Biosystems) with SYBR Green Universal PCR Master Mix. The same primers described above were used. Values for RN13 were normalized to values for GAPDH, and values obtained from the control untreated samples were designated as 1. Experiments were performed in triplicate.

Chromatin Immunoprecipitation—5 × 10^6 cell eq of chromatin (2–2.5 kilobase pairs (kb) in size) were immunoprecipitated with 10 μg of anti-phospho-DNp63α antibody as described (34, 37). After reversal of formaldehyde cross-linking and RNase A and proteinase K treatments, immunoprecipitate-enriched DNAs were used for PCR amplification. PCR consisted of 40 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s using Taq DNA polymerase. The PCR primers used for ChIP were as follows: for the TP63-responsive element, 5'-TGGGGTGCAAGGGCCTACT-3' (sense, -1400) and 5'-CAGGGTCTGCTCTGAAT-3' (antisense, -1120); for the NF-kB-responsive element, 5'-TGAAGGCCCTCTAAGGACC-3' (sense, -1030) and 5'-GACCCCTCTTGACCAGC-3' (antisense, -810); and for the NF-kB-responsive element, 5'-GACTCCACCCCTCAGAG-3' (sense, -750) and 5'-ACAGCTGACACATATCA-3' (antisense, -530); for the TP63-responsive element, 5'-CTGAACCCCTTCCTCCGG-3' (sense, -590) and 5'-CCCTGCTCCCTATGACAC-3' (antisense, -210); and for the NF-Y-responsive element, 5'-GTCCAAGGGCCCTACAGC-3' (sense, -190) and 5'-CAGGATGACGCCTAGGCT-3' (antisense, +90).
As a negative control, the binding of TF was tested with the nonspecific region primers representing the RPN13 ORF (+301 to +570). To quantify the binding of TFs to the RPN13 gene promoter sequences, we used qPCR with the abovementioned primers for various TFs. ChIP-PCR values were normalized to GAPDH values. For each TF, values obtained from the input samples were designated as 1. Experiments were performed in triplicate.

**RESULTS**

Cisplatin Induces the Phospho-ΔNp63α-dependent Expression of RPN13 in HNSCC Cells—Using the mRNA expression array and ChIP-on-chip array, we previously showed that exposure of HNSCC cells to 10 μg/ml cisplatin for 16 h leads to up-regulation of many mRNA transcripts associated with the DNA damage, cell cycle, and apoptosis pathways (37). Among these mRNA targets, we found RPN13 (also known ADRM1). We previously showed that cisplatin treatment induces the ATM-dependent phosphorylation of ΔNp63α, subsequently modulating various genes implicated in cell survival (33, 35–37). Using the isogenic HNSCC cell lines expressing wild-type ΔNp63α or ΔNp63α-S385G (ΔNp63α with an altered ability to be phosphorylated by ATM kinase), we previously showed the failure of ΔNp63α-S385G to regulate gene transcription (36, 37).

We first found that cisplatin treatment substantially increased the RPN13 mRNA levels in wild-type ΔNp63α cells but not in ΔNp63α-S385G cells as shown by RT-PCR assay (Fig. 1A). By qPCR, we further found that cisplatin treatment increased the RPN13 mRNA levels in wild-type ΔNp63α cells by 12.4 ± 0.9-fold (Fig. 1B). Upon inspection of the 1.5-kbp RPN13 promoter sequence (UCSC Genome Bioinformatics) using TFSEARCH software, we observed that the specific TF cognate sequences are present in the 1.5-kbp human RPN13 promoter (Fig. 2).

To further examine the role of these TFs in the regulation of RPN13 gene expression, we treated wild-type ΔNp63α cells (Fig. 3, left panels) and ΔNp63α-S385S cells (right panels) with control medium and cisplatin (10 μg/ml for 16 h). We found that cisplatin treatment induced phospho-ΔNp63α, NF-YA, and DDIT3 protein levels in wild-type ΔNp63α cells by 12.4 ± 0.9-fold (Fig. 1B). Upon inspection of the 1.5-kbp RPN13 promoter sequence (UCSC Genome Bioinformatics) using TFSEARCH software, we observed that the specific TF cognate sequences are present in the 1.5-kbp human RPN13 promoter (Fig. 2).

Cloning and Initial Analysis of the Human RPN13 Promoter—To define the molecular mechanism of cisplatin-induced RPN13 gene expression in HNSCC cells and a potential involvement of phospho-ΔNp63α, we cloned and analyzed the human RPN13 gene promoter. We screened a human placenta genomic library in the Lambda FIX II vector with a 1.3-kbp cDNA probe encoding full-length human RPN13 (28). A total of 1 × 10⁶ plaques were screened, yielding
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A. DeltaNp63alpha wild type S385G

ΔNp63α, 270 bp

GAPDH, 800 bp

B. ΔNp63α (wt) ΔNp63α-S385G

RPN13, 50, and

GAPDH, 800 bp

ΔNp63α, 270 bp

ΔNp63α-S385G

MATERIALS AND METHODS

To map the TSS, the 5′-RACE anchor primer, 333 and

334 bp of

ΔNp63α coding region (Fig. 4A). Nucleotide

sequence analysis of a 3.8-kbp EcoRI subclone and a 5.5-

kbp PstI subclone demonstrated that these clones contained

620 bp and 1.7 kbp, respectively, of the 5′-flanking region of the

RPN13 gene. Analysis of the 1.7-kbp fragment revealed a 230-bp-long intron found in the coding region of the

RPN13 gene (located between +333 and +334 bp of the ORF).

RPN13 Promoter Regions Involved in the Response to Cisplatin Treatment—We next carried out a functional analysis of the human RPN13 promoter. We designed a series of progressive 5′-deletion constructs that span the following RPN13 promoter regions: −1750/+50, −1280/+50, −1192/+50, −1043/+50, −901/+50, −608/+50, and −331/+50 bp (Fig. 5C). The resulting 5′-progressive deletions were subcloned into the pGL3 luciferase reporter expression cassette (as described under “Experimental Procedures”).

RPN13 promoter was found on the UCSC Genome Bioinformatics human genome web site, and certain potential TF-responsive elements (RE) were defined using TFSEARCH software. TF sequences are shown in boldface. The TSS is shown as an uppercase letter. The following responsive elements were located in the RPN13 promoter: TP65 (−1376/−1354, −1231/−1216, −1189/−1167, and −500/−491), NF-Y/DDIT3 (−1267/−1246, −93/−71, and −65/37), NF-xB (−995/−985 and −724/−716), STAT (−857/−843), and GAS (gamma-activated site; −971/−862).

FIGURE 4. A diagram showing the human RPN13 promoter regions (ΔNp63α, S385G) and the corresponding luciferase activity (ΔNp63α, S385G) in wild-type HNSCC cells (Fig. 4B). RNA was reversed-transcribed using the RPN13-specific primer (+894 to +875 bp of the RPN13 ORF) and then amplified with the RPN13-specific primer (+423 to +399 bp) and the universal 5′-RACE anchor primer, yielding a single band of ~459 bp (Fig. 5A). The resulting PCR product was cloned and sequenced, and it showed identity to the RPN13 cDNA sequence (Fig. 5B), suggesting that the TSS is a G residue (~70), as confirmed by the primer extension assay (Fig. 5B).

FIGURE 5. Partial sequencing of the XbaI clones revealed that the resulting PCR product was cloned and sequenced, and it showed identity to the RPN13 cDNA sequence (Fig. 5B).
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was increased (by 8.5 ± 0.7-fold) in both wild-type ∆Np63α and ∆Np63α-S385G cells exposed to control medium (Fig. 6, A and B, sample 3). Subsequent 5′-deletions of the RPN13 promoter from −1750 to −1280 nucleotides decreased the basal luciferase activity by 7.6 ± 0.6-fold in both cell lines (Fig. 6, A and B, sample 5), which remained substantially similar with the −1192/+50 construct (6.8 ± 0.5-fold), −1043/+50 construct (5.8 ± 0.4-fold), −901/+50 construct (5.2 ± 0.5-fold), −608/+50 construct (3.5 ± 0.3-fold), and −331/+50 construct (2.6 ± 0.2-fold) as shown (samples 7, 9, 11, 13, and 15).

After cisplatin treatment, the RPN13 gene promoter (∆Np63α-S385G cells) driven luciferase activity strongly increased in wild-type ∆Np63α cells (26.7 ± 1.5-fold) while minimally increasing in ∆Np63α-S385G cells (8.9 ± 0.7-fold) compared with the measurements obtained with cells exposed to control medium (Fig. 6, A and B, sample 3 versus 4). Similarly, 5′-deletions of the 1750-bp RPN13 promoter to −1280/+50 nucleotides decreased the cisplatin-induced luciferase activity by 21.3 ± 1.3-fold (Fig. 6A, sample 6), to −1192/+50 nucleotides by 15.2 ± 1.1-fold (sample 8), to −1043/+50 nucleotides by 10.2 ± 0.8-fold (sample 10), to −901/+50 nucleotides by 9.6 ± 0.6-fold (sample 12), to −608/+50 nucleotides by 9.4 ± 0.5-fold (sample 14), and to −331/+50 nucleotides by 4.6 ± 0.3 (sample 16).

We further observed that, in ∆Np63α-S385G cells treated with cisplatin, the luciferase activity driven by the ∆Np63α construct was decreased by 8.2 ± 0.6-fold (Fig. 6B, sample 6), by the −1192/+50 construct by 7.7 ± 0.5-fold (sample 8), by the −1043/+50 construct by 7.6 ± 0.4-fold (sample 10), by the −901/+50 construct by 6.5 ± 0.4-fold (sample 12), by the −608/+50 construct by 5.5 ± 0.4-fold (sample 14), and by the −331/+50 construct by 4.5 ± 0.3-fold (sample 16). These data suggest that the ability of HNSCC cells to mediate the ATM-dependent phosphorylation of ∆Np63α is essential for phospho-∆Np63α protein binding to certain RPN13 promoter regions in vivo and is critical for cisplatin-induced RPN13 transcriptional regulation. Cisplatin-induced Phospho-∆Np63α Interacts with Other Transcription Factors That Regulate the RPN13 Promoter

To examine the molecular mechanism underlying RPN13

FIGURE 3. Immunoblot analysis of the protein levels of RPN13 and TFs (tested with the indicated antibodies) potentially regulating RPN13 expression. Total, nuclear, and cytoplasmic lysates were used as indicated. The loading control used for total and cytoplasmic lysates was β-actin, and that for nuclear lysates was TOP2A. CIS, cisplatin.
gene transcriptional regulation, we tested whether the phospho-ΔNp63α protein along with other TFs (defined in the human RPN13 promoter sequence) (Fig. 2) were endogenously bound to the RPN13 promoter. We used the ChIP assay with antibodies to ΔNp63α, phospho-ΔNp63α, DDIT3, and NF-κB (p65 subunit) on wild-type ΔNp63α or ΔNp63α-S385G cells exposed to control medium or 10 μg/ml cisplatin for 16 h. We found that cisplatin treatment of wild-type ΔNp63α cells induced phospho-ΔNp63α protein binding to the RPN13 promoter sequences (Fig. 7A). The binding of the phospho-ΔNp63α protein was dramatically increased in wild-type ΔNp63α cells compared with the non-phosphorylated ΔNp63α protein in ΔNp63α-S385G cells (Fig. 7A, upper (input) and lower (ChIP) panels). Similarly, cisplatin dramatically induced the binding of DDIT3 to the RPN13 promoter (Fig. 7A). However, NF-κB (p65 subunit) binding to the RPN13 promoter minimally increased in wild-type ΔNp63α cells upon cisplatin exposure (Fig. 7A). In contrast, the binding of ΔNp63α, phospho-ΔNp63α, and DDIT3 to the RPN13 promoter was totally abolished in ΔNp63α-S385G cells, whereas NF-κB (p65 subunit) binding essentially remained (Fig. 7B, upper (input) and lower (ChIP) panels). The TFs tested showed no binding to the nonconspecific regions of the RPN13 promoter (data not shown). By qPCR, we assessed the quantitative differences in TF binding to the RPN13 promoter in wild-type ΔNp63α and ΔNp63α-S385G cells under control and cisplatin-treated conditions (Fig. 7C). We observed that cisplatin treatment substantially increased the binding of ΔNp63α, phospho-ΔNp63α, and DDIT3 by 10.5–14.1-fold in wild-type ΔNp63α cells while having a minimal effect in ΔNp63α-S385G cells (Fig. 7C).

To further examine the mutual protein-protein interactions of the various TFs bound to the RPN13 promoter, we coprecipitated protein-protein complexes with antibody to ΔNp63α in wild-type ΔNp63α and ΔNp63α-S385G cells treated with control medium or 10 μg/ml cisplatin for 16 h. We found that cisplatin treatment induced complex formation between phospho-ΔNp63α, DDIT3, and NF-YA, whereas complexes between ΔNp63α and NF-κB (p65 subunit) or STAT3 essentially remained unchanged in cells upon cisplatin exposure (Fig. 8, left panels). However, in ΔNp63α-S385G cells, neither control medium nor cisplatin led to complex formation between ΔNp63α and other regulators of RPN13 transcription (Fig. 8, right panels).

Cisplatin Induces the Phospho-ΔNp63α/RPN13-dependent Proteasome Degradation of NOS2—RPN13 was previously found to bind NOS2 and to inactive its function through...
**FIGURE 7.** Cisplatin induces the binding of TFs to the RPN13 promoter in vivo. Cells were treated with control medium (−) or 10 μg/ml cisplatin (CIS; +) for 16 h. A and B, wild-type ΔNp63α and ΔNp63α-S385G cells, respectively. ChIP was performed with the indicated antibodies as described under “Experimental Procedures.” RT-PCR fragments produced are shown with arrows, and the sizes are indicated. IP, immunoprecipitation. C, qPCR analysis of TF binding to RPN13 promoter sequences. Values for RPN13 were normalized to values for GAPDH. For each TF, values obtained from the input samples were designated as 1. Experiments were performed in triplicate. Numerical values show the -fold change between control (−) and CIS-treated (+) samples.

**FIGURE 8.** Cisplatin induces complex formation between various TFs involved in RPN13 gene transcriptional regulation. The ΔNp63 protein complexes with various TFs were immunoprecipitated (IP) with the indicated antibodies. Separate experiments are shown as separate images. CIS, cisplatin.

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Inhibition of NOS2 dimerization and targeting it to the proteasome-dependent degradation pathway (5–8, 22, 39). We examined whether exposure of HNSCC cells to cisplatin treatment would lead to NOS2 degradation and subsequently inhibition of NOS2 activity. To access the role of phospho-ΔNp63α in this process, we treated wild-type ΔNp63α and ΔNp63α-S385G cells with control medium or 10 μg/ml cisplatin for 16 h. Although detectable induction of NOS2 gene transcription was shown to be achieved by exposure of cells to IFNγ (21, 22, 40), we first tested this treatment with HNSCC cells, observing NOS2 expression exclusively upon IFNγ treatment (supplemental Fig. 1A). We further tested whether NOS1 (neuronal) or NOS3 (endothelial) can be detected in HNSCC cells upon both IFNγ and cisplatin exposure. Only negligible amounts of both NOS1 and NOS3 were found in HNSCC cells under these experimental conditions (supplemental Fig. 1B). Thus, to ensure proper induction of both RPN13 and NOS2, HNSCC cells were also treated with 20 units/ml IFNγ for 16 h as described previously (21, 22, 40).

We found that cisplatin treatment along with IFNγ exposure dramatically induced RPN13 expression in wild-type ΔNp63α cells (Fig. 9A, left panels) in contrast to ΔNp63α-S385G cells (right panels). However, NOS2 expression substantially decreased in wild-type ΔNp63α cells (Fig. 9A, left panels), whereas no changes were observed in ΔNp63α-S385G cells (right panels). We further found that RPN13-NOS2-UCH37 protein complex formation substantially increased in wild-type ΔNp63α cells upon cisplatin exposure (Fig. 9A, left panels) but minimally increased in ΔNp63α-S385G cells (right panels). Using the proteasome inhibitor lactacystin, we observed no changes in NOS2 protein levels in both cell lines exposed to control medium or cisplatin treatment (Fig. 9A, lowest panel). Using the radioactive assay for converting arginine to citrulline (21, 22), we examined NO production in HNSCC cells exposed to control medium or 10 μg/ml cisplatin for 16 h grown without or with IFNγ. To ensure that we exclusively monitored the calcium-independent activity of NOS2, we tested the total NOS activity in the presence of EDTA as described previously (21, 22, 41). We observed that, in wild-type ΔNp63α cells, IFNγ dramatically increased NOS2 activity in the absence of cisplatin (Fig. 9B, samples 1 and 2), whereas cisplatin treatment substantially modulated IFNγ-induced NOS2 activity (samples 3 and 4). We further found that, in ΔNp63α-S385G cells, both control medium and cisplatin had no effect on the IFNγ-dependent activation of NOS2 activity (Fig. 9B, samples 5–8).

We then examined whether siRNA silencing of RPN13 expression would affect the NOS2 level and activity in wild-type ΔNp63α cells treated with control medium or 10 μg/ml cisplatin for 16 h. We found that, in the presence of scrambled siRNA, IFNγ and cisplatin increased RPN13 and decreased NOS2 expression, whereas RPN13 siRNA inhibited RPN13 expression and increased NOS2 expression (Fig. 10A), supporting the notion that RPN13 is intimately involved in NOS2 inactivation. Because we tested the total NOS activity under calcium-independent conditions and only negligible amounts of both NOS1 and NOS3 were found in HNSCC cells (supplemental Fig. 1, B–D), we concluded that only NOS2 activity...
was monitored under these experimental conditions. Thus, our data showed that scrambled siRNA had no effect on RPN13-dependent inhibition of NOS (NOS2) activity (Fig. 10, samples 1 and 2), whereas RPN13 siRNA reversed this inhibitory effect on NOS (NOS2) activity (samples 3 and 4).

DISCUSSION

We previously found that HNSCC cells exposed to cisplatin display an increase in the ATM-dependent phosphorylation of ΔNp63α, leading to activation of its transcriptional function (33–37). Using cDNA chip microarray expression and ChIP-on-chip array, we found gene targets induced/down-regulated via a cisplatin/phospho-ΔNp63α-dependent pathway (37). Among those genes, whose expression was up-regulated in HNSCC cells upon cisplatin exposure, we found a human homolog of yeast Rpn13, which serves as a ubiquitin receptor and associates with the deubiquitinating enzyme UCH37, recruiting the latter to the 26 S proteasome (24–27).

We previously showed that RPN13 physically interacts with NOS2, modulating its activity through blocking its homodimerization and potentially targeting nascent NOS2 polypeptides into a membrane-bound location (21–23). NOS2 inactivation is also likely to be achieved by targeting this enzyme into a proteasome-dependent degradation pathway, modulating NOS2 turnover and protecting cells against long-term and high-output NO production by this powerful enzyme (5–8).

Accumulating evidence supports the notion that high-level NO might lead to a pro-apoptotic response, whereas low-level NO would likely play an anti-apoptotic role (10, 16–18). NO affects cellular decisions of life and death either by turning on apoptotic pathways or by shutting them off (10, 16–18). Pro-apoptotic pathways of NO appeared likely to be compatible with TP53 intrinsic mitochondria-dependent apoptosis (10, 16–18, 42–50). Anti-apoptotic actions of NO range from an immediate interference with pro-apoptotic signaling cascades to long-lasting effects based on expression of cell-protective proteins, such as the ability of NO to block caspases by S-nitrosylation (10, 16–18, 50). High concentrations of NO and
its metabolites (peroxynitrite and nitric oxide) cause damage to DNA and proteins, leading to TP53 accumulation, whereas TP53 mediates transcriptional transrepression of NOS2, thereby establishing a regulatory feedback loop (11–15, 41, 45–50). On the other hand, low NO concentrations act on pathways activating TFs, such as NF-κB and AP-1, thereby influencing gene expression (50). During inflammatory reactions NOS2 produces a substantial amount of NO for prolonged periods of time, leading to a nitrosative stress directly affecting NF-κB, AP-1, OCT-1, hypoxia-inducible factor 1, TP53, and c-MYB or modulating the accessibility of promoters to transcription initiation via increased DNA methylation or histone deacetylation (45–50).

NO signaling is crucial for effecting long-lasting changes in cells, including gene expression, cell cycle arrest, cell death, and cell differentiation (9, 16–18, 51, 52). Using cDNA microarray technology to study the kinetics of gene activation by NO, Enikolopov and co-workers (9, 51, 52) determined that NO induces three distinct waves of gene activity. The first wave is induced within 30 min of exposure to NO and represents the primary gene targets of NO (9, 51, 52). It is followed by subsequent waves of gene activity that may reflect further modulation of TP53 phosphorylation and stability (41). NO is a potent activator of TP53 through rapid response associated with cancer, changing the cellular microenvironment, reactive oxygen species production, and sets of genes ultimately induced by both hypoxia and NO in human cancer (11, 12, 16–18). At the same time, TP53 was shown to correlate with poor patient survival after platinum chemotherapy (54, 55). Depletion of endogenously produced NO was shown to potentially enhance cisplatin-induced death of melanoma cells (54, 55). Other approaches, including altering protein–protein interactions and proteasome inhibition, would also be considered as future venues to study molecular and cellular mechanisms potentially underlying tumor resistance to chemotherapy (56–58).

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