Cell Cycle 12:4, 684–697; February 15, 2013; © 2013 Landes Bioscience

Phospho-ΔNp63α/microRNA feedback regulation in squamous carcinoma cells upon cisplatin exposure

Yiping Huang,[1] Dafna Kesselman,[1] Darya Kizub,[1] Rafael Guerrero-Preston[2] and Edward A. Ratovitski[1,2,*]

[1]Department of Dermatology; Johns Hopkins University School of Medicine; Baltimore, MD USA; [2]Department of Otolaryngology/Head and Neck Surgery; Johns Hopkins University School of Medicine; Baltimore, MD USA

Keywords: p53, p63, cisplatin, squamous cell carcinomas, DNA/protein interactions, microRNAs

Abbreviations: ARF, ADP-ribosylation factor; ATF, activation transcription factor; ATM, ataxia telangiectasia mutated; BANP, BTG3 associated nuclear protein; BCOR, BCL6 corepressor; BHLH, basic helix-loop-helix; CDKN, cyclin-dependent kinase inhibitor; C/EBP, CCAAT/enhancer binding protein; CCTF, CCCTC-binding factor; ChIP, chromatin immunoprecipitation; CIS, cisplatin; Con, control; CITED, CBP/p300-interacting transactivator; CRTC, CREB-regulated transcription coactivator; CTBP, c-terminal binding protein; DDIT, DNA-damage-inducible transcript; EP, E1A-binding protein; EZH, enhancer of zeste homolog; FL, FLAG; HDAC, histone deacetylase; GTF, general transcription factor; HSF, heat shock factor; IP, immunoprecipitation; iTRAQ, isobaric tags for relative and absolute quantification; JHMI, Johns Hopkins Medical Institutions; KAT, K(lysine) acetyltransferase; LC, liquid chromatography; MECP, methyl CpG binding protein; miR, microRNA; MS, mass spectrometry; NF, nuclear factor; p, phosphorylated; qPCR, quantitative PCR; RU, relative units; SCC, squamous cell carcinoma; SCX, strong cationic exchange; SP, specificity factor; SRY, sex determining region Y; TBPL, TATA-binding protein-Like; TFAP, transcription factor activation protein; TP, tumor protein; YAP, Yes-associated protein; ZBTB, zinc finger and BTB domain

Our previous reports showed that the cisplatin exposure induced the ATM-dependent phosphorylation of ΔNp63a, which is subsequently involved in transcriptional regulation of gene promoters encoding mRNAs and microRNAs in squamous cell carcinoma (SCC) cells upon cisplatin-induced cell death. We showed that phosphorylated (p)-ΔNp63a plays a role in upregulation of pro-apoptotic proteins, while non-p-ΔNp63a is implicated in pro-survival signaling. In contrast to non-p-ΔNp63a, p-ΔNp63a modulated expression of specific microRNAs in SCC cells exposed to cisplatin. These microRNAs were shown to attenuate the expression of several proteins involved in cell death/survival, suggesting the critical role for p-ΔNp63a in regulation of tumor cell resistance to cisplatin. Here, we studied the function of ΔNp63a in transcriptional activation and repression of the specific microRNA promoters whose expression is affected by cisplatin treatment of SCC cells. We quantitatively studied chromatin-associated proteins bound to tumor protein (TP) p63-responsive element, we found that p-ΔNp63a along with certain transcription coactivators (e.g., CARM1, KAT2B, TFAP2A, etc.) necessary to induce gene promoters for microRNAs (630 and 885-3p) or with transcription corepressors (e.g., EZH2, CTBP1, HDACs, etc.) needed to repress promoters for microRNAs (181a-5p, 374a-5p and 519a-3p) in SCC cells exposed to cisplatin.

Introduction

Acquired or intrinsic resistance of cells to the drug limits the use of cisplatin as an inducer of cell death in cancer chemotherapy.[1,2] Cisplatin treatment induces DNA damage stress, oxidative and endoplasmic reticulum stresses.[3] Several mechanisms are involved in cisplatin resistance, such as decreased intracellular drug accumulation and mismatch-repair activity and increased levels of cellular thiols and nucleotide excision-repair activity as well as altered expression of regulatory proteins involved in signal transduction pathways that control the cell death/cell survival pathways.[3]

Many genes are differentially expressed in sensitive and resistant tumor cells and involved in transcriptional regulation of mRNA and microRNA downstream targets implicated in DNA repairs and/or signal transduction, modulation of cell death and cell cycle arrest, and metabolomics.[3-5] Among key molecules whose expression is altered in cisplatin-resistant cells as compared with cisplatin-sensitive cells are the tumor proteins (TP) 53, TP63 and TP73, proto-oncoprotein c-Myc, Y-BoX binding protein-1 (YBX1), CCAAT-binding nuclear factor (NF)-Y, activating transcription factor (ATF) 4 and 5, CLOCK, single-stranded recognition protein (SSRP)-1 and some others, which by functioning as transcription factors influence cellular sensitivity to cisplatin

*Correspondence to: Edward A. Ratovitski; Email: eratovi1@jhmi.edu
Submitted: 12/21/12; Accepted: 01/13/13
http://dx.doi.org/10.4161/cc.23598

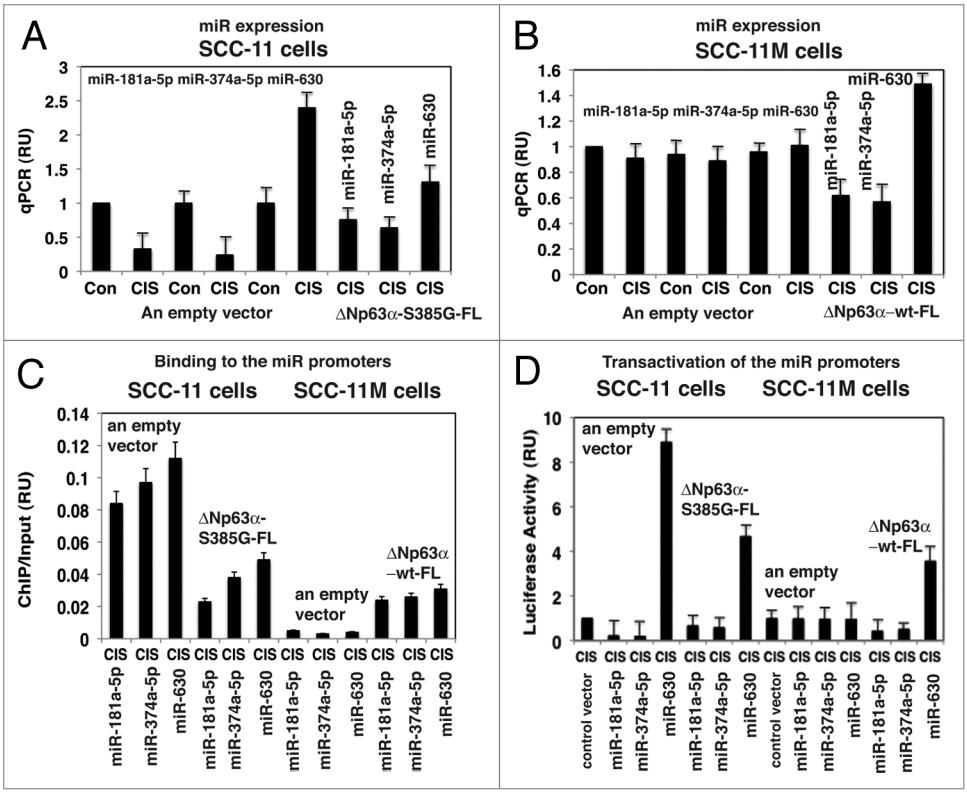

Figure 1. P-ΔNp63α regulated transcription of the specific microRNA promoters in SCC-11 cells upon cisplatin exposure. SCC-11 cells (**A**) and SCC-11M (**B**) cells were transfected with an empty vector and with the ΔNp63α-S385G-FL or ΔNp63α-wt-FL expression cassettes for 24 h, exposed to control media or 10 g/ml cisplatin (CIS) for 12 h and then tested for specific microRNA expression using qPCR (**A and B**) qPCR experiments were performed in triplicate with +SD as indicated (< 0.05). (**C**) Resulting SCC-11 and SCC-11M cells were used for ChIP analysis to identify the binding of ΔNp63α to the specific microRNA promoters. The amount of immunoprecipitated-enriched DNA in each sample (ChIP) is represented as signal relative to the total amount of input chromatin DNA (input) using the same primers for the specific promoter region. (**D**) SCC-11 cells and SCC-11M cells were also transfected with 100 ng of the control promoter-less pLightSwitch_Prom plasmid and the pLightSwitch_Prom plasmids containing promoter sequences of specific microRNAs (as indicated) and luciferase reporter gene as indicated. Cells were exposed to control medium without cisplatin (Con) and medium with 10 μg/ml cisplatin (CIS) for 12 h. RenSP Renilla luciferase reporter activity assays were conducted in triplicate (+SD are indicated, $p < 0.05$). Data presented as relative to data obtained from the control untreated cells containing the promoter-less reporter plasmid designated as 1.

exposure.[5-24] Loss of normal TP53 function or TP53 modifications confer resistance to cisplatin in various human cancer cells, while TP73 overexpression is associated with cisplatin resistance, and post-translational modifications of TP63 or lack thereof might contribute to tumor cell response to cisplatin exposure.[10-20]

The role for TP63 in cisplatin resistance is still under much scrutiny, and thereby its role in transcriptional regulation of specific genes implicated in cell survival, metabolomics and autophagy needs to be further examined. We previously reported that the exposure of squamous cell carcinoma (SCC) cells to cisplatin treatment induced the ataxia telangiectasia mutated (ATM) kinase-dependent production of phopshorylated (p)-ΔNp63α. We further reported that p-ΔNp63α and non-p-ΔNp63α (ΔNp63α-S385G protein, which is not phosphorylated by ATM kinase) differentially regulated transcription of mRNAs and microRNAs (miR) targets implicated in control of cell death and cell survival.[20,25-28] Global analysis of cisplatin-modulated gene expression in sensitive SCC cells and resistant SCC cells revealed a number of genes shown to respond to p-ΔNp63α or non-p-ΔNp63α, which thereby are likely targets for cisplatin sensitivity or resistance.[20] We then showed that both p-ΔNp63α and non-p-ΔNp63α are capable of differentially interacting with many proteins involved in signaling pathways of cell death/survival, including other transcription factors.[29] Moreover, we found that sensitive SCC cells express a dramatically greater ratio of p-ΔNp63α over non-p-ΔNp63α than resistant SCC cells.[30]

Using the combined DNA pull-down/iTRAQ (isobaric tag for relative and absolute quantitation) approach allowing the global analysis of transcription factors and chromatin accessory proteins bound to specific promoter,[31] we defined the critical components necessary to induce or repress ΔNp63α-dependent gene expression in SCC cells upon cisplatin exposure.

Results

P-ΔNp63α directly regulates the microRNA promoters in SCC cells upon cisplatin exposure. Our previous reports showed that the cisplatin-induced and ATM-dependent phosphorylation of ΔNp63α led to a dramatic deregulation of microRNA transcription and processing in SCC cells.[17,23] Specifically, a few microRNAs were downregulated [e.g., miR-181a-5p (181a), miR-374a-5p (374a) and miR-519a-3p (519a)], while a couple of microRNAs were upregulated (e.g., miR-630 and miR-885-3p) by p-ΔNp63α. Our subsequent reports showed that selected microRNAs modulated the expression of proteins implicated in signaling leading to apoptosis, cell cycle arrest, autophagy and DNA damage response.[24,25] However, the mechanistic nature of the p-ΔNp63α -dependent transcriptional regulation leading to activation or repression of microRNA expression in SCC cells upon cisplatin exposure remains unclear.

To examine the effect of p-ΔNp63α and non-p-ΔNp63α on the expression of tested microRNAs, we exposed SCC-11 cells and SCC-11M cells to control medium and 10μg/ml cisplatin for 12h. Using quantitative (q)-PCR analysis we showed that cisplatin induced the downregulation of miR-181a-5p and miR-374a-5p and upregulation of miR-630 in SCC-11 cells (**Fig. 1A**).

No such changes were found in SCC-11M cells under these experimental conditions (**Fig. 1B**). Moreover, the ΔNp63α-S385G-FL forced expression in SCC-11 cells substantially modulated downregulation of miR-181a-5p and miR-374a-5p, or upregulation of miR-630 in spite of cisplatin treatment (**Fig. 1A**). However, the ΔNp63α-FL forced expression in SCC-11M cells exposed to cisplatin mimicked the effect of the drug on SCC-11 cells (**Fig. 1B**), suggesting the critical role for the ΔNp63α phosphorylation in regulation of tested microRNA levels. These data essentially confirmed our previous observations.[23]

Using chromatin immunoprecipitation (ChiP) analysis, we further tested the ability of p-ΔNp63α to bind the selected microRNA promoters in both SCC-11 cells and SCC-11M cells exposed to cisplatin (**Fig. 1C**). We thus showed that the phosphorylation of ΔNp63α is necessary for the binding to the specific microRNA promoters, since transfection of SCC-11 cells with the exogenous ΔNp63α-S385G-FL construct dramatically attenuated this binding, while transfection of SCC-11M cells with the exogenous ΔNp63α-FL construct substantially increased this binding (**Fig. 1C**).

We next tested, whether p-ΔNp63α affects the luciferase gene expression driven by miR-181a-5p, miR-374a-5p and miR-630 gene promoters. As described in the Materials and Methods section, these custom-made constructs were introduced into both SCC-11 cells and SCC-11M cells subsequently exposed to 10μg/ml cisplatin for 12h (**Fig. 1D**). As control samples, the SCC-11 cells and SCC-11M cells transfected with an empty pLightSwitch_Prom, were used (**Fig. 1D**). In some experiments, SCC-11 cells were transfected with the exogenous ΔNp63α-S385G-FL construct, while SCC-11M cells were transfected with the exogenous ΔNp63α-FL construct (**Fig. 1D**). We showed that while the luciferase activities of pLightSwitch_miR-181a-5p-Prom and pLightSwitch_miR-374a-5p-Prom were downregulated, the luciferase activity of pLightSwitch_miR-630-Prom was upregulated in SCC-11 cells, but no changes were observed in SCC-11M cells (**Fig. 1D**). However, addition of ΔNp63α-S385G-FL to SCC-11 cells increased the luciferase gene activity under miR-181a-5p and miR-374a-5p promoters and decreased the luciferase activity under miR-630 promoter (**Fig. 1D**). At the same time, addition of ΔNp63α-FL to SCC-11M cells decreased the luciferase gene activity under miR-181a-5p and miR-374a-5p promoters and increased the luciferase activity under miR-630 promoter (**Fig. 1D**).

Interactome analysis of transcription factors bound to TP63-responsive elements in microRNA promoters. To examine what transcription factors and/or chromatin accessory proteins bound to microRNA promoters along with ΔNp63α near or on TP63-responsive element, we employed the DNA/protein bound iTRAQ-labeled technology coupled with the liquid chromatography (LC)/double mass-spectrometry (MS/MS) analysis.[28] SCC-11 cells (three samples) and SCC-11 M cells (two samples) were treated with 10 μg/ml cisplatin for 12 h, and then nuclear lysates were obtained from 5×10^9 cells, which were subsequently incubated with MagnaBind streptavidin beads with the 25nmol bead-bound complementary double-stranded oligonucleotides (50 base pairs) corresponding to the specific regions of the specific promoters for miR-181a-5p, miR-519a-3p, miR-374a-5p, miR-630 and miR-885-3p encompassing TP63-responsive element (**Fig. 2**; **Fig. S1–5**), as described in the **Supplemental Methods** section.

We quantitatively compared the TP63-bound protein complexes in SCC-11 cells or SCC-11M cells exposed to cisplatin, as previously described.[26] For iTRAQ labeling, 200 μg of eluted protein from large-scale DNA pull-downs were digested with trypsin, and peptides were purified and labeled with the following iTRAQ reagents: products isolated from SCC-11 cells exposed to 10μg/ml cisplatin were labeled with iTRAQ reagents (115, 116 and 117), whereas products isolated from SCC-11M cells exposed to cisplatin were labeled with iTRAQ reagents (113 and 114), respectively. The specific mixes were fractionated by strong cationic exchange high-pressure LC followed by MS/MS analysis, as previously described. Among proteins bound to TP63-responsive element that met the stringent statistical criteria (e.g., iTRAQ ratio > 1.25 and < 0.75), we identified 38 proteins [iTRAQ ratios ranged from 0.219 (depleted) to 8.854 (enriched), **Tables S1–5**]. As shown, the miR-181a-5p promoter sequence was enriched with 12 proteins, and depleted with three proteins, while the miR-519a-3p and miR-374a-5p promoter sequences were enriched with 12 and 10 proteins, respectively (and depleted with two and five proteins, respectively) in SCC-11 cells compared with SCC-11M cells (**Table S1–3**). At the same time, the miR-630 and miR-885-3p promoter sequences were enriched with 13 and 12 proteins, respectively (and depleted with five and four proteins, respectively) in SCC-11 cells compared with SCC-11M cells (**Table S4 and S5**). To confirm data obtained from the quantitative MS analysis, we performed immunoblotting assays with proteins bound to TP63-responsive elements in SCC-11 cells compared with SCC-11M cells exposed to cisplatin treatment. We showed that many proteins identified by quantitative MS are indeed differentially bound to TP63-responsive elements found in the selected microRNA promoters (**Fig. 2**).

In total ~18 proteins might be potentially involved in transcription repression of the selected microRNA promoters (miR-181a-5p, miR-519-3p and miR-374a-5p; **Tables S1–S3**), while totally ~16 proteins could play a role in transcription activation of the microRNA promoters (miR-630 and miR-885-3p; **Tables S4 and S5**) in SCC-11 cells exposed to cisplatin (**Fig. 3**). At the same time, we found 10 proteins that might function in transcription activation, while eight proteins are likely to act as transcriptional repressors in SCC-11M cells exposed to cisplatin (**Fig. 4**).

Notably, in addition to p-ΔNp63α (known to be phosphorylated by ATM kinase), which essentially recognized TP63-responsive element in the tested microRNA promoters, the following proteins were a part of the complex that support activation of the miR-630 and miR-885-3p promoters: heat shock factor 1 (HSF1), general transcription factor 2B (GTF2B), proto-oncogene c-REL, specificity factors SP1 and 3, activating transcription factor 2 (ATF2), CREB-regulated transcription coactivator 2 (CRTC2), Cbp/p300-interacting transactivator 2 (CITED2), specificity factor (SP1 and 3), E1A-binding protein, p300 (EP300), TATA-binding protein-like 1 (TBPL1), proto-oncogene c-MYB, DNA damage-inducible transcript 3

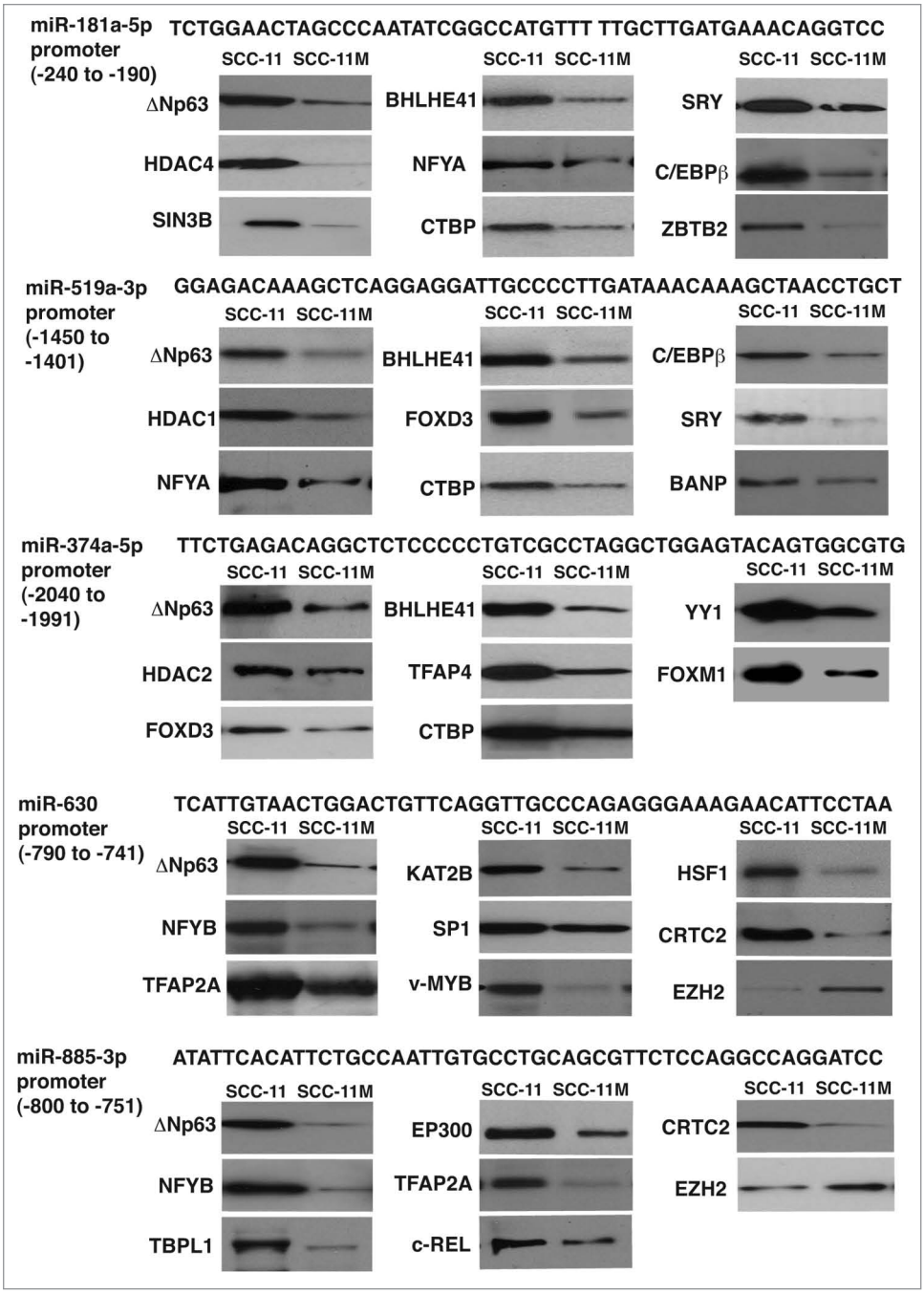

Figure 2. Various transcription factors bound to the specific microRNA promoters in SCC upon cisplatin exposure. SCC-11 cells and SCC-11M cells were exposed to 10 μg/ml cisplatin for 12 h. Nuclear lysates were incubated with the 50 bp sequences (locations are indicated in parentheses) derived from the specific microRNA promoters (miR-181a-5p, miR-519-3p, miR-374a-5p, miR-630 and miR-885-3p) containing the predicted TP63 binding site. Proteins bound to the tested sequences were analyzed by iTRAQ (LS/MS/MS) and validated by immunoblotting assays with the indicated antibodies.

(DDIT3), K(lysine) acetyltransferase 2B (KAT2B, PCAF), transcription factor activation protein 2A (TFAP2A) and nuclear factor YB (NFYB) as shown in **Figure 3A**. At the same time, the following proteins took a part in the repression complex for the miR-181a-5p, miR-519a-3p and miR-374a-5p promoters: BTG3-associated nuclear protein (BANP, SMAR1), Yin and Yang 1 (YY1), Forkhead box M1 FOXM1, FOXD3, sex-determining region Y SRY, CCAAT/enhancer binding protein β (C/EBPβ), histone deacetylase (HDAC1, HDAC2, HDAC4), methyl CpG binding protein 2 (MECP2), CCCTC-binding factor (CTCF), SIN3 transcription regulator homolog B (SIN3B), C-terminal binding protein 1 (CTBP1), basic helix-loop-helix family member E41 (BHLHE41), zinc finger and BTB domain containing 2 ZBTB2, nuclear factor YA (NFYA) and transcription factor activation protein 4 (TFAP4) as shown in **Figure 3B**. On the other hand, non-p-ΔNp63α was shown to bind to the following proteins likely involved in gene activation: GTF2B, CRTC2, CITED2, SP1, TBPL1, TFAP2A, KAT2B, K(lysine) acetyltransferase 5 (KAT5) and coactivator-associated arginine methyltransferase 1 (CARM1) shown in **Figure 4A**, while transcription repressors bound to non-p-ΔNp63α include enhancer of zeste homolog 2 (EZH2), HDAC1, HDAC2, CTCF, SIN3B, suppressor of zeste 12 homolog (SUZ12) and C-terminal binding protein 1 (CTBP1) as shown in **Figure 4B**.

MicroRNAs modulate the protein levels of TP63 and other transcription factors in SCC cells upon cisplatin exposure. Using miRDB-MicroRNA target prediction and functional study database, we found that the microRNAs previously shown to be induced or repressed by p-ΔNp63α in SCC-11 cells upon cisplatin exposure, in fact, showed a homology to the respective "seed" sequences at the ΔNp63α mRNA 3'-untranslated region (UTR, target prediction scores ranging from 53 to 71 as shown in **Fig. 5A**). These observations suggested that the specific microRNAs (e.g., miR-181a-5p, miR-374a-5p, miR-519a-3p, miR-630 and miR-885-3p) are likely to modulate the expression of ΔNp63α protein in SCC-11 and/or SCC-11M cells.

First, we showed that when SCC-11 cells and SCC-11M cells were transfected with the scrambled RNA, the cisplatin treatment caused the downregulation of ΔNp63α level in SCC-11 cells, but failed to do so in SCC-11M cells suggesting that

p-ΔNp63α -dependent expression of microRNA species might play a role in this process (**Fig. 5B**). We further examined the direct effect of mimics and inhibitors for miR-181a-5p, miR-374a-5p, miR-519a-3p, miR-630 and miR-885-3p on the luciferase activity controlled by TP63 3'-UTR using the pLightSwitch_3UTR-TP63 plasmid. We thus found that mimics for miR-181a-5p, miR-374a-5p, miR-519a-3p, miR-630 and miR-885-3p repressed the pLightSwitch_3UTR-TP63 luciferase activity, while inhibitors for these microRNAs induced the luciferase activity in SCC-11 cells and SCC-11M cells grown in control medium (**Fig. 5B and C**). We further found that the ΔNp63α protein levels were differentially attenuated by selected microRNAs tested on SCC-11 cells (ranging from 0.11 to 0.82 from scrambled control designated as 1 and normalized for the β-actin levels in total lysates), as shown in **Figure 5D**.

We next examined whether selected microRNAs might affect the expression of transcription factors and chromatin accessory proteins shown to bind the TP63-responsive element in the microRNA promoters. Again, using miRDB-microRNA target prediction and functional study database, we found that the 3'-UTR for several mRNAs of interest contained the respective "seed" sequences (target prediction scores ranging from 65 to 91, as shown in **Fig. 6A**). We then tested whether, in fact, specific microRNA species could affect the luciferase activity regulated by 3'-UTR sequences derived from ATM, CARM1, EP300, NFYB, BHLHE41, KAT2B, EZH2 and TBPL1. SCC-11 cells were transfected with the control scrambled RNA along with the empty pLightSwitch_3UTR vector (**Fig. 6B**). SCC-11 cells were also transfected with the pLightSwitch_3UTR-ATM, pLightSwitch_3UTR-BHLHE41, pLightSwitch_3UTR-CARM1, pLightSwitch_3UTR-NFYB, pLightSwitch_3UTR-KAT2B, pLightSwitch_3UTR-EP300, pLightSwitch_3UTR-EZH2 and pLightSwitch_3UTR-TBPL1 (**Fig. 6B**). We observed that while individual microRNAs had a slight inhibitory effect on the ATM-luciferase activity, the combination of all three microRNAs (miR-181a-5p, miR-374a-5p and miR-519a-3p) dramatically reduced the ATM-luciferase activity (**Fig. 6B**). MiR-519a-3p miR-885-3p, miR-374a-5p, miR-181a-5p and miR-630 were shown to markedly reduce the luciferase activities of the BHLHE41, CARM1, NFYB, KAT2B, EP300, EZH2 and TBPL1 vectors (**Fig. 6B**).

We next tested the effect of cisplatin treatment, as well as tested microRNAs on levels of the selected target proteins (e.g., ATM, BHLHE41, EZH2 and CARM1) in total lysates of SCC-11 cells and SCC-11M cells (**Fig. 7**). We also used siRNA directed against ATM, BHLHE41, EZH2 and CARM1 to modulate the expression of tested proteins in SCC-11 cells and SCC-11M cells (**Fig. 7**). We found that cisplatin induced the expression of ATM and BHLHE41 in SCC-11 cells transfected with the scrambled siRNA. However, miR-181a-5p together with miR-374a-5p and miR-519-3p or miR-519a-3p offseted the cisplatin effect on these proteins, respectively (**Fig. 7A and C**). Similarly, siRNAs against ATM and BHLHE41 attenuated the expression of both tested proteins (**Fig. 7A and C**). We further observed that cisplatin induced the expression of EZH2 and

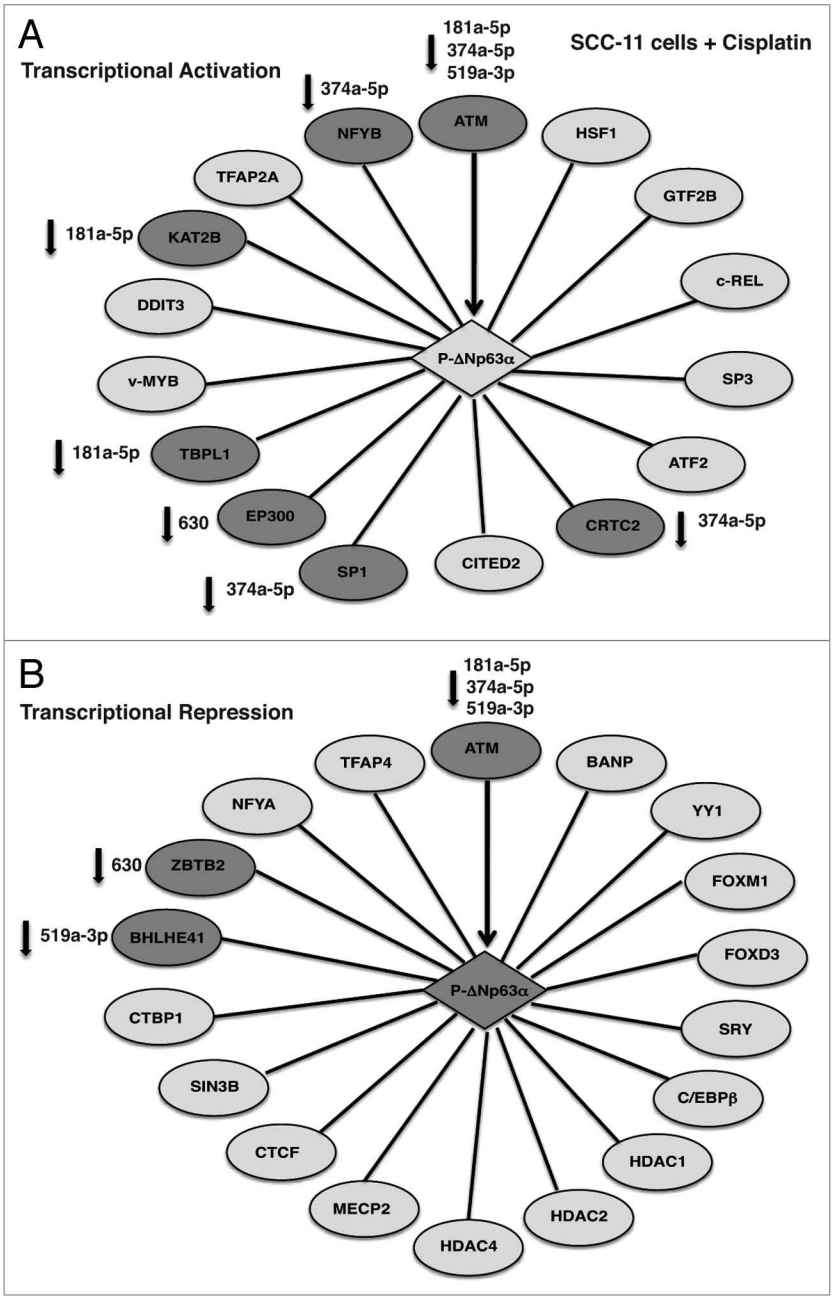

Figure 3. Schematic representation of p-ΔNp63α-dependent protein interaction network involved in transcriptional activation (**A**) or repression (**B**) of the specific microRNA promoters in SCC-11 cells upon cisplatin exposure. Proteins that are potentially affected by specific microRNAs highlighted in darker shades and specific microRNAs indicated.

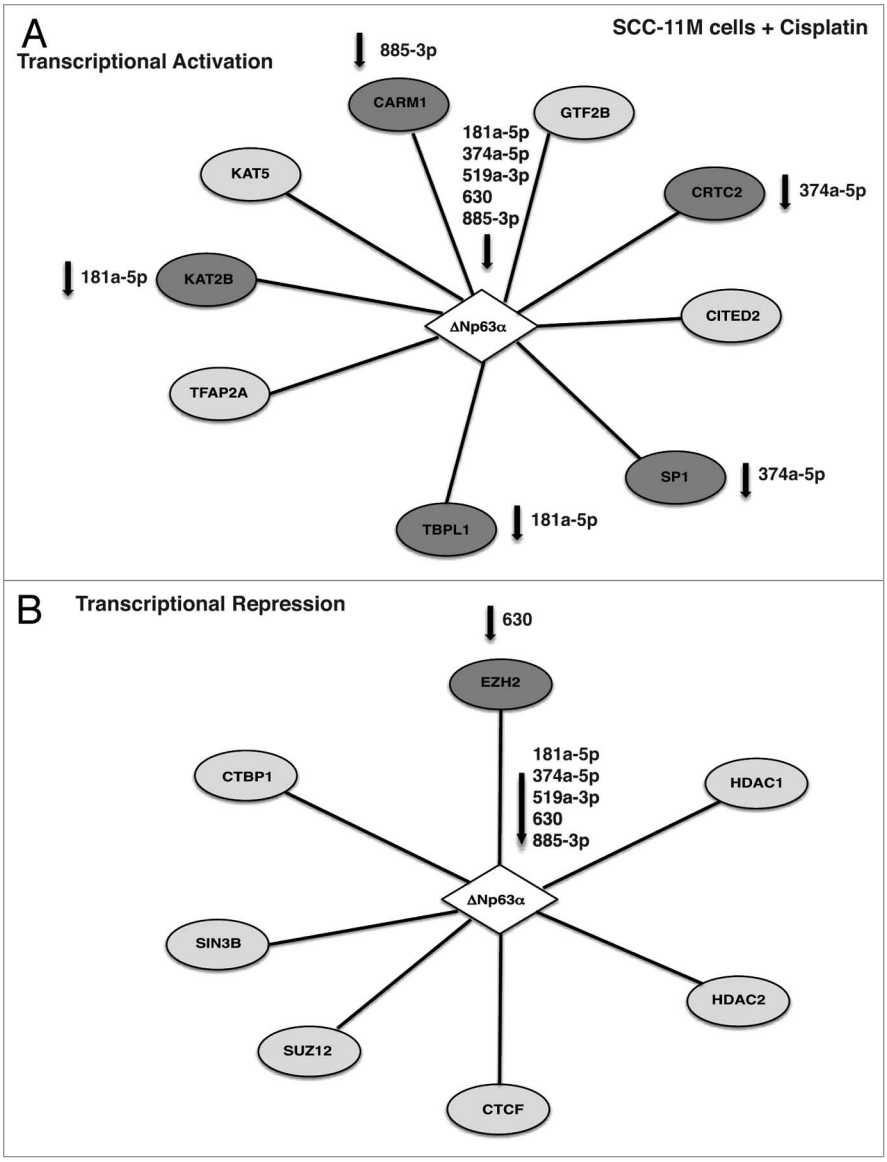

Figure 4. Schematic representation of ΔNp63α-dependent protein interaction network involved in transcriptional activation (**A**) or repression (**B**) of the specific microRNA promoters in SCC-11M cells upon cisplatin exposure. Proteins that are potentially affected by specific microRNAs highlighted in darker shades and specific microRNAs indicated.

CARM1 in SCC-11M cells transfected with the scrambled siRNA. However, miR-630 or miR-885-3p counteracted the cisplatin effect on these proteins, respectively (**Fig. 7B and D**). Similarly, siRNAs against EZH2 and CARM1 decreased the expression of both tested proteins (**Fig. 7B and D**). Although, the cisplatin exposure of SCC-11 cells caused a dramatic decrease in cell viability, siRNAs against both ATM and BHLHE41 partially reversed this cisplatin effect on sensitive SCC-11 cells, rendering them to become more resistant to cisplatin treatment (**Fig. 7E**). On the other hand, the cisplatin exposure of SCC-11M cells had a more moderate effect on the cell viability, while siRNAs against both CARM1 and EZH2 rendered the resistant SCC-11M cells to be more sensitive to cisplatin-induced cell death (**Fig. 7F**).

Discussion

Defects in the DNA damage response signaling (e.g., ATM signaling) often lead to an increased susceptibility to cancer and thereby represent novel therapeutic targets.[10,13,32-35] Exposure of tumor cells to cisplatin chemotherapy often induces DNA damage associated with expression of mRNAs whose encoded proteins implicated in cell cycle arrest and apoptosis.[16,18,32-35] Moreover, numerous microRNAs involved in regulation of transcription and protein stability are also deregulated in cancer cells exposed to cisplatin treatment.[4,5,26,36-40]

We previously reported that SCC cells enabling the ATM-dependent phosphorylation of ΔNp63α upon cisplatin exposure showed altered expression of the specific mRNAs or microRNAs, displaying reduction of the miR-181a-5p, miR-519-3p and miR-374a-5p levels and induction of the miR-630 and miR-885-3p levels.[26] We further found that the p-ΔNp63α protein, in association with NFY proteins, binds to the specific mRNA and microRNA promoters in SCC-11 cells, while it failed to bind these promoters in SCC-11M cells.[25,26] We found here that the cisplatin-induced p-ΔNp63α decreased the reporter activity driven by the miR-181a-5p and miR-374a-5p promoters, while increased the activity of reporter luciferase gene fused to the miR-630 and miR-885-3p promoters.

In this study, we focused on the cisplatin-induced TP63/microRNA functional relationship in SCC cells attempting to address the question why p-ΔNp63α reduced transcription of some microRNAs and induced others. Since, expression of microRNAs is maintained by RNA polymerase II and III transcription machinery, we suggested that the regulatory role of p-ΔNp63α is intimately intertwined with other transcription factors and other chromatin accessory proteins.

Since TP63-responsive elements in the tested microRNA promoters are surrounded by other transcription factor cognate binding sites (**Figs. S1–5**), we analyzed the protein complexes formed between ~50 oligos derived from the specific regions of the microRNA promoters in question (miR-181a-5p, miR-519-3p, miR-374a-5p, miR-630 and miR-885-3p, **Fig. 2**), as described elsewhere.[31] We used a pull-down of proteins (from SCC-11 cells and SCC-11M cells exposed to cisplatin) bound to 50bp oligos followed by tryptic digestion. Resulting peptides were labeled with iTRAQ reagents and subsequently subjected to LC/MS/MS fractionation and characterization, as was previously described for TP53 and ΔNp63α-interacting proteins. Using the

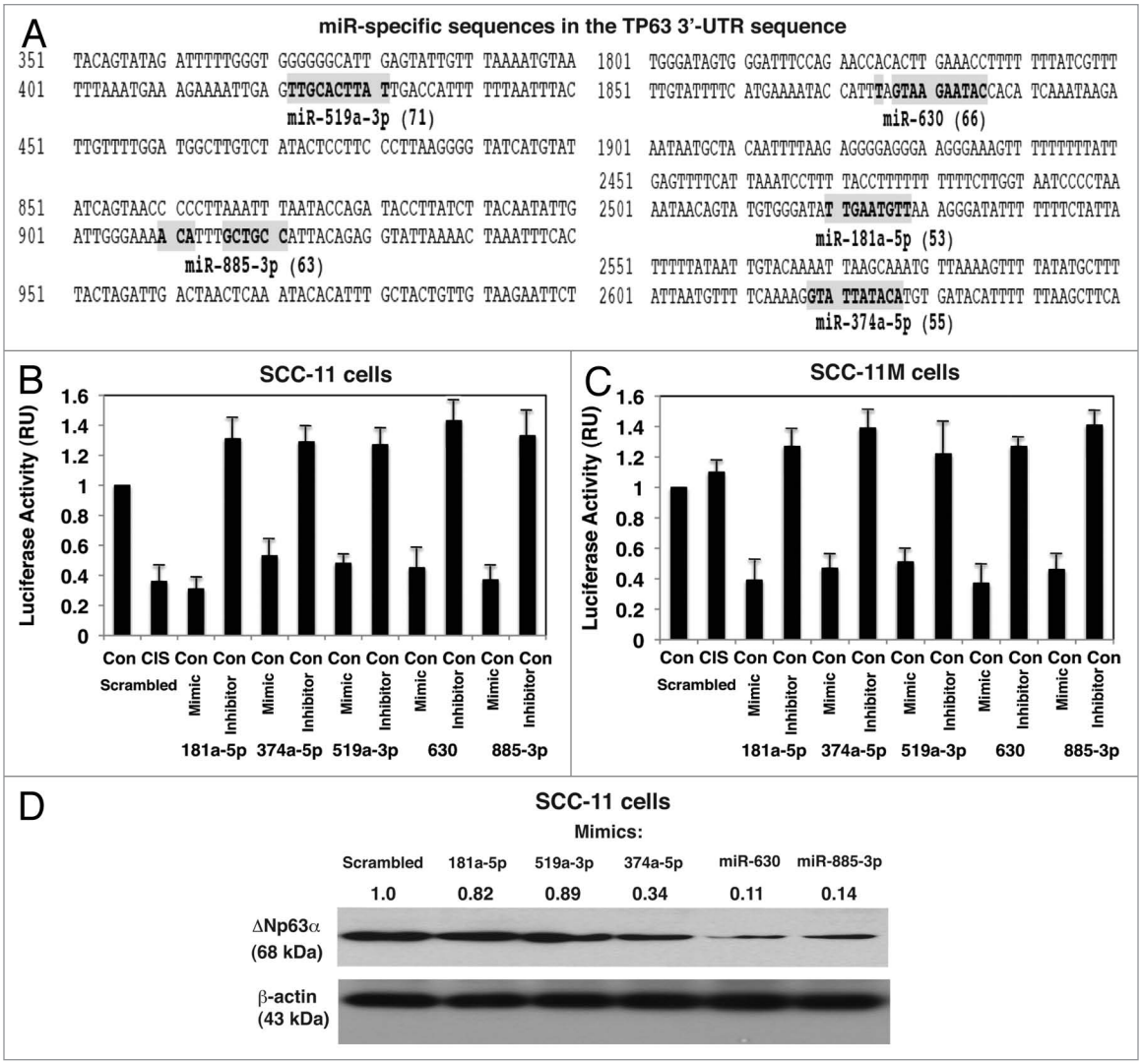

Figure 5. Specific microRNA mimics and inhibitors modulated the expression of ΔNp63α via its 3'-UTR sequences upon cisplatin exposure. (**A**) Predicted "seed" sequences for specific miRs in the TP63 3'-UTR with the target prediction scores in parentheses. SCC-11 cells (**B**) and SCC-11M cells (**C**) were transfected with the LightSwitch_3UTR vector for the TP63 3'-UTR along with the scrambled microRNA, or mimics and inhibitors for miR-181a-5p, miR-374a-5p, miR-519-3p, miR-630 and miR-885-3p for 36 h. Cells were treated with control medium without cisplatin (Con) or medium with 10 μg/ml cisplatin (CIS) for additional 12 h and then tested for the RenSP Renilla luciferase reporter activity. Measurements (in triplicate) for the luciferase activity presented as relative units (RU). Values obtained from cells transfected with the scrambled RNA and treated with control medium were designated as 1. (**D**) Total lysates from the resulting SCC-11M cells from (**C**) treated with control medium were subsequently analyzed by immunoblotting with antibodies to ΔNp63α and β-actin. Relative levels of ΔNp63α normalized for β-actin levels were quantified and shown above immunoblot images. Levels of ΔNp63α in cells with the scrambled microRNA were designated as 1.

combination of DNA binding and quantitative mass spectrometry identification of proteins bound to specific promoter DNAs, we defined the critical components necessary to induce or repress p-ΔNp63α-dependent microRNA gene expression in SCC cells upon cisplatin exposure, including transcription factors, regulators and chromatin accessory proteins bound to the specific microRNA promoters. We thus found that the following proteins might be involved in transcriptional induction of the miR-630 and miR-885-3p promoters in SCC-11 cells upon cisplatin exposure: HSF1, GTF2B, c-REL, SP1 and 3, ATF2, CRTC2, CITED2, EP300, TBPL1, v-MYB, DDIT3, KAT2B, TFAP2A and NFYB. We further found that the following proteins could take a part in the repression complex for the miR-181a-5p, miR-519a-3p and miR-374a-5p promoters in SCC-11 cells treated with cisplatin: BANP, YY1, FOXM1, FOXD3, SRY, C/EBPβ, HDAC1, HDAC2, HDAC4, MECP2, CTCF, SIN3B, CTBP1, BHLHE41, ZBTB2, NFYA and TFAP4. On the other hand, the following proteins are likely to be involved in gene activation of the specific promoters in SCC-11M cells treated with cisplatin: GTF2B, CRTC2, CITED2, SP1, TBPL1, TFAP2A, KAT2B, KAT5 and CARM1, while transcription repressors include EZH2, HDAC1, HDAC2, CTCF, SIN3B, SUZ12 and CTBP1.

We further observed that the selected microRNAs (miR-181a-5p, miR-519-3p, miR-374a-5p, miR-630 and miR-885-3p)

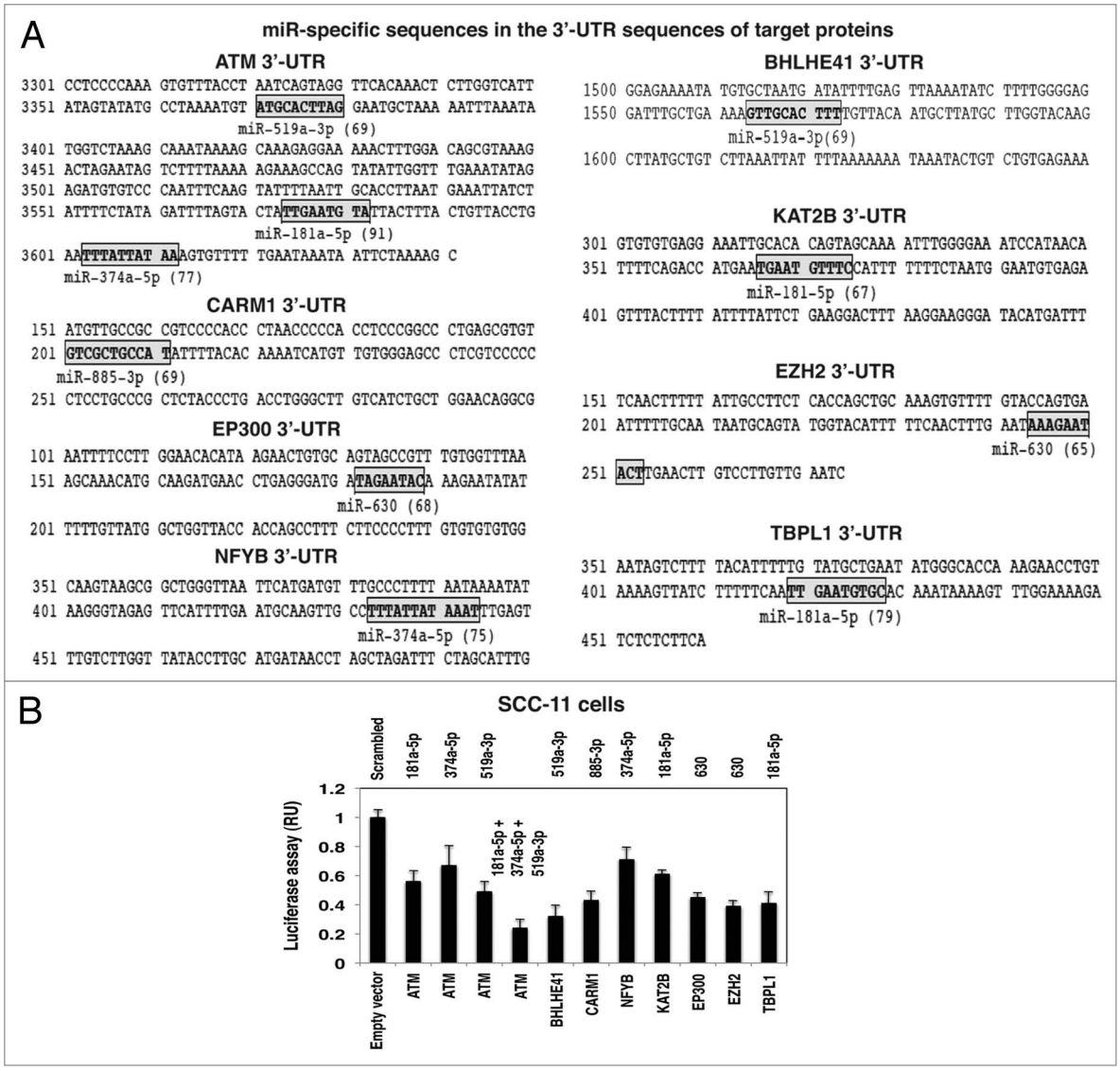

Figure 6. Specific microRNA mimics modulated the expression of the ΔNp63α protein interacting targets via their 3'-UTR sequences. (**A**) Predicted "seed" sequences for specific microRNAs in the protein target 3'-UTRs with the target prediction scores in parentheses. (**B**) SCC-11 cells were transfected with the LightSwitch_3UTR plasmids for the indicated protein 3'-UTRs along with the scrambled microRNA and mimics for miR-181a-5p, miR-374a-5p, miR-519-3p, miR-630 and miR-885-3p for 36 h. Cells were then tested for the RenSP Renilla luciferase reporter activity. Measurements (in triplicate) for the luciferase activity presented as relative units (RU). Values from cells transfected with the scrambled RNA were designated as 1.

modulated the stability of specific transcription factors (ATM, CARM1, EP300, NFYB, BHLHE41, KAT2B, EZH2 and TBPL1), including TP63 shown by the prediction target analysis of "seed" sequences, luciferase reporter and immunoblotting assays in SCC cells. Finally, we showed that siRNA knockdown of selected targets (modulated by microRNAs whose transcription is regulated by p-ΔNp63α), notably ATM and BHLHE41 might render sensitive SCC-11 cells to become more resistant to cisplatin-induced cell death. However siRNA silencing of EZH2 and CARM1 would render more resistant SCC-11M cells to be more sensitive to cisplatin-induced cell death.

Taken together, we demonstrated that the p-ΔNp63α protein can transcriptionally regulate the microRNA gene promoters by forming protein complexes with other transcriptional and chromatin-associated factors, while total ΔNp63α levels, and p-ΔNp63α levels (via downregulation of ATM) are maintained through a microRNA-mediated post-transcriptional/translational machinery, thereby providing a regulatory feedback for selected microRNAs and their respective promoters.

Control of gene expression is exerted at a number of different levels, one of which is the accessibility of gene promoters to the transcriptional machinery.[41-52] Intriguingly, among many proteins forming complexes with p-ΔNp63α bound to the TP63-responsive element in the tested microRNA promoters, one can find specific transcription coactivators (CARM1, CITED2, CTRC2, DDIT3, c-REL, SP1, SSRP1, TFAP2A and YAP) or corepressors (BHLHE41, CTBP1, EZH2, YY1 and ZBTB20), histone acetyltransferases (EP300, KAT2B)

and histone deacetylases (HDAC1, 2 and 4) are also likely to be associated with drug-induced cell death and especially with cisplatin resistance of tumor cells.[46,47,53-59]

Among transcription activators, the CARM1-mediated arginine methylation is shown to play a role in regulation of histone acetylation and transcription: facilitating transcription by discharging corepressors from chromatin and thereby has been linked to transcriptional regulation, cell cycle regulation and DNA repair.[60-62] CARM1 is a novel transcriptional coactivator of nuclear factor kappa B (NFκB) and functions as a promoter-specific regulator of NFκB recruitment to chromatin. CARM1 forms a complex with EP300 and NFκB in vivo and interacts directly with the NFκB subunit p65 in vitro. Moreover, CARM1 synergistically coactivates NFκB-mediated transactivation, in concert with the transcriptional coactivators, EP300 and / CREB-binding protein (CREBBP), as previously described.[60]

Second, Yes-associated Protein (YAP) transcriptional coactivator has been implicated in tumorigenesis by regulating cell proliferation and apoptosis.[63,64] YAP is phosphorylated in response to genotoxic stress induced by cisplatin treatment.[63] Physical association of YAP and ΔNp63α was markedly enhanced by cisplatin. YAP coactivator activity correlated with its state of phosphorylation and sensitivity to cisplatin-induced apoptosis.[64]

Third, short-hairpin RNA against Cited2 transcriptional modulator sensitized cancer cells to cisplatin, suggesting that acquired cisplatin resistance of cancer cells could be reversed by Cited2 silencing.[65] Fourth, histone acetyltransferase genes, Kat2b (Pcaf), Clock and Tip60, are overexpressed, subsequently inducing the expression of DNA repair genes in cisplatin-resistant cells.[54] Finally, Tfap2a was identified as a strong independent predictive marker for a good response and survival after cisplatin-containing chemotherapy.[22] siRNA-mediated knockdown of Tfap2a increased the cell proliferation and rendered the cancer cells to become more resistant to cisplatin.[22]

Among transcription repressors, EZH2, a specific histone-3 lysine-27 (H3K27) methyltransferase, plays a critical role in tumorigenesis and cancer progression through epigenetic gene silencing and chromatin remodeling.[53,66-69] EZH2 was overexpressed in cisplatin-resistant ovarian cancer cells compared with cisplatin-sensitive cells.[53] Knockdown of EZH2 by siRNA resensitized drug-resistant ovarian cancer cells to cisplatin and decreased the level of H3K27 trimethylation.[53] Loss of EZH2 also enhanced sensibility of tumor xenografts to cisplatin and inhibited tumor growth in vivo.[53] EZH2 is one of the potent

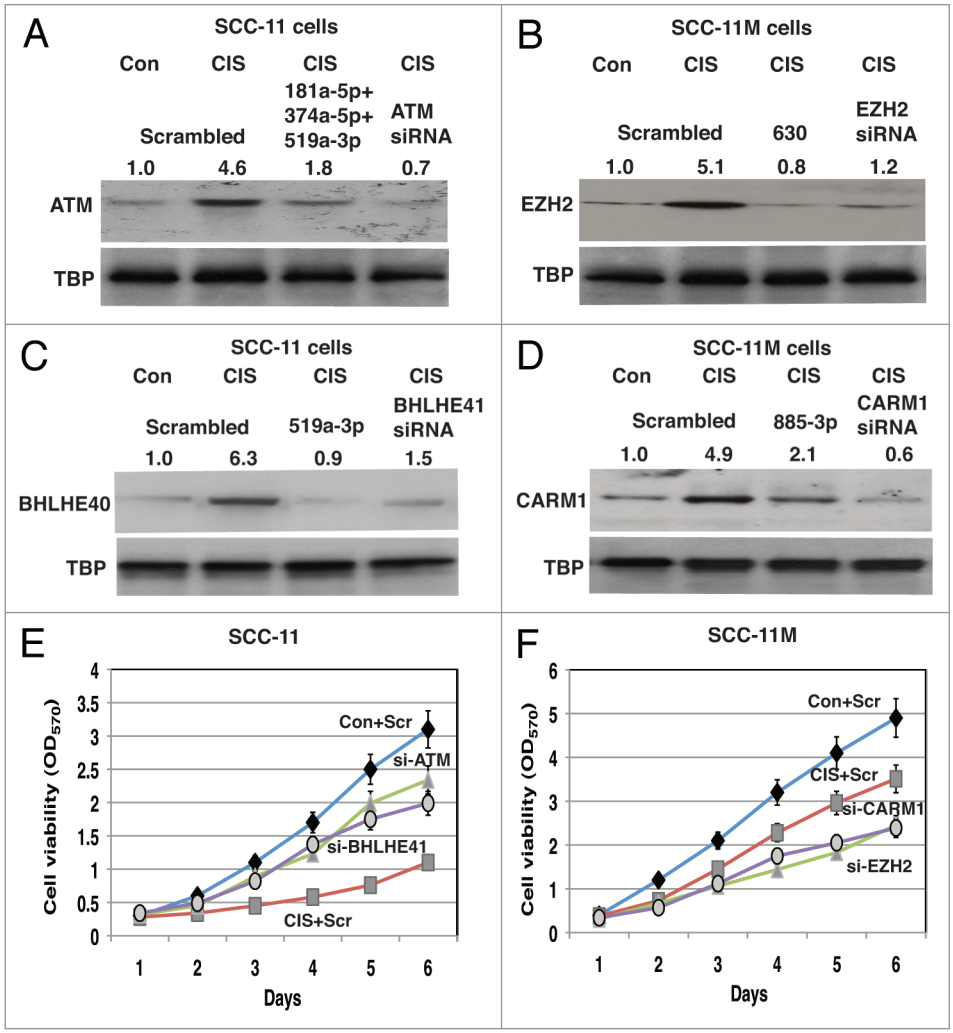

Figure 7. Specific microRNA mimics modulated expression of the ΔNp63α protein interacting targets in SCC cells exposed to cisplatin and affected cell viability. Immunoblotting assay. SCC-11 cells (**A and C**) and SCC-11M cells (**B and D**) were transfected with the scrambled microRNA and indicated microRNA mimics for 36 h. Cells were then treated with control medium without cisplatin (Con) or medium with 10 μg/ml cisplatin (CIS) for additional 12 h and nuclear lysates were tested for indicated endogenous proteins. Loading levels were tested using a TBP antibody. Relative protein levels normalized for the TBP levels were quantified and shown above immunoblot images. Protein levels in cells with the scrambled miR were designated as 1. Cell viability assay. (**E**) SCC-11 cells were transfected with the scrambled siRNA (Scr) and siRNAs against ATM (si-ATM) or BHLHE41 (si-BHLHE41). (**F**) SCC-11M cells were transfected with the scrambled siRNA (Scr) and siRNAs against CARM1 (si-CARM1) or EZH2 (si-EZH2). Resulting cells were cultured in the presence (CIS) or absence (Con) of the 10 μg/ml cisplatin for indicated times. 10^4 cells/well in 96-well plates were then incubated in serum-free medium with 5 μg/ml of the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide in the dark for 4 h at 37°C. Cells were lysed and incubated for 2 h at 37°C, and the measurements were obtained on a Spectra Max-250 plate reader. Each assay was repeated at three times in triplicate. The bars are the mean ± SD of triplicate; $p < 0.05$, t-test.

regulators of the accessibility of gene promoters and a part of the Polycomb repressive complex 2 (PRC2) along with SUZ12 and EED catalyzing trimethylation of H3K27 and subsequently recruiting other DNA methyltransferases and histone deacetylases, resulting in transcriptional repression.[68,69] EZH2 overexpression promotes the proliferation and invasion of epithelial ovarian and prostate cancer cells, contributing to cell resistance to cisplatin exposure and suggesting that EZH2 is a potential target for developing cancer therapeutics.[66,67]

Second, FOXM1, an oncogenic transcription factor, promotes tumorigenesis by regulating genes associated with cell cycle progression and cell proliferation, and its inhibition has been shown to sensitize cancer cells to apoptosis.[55,56] The anti-FoxM1 siRNA can be functional when administered into tumors in vivo and holds potential as part of a therapy for cancer treatment.[70]

Third, BHLHE41 (DEC2/SHARP1) is a basic helix-loop-helix transcription repressor involved in the regulation of apoptosis, cell proliferation and cisplatin resistance.[71-73] The expression of BHLHE41 was upregulated by cisplatin, while its forced expression inhibited pro-apoptotic facilitator BCL2-interacting protein (BIM), thereby blocking apoptosis. Interestingly, CTBP1 corepressor was shown to be a component of the RBP-Jκ/SHARP-co-repressor complex, which augmented the SHARP-mediated transcription repression.[75,76]

Fourth, ZBTB2, a POK family transcription factor, repressed transcription of the ADP-ribosylation factor (ARF), TP53 and cyclin-dependent kinase inhibitor 1A (CDKN1A) genes.[77] ZBTB2 was shown to interact with SP1 and TP53, thereby inhibiting SP1-induced and TP53-dependent transcription activation. ZBTB2 also interacted with the complex of HDAC3, BCL6 corepressor (BCOR) and nuclear receptor corepressors (NCOR1 and 2), leading to an additional transcription repression.[77] Although the forced ZBTB2 expression stimulated cell proliferation, its knockdown decreased cell proliferation.[77]

Finally, recent reports showed strong evidence that HDACs are implicated in transcription repression and cisplatin resistance.[78-82] Following DNA damage, HDAC4 becomes recruited on NFY-dependent repressed G_2/M gene promoters through a TP53-dependent mechanism.[78] Platinum therapy induced a significantly enhanced apoptosis in resistant ovarian cancer cells transfected with HDAC4 siRNA, suggesting that HDAC4 is likely to be a beneficial target to counter platinum resistance in ovarian cancer.[78]

ΔNp63α was specifically shown to associate with HDAC1 and HDAC2 to form an active transcriptional repressor complex that can be targeted to therapeutic advantage.[82] Cisplatin chemotherapy as well as HDAC inhibitors promoted dissociation of ΔNp63α and HDAC from the pro-apoptotic gene *Puma* promoter, in turn leading to increased histone acetylation, *Puma* expression and apoptosis.[82]

Our study established a new functional link between p-ΔNp63α and the deregulated microRNA promoters in SCC cells exposed to cisplatin, suggesting that a complex transcriptional machinery involving p-ΔNp63α could potentially act as a regulator of death or survival of SCC cells during chemotherapy. Thus, therapeutic compounds deactivating ΔNp63α phosphorylation and/or its downstream microRNA targets could be used in combination with cisplatin to induce optimal tumor regression of human cancers that overexpress p-ΔNp63α.

Transcriptional regulation of both mRNA and microRNA genes is maintained by multiple layers of molecular control including binding of transcription factors to promoter sequences and RNA polymerase initiation complex, modifications (acetylation/deacetylation, phosphorylation/dephoshorylation, methylation/demethylation) of DNA and histones, gene accessibility via nucleosome and chromatin remodeling, and transcriptional cycling.[41,42,44,47,52] Each of these regulatory layers plays a critical role in activation/repression of target gene promoters and future investigations needed to clarify their contributions to the mRNA and microRNA regulatory network under chemotherapeutic treatments.

Materials and Methods

Antibodies. We used a rabbit polyclonal antibody Ab-1 directed against human ΔNp63 (EMD Chemicals), and monoclonal antibodies against human β-actin (Sigma) and TATA-binding protein (TBP, 1TBP18, ab818, Abcam). Mouse monoclonal antibodies to p63 (4A4, sc-8431), to SIN3B (H-4, sc-1314), to C/EBPβ (47A1, sc-56637), to TFAP2A (H-79, sc-8975), to c-MYB (3H2746, sc-73247), to TBPL1 (C-16, sc-10105) and to ATM (ATM 11G12, sc-53173) were obtained from Santa Cruz Biotechnology. We also used rabbit polyclonal antibodies against human NFYA (NBP1-19146), HDAC2 (NB100-2232, Novus), CtBP1 (NBP1-44886), FOXD3 (NB100-78525), TFAP4 (NBP1-46201), CARM1 (NB100-920) and a monoclonal antibody against BHLHE41 (SHARP1, 4H6, H00079365-M01), all purchased from Novus Biologicals. Antibodies to NFYB (PAB0659), to (PAB12512), to HDAC1 (PAB0647), to SRY (clone SRY.G12, MAB8814) were all obtained from Abnova. We then used the following rabbit polyclonal antibodies from Bethyl Laboratories: anti-FOXM1 (A301-532A), anti-YY1 (A302-778A), anti-PCAF (KAT2B, A301-666A), anti-SP1 (A300-133A), anti-HSF1 (A303-174A), anti-TORC2 (CRTC2, A300-637A), anti-ZBTB2 (A303-262A), anti-SMAR1/BANP (A300-278A) and anti-c-REL (A301-825A) and antibodies against EP300 (554215) and EZH2 (612666) from BD Transduction Laboratories. Custom rabbit polyclonal antibody against phosphorylated peptide encompassing the ΔNp63α protein sequence (ATM motif, NKLPSV-pS-QLINPQQ, residues 379-392) was purified against the phosphorylated peptide vs. non-phosphorylated peptide.[20]

Cells and reagents. The cell line SCC-11 (expressing wt-TP53, wt-TP63 is amplified and ΔNp63α is overexpressed) was characterized, tested and authenticated by a short tandem repeat profiling analysis using the AmpFISTR Identifiler PCR Amplification Lit (Applied Biosystems) at the JHMI Fragment Analysis Facility.[20,25-29] The stable SCC cell lines expressing wild type ΔNp63α (SCC-11) or ΔNp63α-S385G (SCC-11M) were generated using Flp-In technology.[20] Cells were maintained in RPMI medium 1640 and 10% fetal bovine serum and incubated

with control medium without cis-diamminedichloro-platinum-dichloride (cisplatin, CIS, Sigma, P4394) or medium with 10 μg/ml cisplatin (Sigma) for the indicated time periods. Cells were lysed with 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.5% Brij-50, 1 mM PMSF, 0.5 mM NaF, 0.1 mM Na_3VO_4, 2× complete protease inhibitor cocktail, sonicated for 10 sec intervals, and spun for 30 min at 15,000× g. Total and nuclear supernatants were analyzed by immunoblotting, and the levels of tested proteins were normalized against β-actin or TBP levels, respectively. Blots were scanned and quantified by the Image Quant software version 3.3 (Molecular Dynamics). Values were expressed as percentage of a control sample (defined as 1).

Isolation of nuclear 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, 0.1 mM EGTA) added with protease inhibitors (Sigma). After resuspension, 0.6% Triton X-100 (final concentration) was added and the nuclei were pelleted by centrifugation at 2,500–3,000×g for 10 min at 4°C. Nuclear pellets were resuspended in the extract buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.4 M NaCl, 0.1mM EDTA, 0.1 mM EGTA), rocked for 15 min at 4°C and nuclear lysate (supernatant) was recovered by centrifugation at 10,000 × g for 5 min at 4°C as described elsewhere.[29]

qPCR of microRNAs. For validation of differential expression of microRNAs, we isolated total small RNAs using mirVana miRNA Isolation kit (#AM1560, Applied Biosystems). We then used the High Capacity cDNA Reverse Transcription kit (#4374966, Applied Biosystems) to produce single-stranded cDNA probes. Next, we applied a quantitative two-step qRT-PCR using the TaqMan MicroRNA Assay Kit TaqMan® U47 (#4380911) and TaqMan® Gene Expression Master Mix, 1-Pack (#4369016) both obtained from Applied Biosystems. For precursor pri-microRNAs, we used the following individual kits: pri-hsa-mir-181a-5p (Hs03302966_pri), pri-hsa-mir-519a-3p (Hs03302632_pri), pri-hsa-mir-374a-5p (Hs03304235_pri), pri-hsa-mir-630 (Hs03304713_pri) and pri-hsa-mir-885-3p (Hs03305150_pri). The reaction conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min with a sample volume of 20 μl. Expression was normalized to the U47 expression (gene ID 26802) and expression levels were determined as the average Ct of the U47 control. This averaged value was used to normalize the sample's Ct. The averaged normalized value for each sample was then entered into this formula = 10 (NL avg/-3.5) and the average microRNA expression was determined using the Mann-Whitney's U test.[26]

MicroRNA mimics and inhibitors and transfection. The following individual microRNA mimics (precursors): hsa-miR-181a-5p (PM10381), hsa-miR-519a-3p (PM12949), hsa-miR-374a-5p (PM12702), hsa-miR-630 (PM11552) and hsa-miR-885-3p (PM12458), and inhibitors: hsa-miR-181a (AM10381), hsa-miR-519a (AM12949), hsa-miR-374a (AM12702), hsa-miR-630 (AM11552) and hsa-miR-885-3p (AM12458) were purchased from Ambion/Applied Biosystems.[26] Cells were transfected for 24 h in a 6-well plate with 100 pmol of the mimic, inhibitor or control in 500 μl serum-free media with 5 l of Lipofectamine-2000 reagent (Invitrogen). Each experiment was performed independently at least three times and in triplicate. Cells were also transfected with scrambled siRNA (SR30004), siRNA against ATM (si-ATM, SR300330) and siRNA against BHLHE41 (SR312407), all from Origene, CARM1 siRNA (sc-44875, Santa Cruz Biotechnology) and SignalSilence Ezh2 siRNA (6509S, Cell Signaling Technology).

Chromatin immunoprecipitation (ChIP). Five × 10^6 cell equivalents of chromatin (2–2.5 kbp in size) were immunoprecipitated (IP) with 10 μg of anti-p-ΔNp63α antibody, as described elsewhere.[26] After reversal of formaldehyde cross-linking, RNA-ase A and proteinase K treatments, IP-enriched DNAs were used for PCR amplification.[26] PCR was performed for 40 cycles (30s at 94°C, 30s at 60°C and 30s at 72°C) using Taq DNA polymerase (Invitrogen). Although the tested promoters contain multiple potential TP63 binding sites, the regions for PCR were selected based on the efficiency of amplification, choosing the highest PCR outcome. The specific regions (containing tested binding sites defined by the web browser: www.cbrc.jp/research/db/TFSEARCH) and non-specific regions (containing no tested binding sites) of selected gene promoters were amplified for ChIP-PCR assay (primers are underlined in **Figs. S1–S5**) and yielding the 250 bp or 150 bp PCR products, respectively. To quantify the binding of p-ΔNp63α to the selected gene promoter sequences (enrichment), we used qPCR. ChIP-PCR values were obtained from the ChIP and Input samples and were normalized to GAPDH PCR values. For each transcription factor, values obtained from the input samples were designated as 1. ChIP/input ratio was plotted from all biological experiments using the Microsoft Excel software. Experiments were performed in triplicate.

Cloning of the reporter plasmids for microRNA promoters. Promoter sequences for miR-181a-5p, miR-374a-5p and miR-630 were amplified using the following primers: for miR-181a-5p, sense - 5'-TCC ATC AAA ACA AAA CGA AAC AAC TCG AAA TAA TTT AGA ATA T-3' and antisense - 5'-TGT GGA GGT TTG CCA AAC TCA GTC GAG CAC GTT CAT CTG CTT-3' yielding the 1,895bp PCR product; for miR-374a-5p, sense - 5'-TCC ATC AAA ACA AAA CGA AAC AAT TAT CGA AGA GAC TTC TAG A-3'and antisense - 5'-CTT TTC TAA CTT ATT CCT ACA GTC GAG CAC GTT CAT CTG CTT-3' yielding the 1,745 bp PCR product; for miR-630, sense - 5'-TCC ATC AAA ACA AAA CGA AAC AAG TTT GAG TGT CAT AAA TCC A-3' and antisense - 5'-TAC TCT TAT TTG GAT CTG TAA GTC GAG CAC GTT CAT CTG CTT-3' yielding the 1,545bp PCR product. The PCR fragments were subcloned into the promoter-less pLightSwitch_Prom vector upstream the luciferase reporter gene (S790005, SwitchGear Genomics) and subsequently used for transfections and reporter assays.

Luciferase reporter assay. We used the 3'-UTR luciferase reporter plasmids for ATM (SC221017, Origene), TP63 (S811809), CRTC2 (S803503), CARM1 (S807909), BHLHE41 (S705709), EP300 (S808354), KAT2B (S810567), EZH2 (S811982), NFYB (S811604) and TBPL1 (S804783) were obtained from SwitchGear Genomics. For the promoter-mediated

luciferase activity assay, and 3'-UTR-mediated luciferase activity assay, a total of 5 × 10^4 cells/well in a 24-well plate were transfected with the control (empty) pLightSwitch_Prom vector (S790005) or with the empty pLightSwitch_3UTR vector (S890005), respectively, using Fugene HD reagent (Roche) as recommended by the manufacturer. Both vectors represent a fully optimized reporter system that includes an improved luminescent reporter gene (RenSP). In addition, cells were transfected with the selected 3'-UTR plasmids as listed above. The LightSwitch Luciferase Assay Reagent (SwitchGear Genomics) enables to monitor luciferase reporter signal according to the manufacturer's protocol. For the 3'-UTR assays, cells were also transfected with 100 ng of the mimics or inhibitors of tested microRNAs. At 36 h, cells were also treated with 10 μg/ml cisplatin or control medium without cisplatin for an additional 12 h. The RenSP Renilla luciferase activity was measured at 480 nm using a luminometer.

Cell viability assay. 10^4 cells/well in 96-well plates were incubated in serum-free medium with 5 μg/ml of the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay, American Tissue Culture Collection) in the dark for 4 h at 37°C as described elsewhere.[21] Cells were lysed and incubated for 2 h at 37°C, and the measurements (A_{570} nm to A_{650} nm) were obtained on a Spectra Max 250 plate reader (Molecular Devices) as described. Each assay was repeated three times in triplicate.

Statistical analysis. Results were expressed as means ± SD from three independent experiments in triplicate. Differences in variables between experimental and control group were assessed by using the Student's t-test. Statistically significant difference was accepted at $p < 0.05$.

Bioinformatics. Putative responsive elements were deifned using the TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) and TRANSFAC (version 7.4, www.generegulation.com/pub/databases.html) databases. For prediction of the microRNA-specific "seed" sequences in target 3'-UTRs, we used the following databases: microRNA.org-Targets and Expression (August 2010, Computational Biology Center, Memorial Sloan-Kettering Cancer Center, www. microrna.org), TargetScan: Prediction of microRNA targets, version 5.2 (June 2011, www.targetscan.org) and miRDB-MicroRNA Target Prediction And Functional Study Database, v3.0 (April 2009, www.mirdb.org). All the targets were selected based on mirSVR scores (from −0.1479 to −0.4518), PhastCons scores (0.4775–0.6217) and target prediction scores (from 55 to 91) assigned by the computational target prediction algorithm.

Concluding Remarks

Drug resistance acquired by tumor cells limits the successful use of cisplatin chemotherapy. Many genes encoding mRNAs and microRNAs are differentially expressed in sensitive and resistant tumor cells, suggesting that the transcriptional regulation of genes for mRNA and microRNAs involved in mechanisms underlying chemoresistance. We demonstrated that the p-ΔNp63α transcriptionally regulates the microRNA gene promoters by forming protein complexes with other transcriptional and chromatin-associated factors, while total ΔNp63α levels are maintained through a microRNA-mediated post-transcriptional/translational machinery, thereby providing a regulatory feedback for selected microRNAs and their respective promoters. Our study established a new functional link between p-ΔNp63α and the deregulated microRNA promoters in SCC cells exposed to cisplatin, suggesting that a complex transcriptional machinery involving p-ΔNp63α could potentially act as a regulator of death or survival of SCC cells during chemotherapy. Thus, therapeutic compounds deactivating ΔNp63α phosphorylation and/or its downstream microRNA targets could be used in combination with cisplatin to induce optimal tumor regression of human cancers that overexpress p-ΔNp63α.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was supported in part by the Flight Attendant Research Institutions grant (#082469 to E.A.R.), and by National Cancer Institute grants K01-CA164092 and U01-CA84986 (R.G-P.).

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/23598

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