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Disruption of TP63-miR-27a* Feedback Loop by Mutant TP53 in Head and Neck Cancer

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Abstract

Background: Alterations in the epidermal growth factor receptor and PI3K pathways in head and neck squamous cell carcinomas (HNSCCs) are frequent events that promote tumor progression. Ectopic expression of the epidermal growth factor receptor–targeting microRNA (miR), miR-27a* (miR-27a-5p), inhibits tumor growth. We sought to identify mechanisms mediating repression of miR-27a* in HNSCC, which have not been previously identified.

Methods: We quantified miR-27a* in 47 oral cavity squamous cell carcinoma patient samples along with analysis of miR-27a* in 73 oropharyngeal and 66 human papillomavirus–positive (HPV+) samples from The Cancer Genome Atlas. In vivo and in vitro TP53 models engineered to express mutant TP53, along with promoter analysis using chromatin immunoprecipitation and luciferase assays, were used to identify the role of TP53 and TP63 in miR-27a* transcription. An HNSCC cell line engineered to conditionally express miR-27a* was used in vitro to determine effects of miR-27a* on target genes and tumor cells.

Results: miR-27a* expression was repressed in 47 oral cavity tumor samples vs matched normal tissue (mean log₂ difference = −0.023, 95% confidence interval = −0.044 to −0.002; two-sided paired t test, P = .03), and low miR-27a* levels were associated with poor survival in HPV+ and oropharyngeal HNSCC samples. Binding of ΔNp63 α to the promoter led to an upregulation of miR-27a*. In vitro and in vivo findings showed that mutant TP53 represses the miR-27a* promoter, downregulating miR-27a* levels. ΔNp63 α and nucleoporin 62, a protein involved in ΔNp63 α transport, were validated as novel targets of miR-27a*.

Conclusion: Our results characterize a negative feedback loop between TP63 and miR-27a*. Genetic alterations in TP53, a frequent event in HNSCC, disrupt this regulatory loop by repressing miR-27a* expression, promoting tumor survival.

Epidermal growth factor receptor (EGFR) is frequently overexpressed in head and neck squamous cell carcinomas (HNSCCs) and is thought to influence therapeutic response. As an important component of a multimodal therapeutic strategy, molecular inhibitors targeting EGFR showed initial promise (1,2). The minimal clinical benefits observed in patients, despite strong biological justification for the use of EGFR inhibitors, suggests that other cellular pathway alterations may lead to baseline or acquired resistance of HNSCC to EGFR-targeted agents (3).

Although HNSCC exhibits high levels of genomic instability, alterations in a relatively small number of pathways act as

drivers of tumor progression (4–6). Alterations in the TP53 family of proteins (TP53, TP63, and TP73) are frequent events in HNSCC (2,4,5), and changes in one member of the TP53 family affects the function of other members (7). Mutations and/or deletions in TP53 are the leading genetic alteration in patients and directly affect response to therapy and overall survival (8,9). ΔNp63 α , one of six transcripts of the TP63 gene, is overexpressed in HNSCC and is essential for its survival (10). ΔNp63 α transcriptionally regulates a host of microRNAs (miRNAs) and genes affecting a wide spectrum of processes, including apoptosis, wound healing, and proliferation (7).

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Dysregulation of miRNA expression in cancers including HNSCC has been linked to tumor development (11–13). We previously identified EGFR as a target of miR-27a (miR-27a-3p) and its complementary strand, miR-27a* (miR-27a-5p) (14). Both miRNAs are transcribed from the miR-23a-24~2-27a locus on chromosome 19 and, depending on the context and cell type, these miRNAs act as oncomiRs or as tumor suppressors (12,13). AKT1 and mTOR, components of the PI3K/AKT1/mTOR pathway, which is the most activated mitogenic network both in human papillomavirus-positive (HPV+) and HPV-negative (HPV-) HNSCC, are direct targets of miR-27a* but not miR-27a (6,14–16). The high frequency of mutations in PI3K, AKT1, and mTOR promotes tumor growth (6,16). Targeting of multiple oncogenes, including EGFR, relevant to HNSCC progression underlies the molecular mechanism for miR-27a* but not miR-27a, affecting HNSCC survival (14).

Our earlier findings in a small patient cohort demonstrated that the expression of miR-27a and miR-27a* is repressed in HNSCC (14). However, the mechanisms mediating miR-27a* repression in HNSCC have not been defined. The goal of the current study was to define mechanisms regulating miR-27a* expression and their effects on HNSCC. We found that increased miR-27a* levels were associated with improved survival in patients with HPV+ HNSCC. We identified Δ Np63 α as an activator of miR-27a* transcription. Δ Np63 α and nucleoporin 62 (NUP62) were validated as novel miR-27a* targets. Thus, Δ Np63 α -miR-27a* forms a feedback loop, whereas mutant TP53-mediated repression of miR-27a* disrupts this regulatory loop.

Methods

Patient Samples

Formalin-fixed, paraffin-embedded tissue samples were obtained using a protocol approved by the institutional review board. We obtained 47 matched normal and tumor samples from oral cavity squamous cell carcinoma (OSCC) patients with primary disease (n = 40) and locoregional recurrence (n = 7) who were treated at The University of Texas MD Anderson Cancer Center. Patients were selected to provide a balance of clinical characteristics including age, stage, and sex. Informed consent from all patients was obtained for tissue used in the study. Tissue sections were examined, selected, and marked for microdissection by a board-certified pathologist (MW).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR analysis on RNA was carried out on a Biorad CFX 96 real-time PCR machine (Biorad, Carlsbad, CA) and data analyzed using Biorad CFX Manager software (Biorad). Each measurement was carried out in triplicate and each experiment was repeated independently for reproducibility. Additional details are provided in the [Supplementary Methods](#) (available online).

MTT Assay

Cells were plated in 96-well plates. The next morning cells were treated with the indicated concentration of doxycycline (Dox) and incubated for 72 hours. MTT reagent was then added for 4 hours, after which the supernatant was replaced with dimethyl sulfoxide, and optical density values were read at 570 nm on a Spectrostar plate reader (BMG Labtech, Cary, NC).

Data represents three experiments with error bars representing SD.

Luciferase Assay

Luciferase assays were carried out in 24-well plates in triplicate and repeated independently. Cells were cotransfected with indicated reporter constructs and 5 ng of Renilla using Lipofectamine 2000 (Thermo Fisher, Carlsbad, CA). Lysates were assayed with the Dual-Glo Luciferase Assay System (Promega, Madison, WI). Firefly activity was normalized to Renilla luciferase activity.

Clonogenic Assay

Clonogenic assays were carried out in triplicate and repeated three times. A representative experiment is shown with error bars representing SD. Details in the [Supplementary Methods](#) (available online).

Statistical Analysis

Experimental data was analyzed with GraphPad Prism (GraphPad Software, La Jolla, CA), Excel (Microsoft Corp, Seattle, WA) and R (version 3.4.1). Analysis of The Cancer Genome Atlas (TCGA) data was performed in R (version 3.4.1). All tests were two-sided and the statistical significance was defined as a P value less .05. Further details are presented in the [Supplementary Methods](#) (available online).

Results

Repression of miR-27a* and Poor Patient Survival in HNSCC

To expand on our previous findings of miR-27a and miR-27a* repression in HNSCC (14), we identified a well-annotated cohort of matched normal and tumor samples from 47 patients with OSCC. qPCR analysis showed that expression of miR-27a* and miR-27a was lower in tumors than in matched normal samples (miR-27a* mean log₂ difference = -0.023, 95% confidence interval [CI] = -0.044 to -0.002; two-sided paired t test, P = .03; [Figure 1A](#); miR-27a mean log₂ difference = -4.01, 95% CI = -6.37 to -1.66; two-sided paired t test, P = .001; [Figure 1B](#)).

The number of patients with HPV+ HNSCC has sharply increased (2,17,18). Analysis of HNSCC samples in TCGA showed higher levels of miR-27a* in HPV+ tumors (n = 66), which have a better prognosis than do HPV- tumors (n = 390) (P = .01) ([Figure 1C](#)). We also found that decreased levels of miR-27a* were associated with poor survival in patients with HPV+ (n = 66, P = .01) HNSCC, who mainly present with oropharyngeal squamous cell carcinoma (SCC; n = 73, P = .002) (OPSCC; [Figure 1, D and E](#), and [Supplementary Tables 1–7](#)). In HPV+ tumors, higher levels of miR-27a* were observed in low-grade tumors (n = 11) (American Joint Committee on Cancer stage I/II) compared with high-grade (stage III/IV) (n = 55) tumors in the TCGA (P = .02) ([Figure 1F](#)).

Δ Np63 α Regulation of miR-27a* Transcription

Transcriptional regulators of the miR-23a-24~2-27a locus have not been defined in HNSCCs. We found that levels of miR-27a were substantially higher than miR-27a* in normal tissue

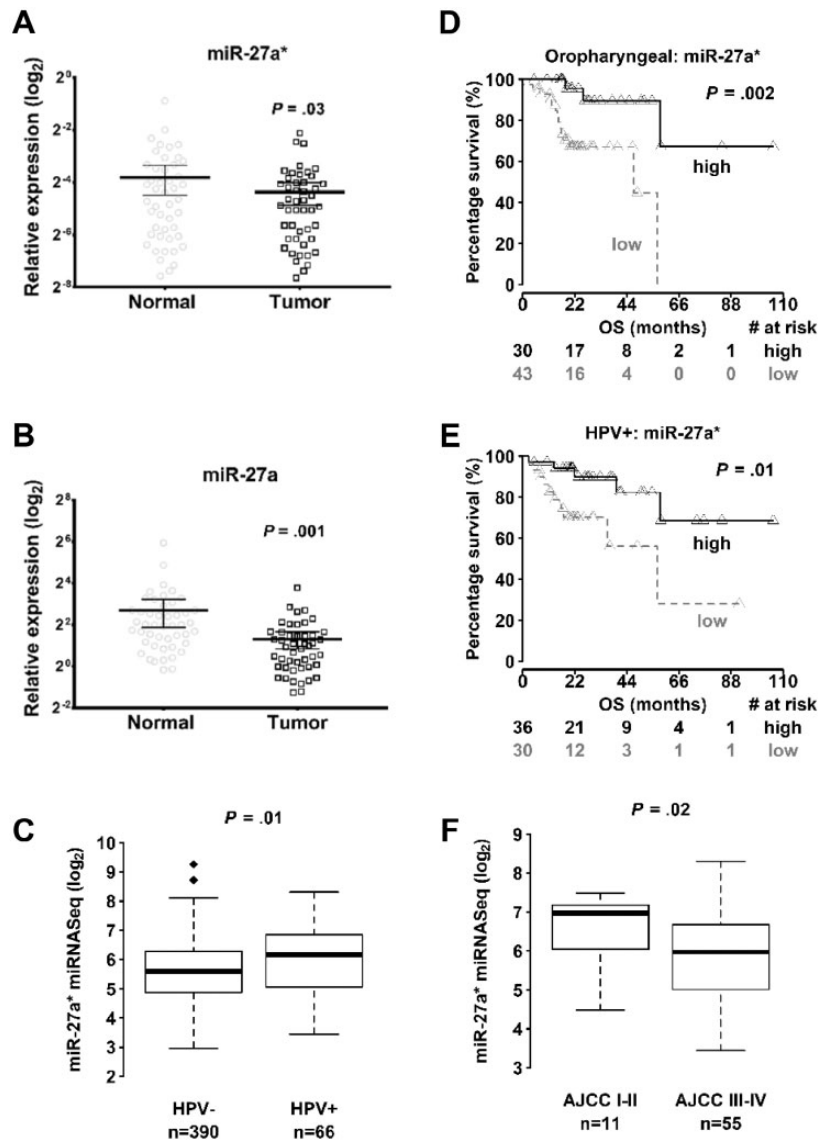


Figure 1. Expression of microRNA-27a* (miR-27a-5p) in head and neck squamous cell carcinoma (HNSCC). Quantitative real-time polymerase chain reaction (qPCR) analysis of miRNA levels from matched normal and tumor samples ($n = 47$) of (A) miR-27a* and (B) miR-27a (miR-27a-3p) expression in oral cavity squamous cell carcinoma (OCSCC) tumors. P values calculated by two-tailed Student t test. Error bars represent mean (95% confidence intervals [CI]). RNU 44 used as an endogenous control. (C) Expression levels of miR-27a* in human papillomavirus-negative (HPV-) ($n = 390$) and HPV-positive (HPV+) ($n = 66$) HNSCC samples from The Cancer Genome Atlas (TCGA) database. Kaplan-Meier overall survival (OS) curves for (D) oropharyngeal squamous cell carcinoma (OPSCC) ($n = 73$) and (E) HPV+ HNSCC ($n = 66$) patients from TCGA expressing high miR-27a* or low miR-27a* levels; # = number. The P value was calculated using the log-rank test. (F) Expression of miR-27a* in American Joint Committee on Cancer (AJCC) stage I-II ($n = 11$) and stage III-IV ($n = 55$) HPV+ tumors in TCGA. The P value was calculated using the Mann-Whitney test. All statistical tests were two-sided.

($P < .001$) and OCSCC samples ($n = 47$) ($P < .001$) (Supplementary Figures S1A and B, available online) and HNSCC cell lines (Supplementary Figure 2A, available online). Having established previously (14) and in the current study (Supplementary Figure 2B, and C, available online) that ectopic expression of miR-27a*, but not miR-23a, -24, or -27a, in HNSCC cells inhibited cellular survival, we focused on identifying mechanisms repressing miR-27a* expression in HNSCC.

Robust expression of Δ Np63 α in HNSCC cells is a primary driver of tumor progression by regulating a miRNA program essential to tumor survival (19). To determine if Δ Np63 α regulates miR-27a*, we suppressed Δ Np63 expression using a Δ Np63-specific small interfering RNA (siRNA) in JHU-029 (TP53 Null) (20), HN31 (TP53 mutant), and UM-SCC-17A (17A; TP53 wild-type) cells

resulting in a decrease in miR-27a* expression (Figure 2, A-C). Knockdown was specific for the Δ Np63 isoform in all cell lines as seen by a depletion of Δ Np63 mRNA (Figure 2, D-F) and Δ Np63 α protein (Figure 2, G-I). An increase in TAp63 mRNA levels was seen in the JHU-029 and HN31 cells (Supplementary Figure 3A, and B available online), and a decrease in TAp63 was seen in 17A cells (Supplementary Figure 3C, available online). To confirm that the change in miR-27a* levels was not due to an increase in TAp63 levels, we used a TP63 siRNA targeting both TA and Δ N isoforms in JHU-029 cells. A decrease in TP63 and TAp63 (Supplementary Figure 3D, available online) was observed, along with a concomitant decrease in miR-27a* levels (Supplementary Figure 3E, available online). This suggests that Δ Np63 α , the dominant isoform expressed in HNSCC (21), regulates miR-27a* expression.

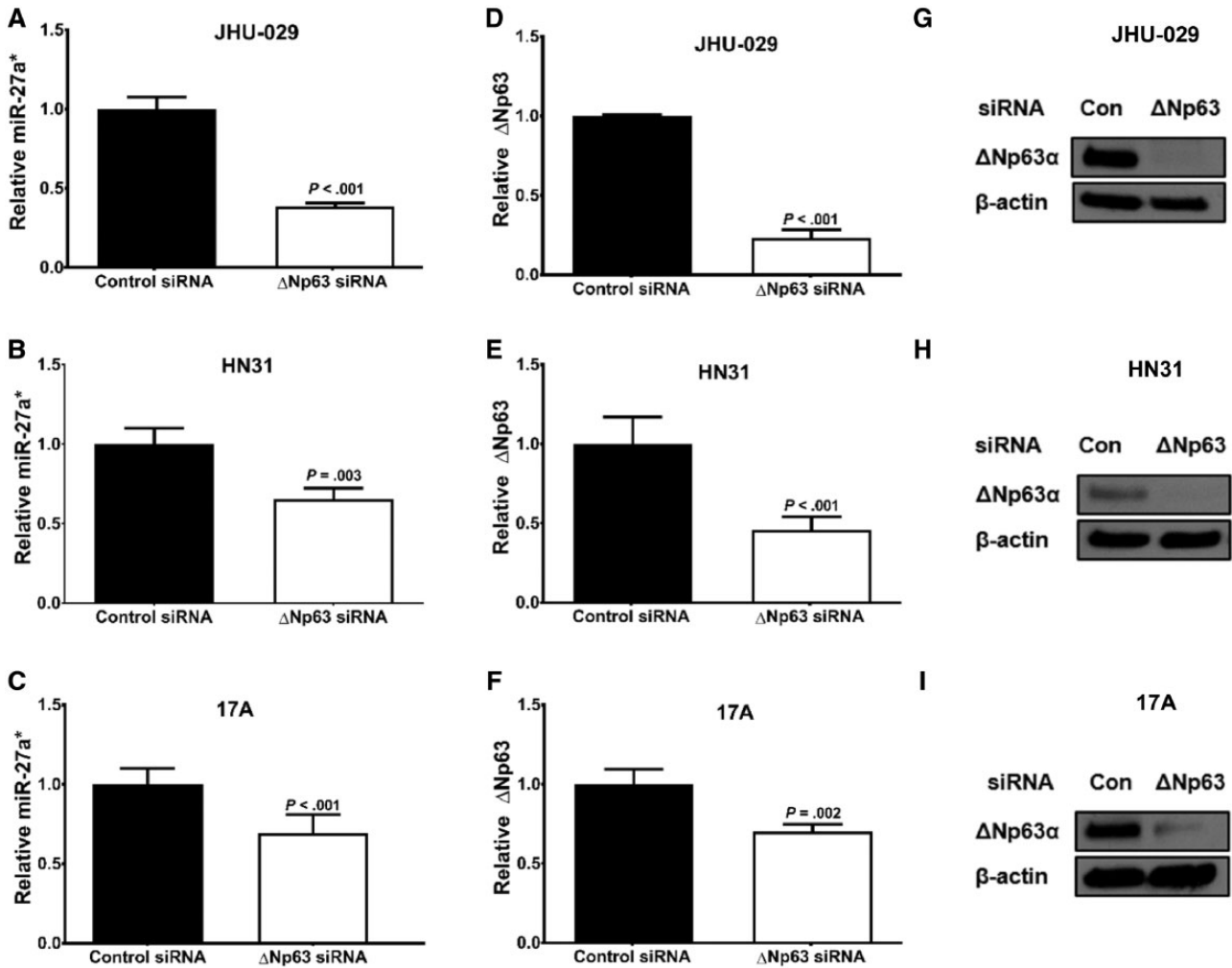


Figure 2. Effects of Δ Np63 α levels on microRNA (miR)-27a* expression in head and neck squamous cell carcinoma (HNSCC) cells. Δ Np63 α was depleted using a Δ Np63-targeting small interfering RNA (siRNA) in the indicated HNSCC cell lines. Expression of miR-27a* in (A) JHU-029, (B) HN31, and (C) UM-SCC-17A (17A) cells and Δ Np63 mRNA in (D) JHU-029, (E) HN31, and (F) 17A cells were analyzed by quantitative real-time polymerase chain reaction (qPCR). Error bars represent mean (SEM). Protein levels were analyzed by immunoblotting in (G) JHU-029, (H) HN31, and (I) 17A cells treated with either control (Con) or Δ Np63 siRNA with β -actin as an endogenous control. Endogenous controls used in qPCR analysis were RNU44/RNU6B for miRNA and glyceraldehyde-3-phosphate dehydrogenase for mRNA.

In silico analysis of the miR-23a-24~2-27a promoter revealed a number of high-affinity putative TP53 and TP63 binding sites (Figure 3A). Quantitative chromatin immunoprecipitation analysis in UM-SCC-22A (22A) cells, which express high levels of Δ Np63 α , showed that Δ Np63 α was recruited to a region upstream of the transcription start site (Figure 3B). HNSCC cells express robust levels of Δ Np63 α , therefore, H1299 cells, which do not have detectable levels of Δ Np63 α protein (22), were cotransfected with luciferase constructs encompassing the Δ Np63 α binding site on the miR-27a* promoter (miR-27a*-luc) and a vector control or Δ Np63 α expression vector. Upregulation of luciferase activity was seen when the Δ Np63 α vector was transfected compared with control vector (Figure 3C). Mutations that abrogated Δ Np63 α binding were introduced in the putative binding site on the mutant miR-27a*-luc (miR-27a*-luc MT) (Figure 3D). JHU-029 cells were cotransfected with the wild-type (WT) or mutant (MT) miR-27a*-luc along with control or TP63 siRNA. Cotransfection of TP63 siRNA resulted in a decrease in WT miR-27a*-luc activity compared with control siRNA; a similar change was not observed with the miR-27a*-luc MT construct

(Figure 3E), demonstrating that Δ Np63 α binds to the miR-27a* promoter activating miR-27a* transcription.

Mutant TP53 Repression of miR-27a* Expression

Mutations in TP53, a frequent event in HNSCC, can greatly affect TP63 activity (4,9,23). TP53-targeting siRNA was used to suppress mutant TP53 in HN31 cells to determine whether mutant TP53 affects Δ Np63 α -directed miR-27a* transcription. A reduction in TP53 mutant protein resulted in an increase in miR-27a* levels (Figure 4A). UM-SCC-1 and PCI-13 cells devoid of TP53 protein were engineered to express mutant TP53, allowing us to explore the role of mutant TP53 in miR-27a* regulation. Expression of mutant TP53 in cells led to a decrease in miR-27a* levels (Figure 4, B and C). These findings strongly suggest that mutant TP53 represses miR-27a* expression.

Mutant TP53 has been reported to promote tumor progression (24). Certain mutations are more disruptive in some cancers than others (25,26). We used the Evolutionary Action (EAp53) scoring system, which has been validated in HNSCC

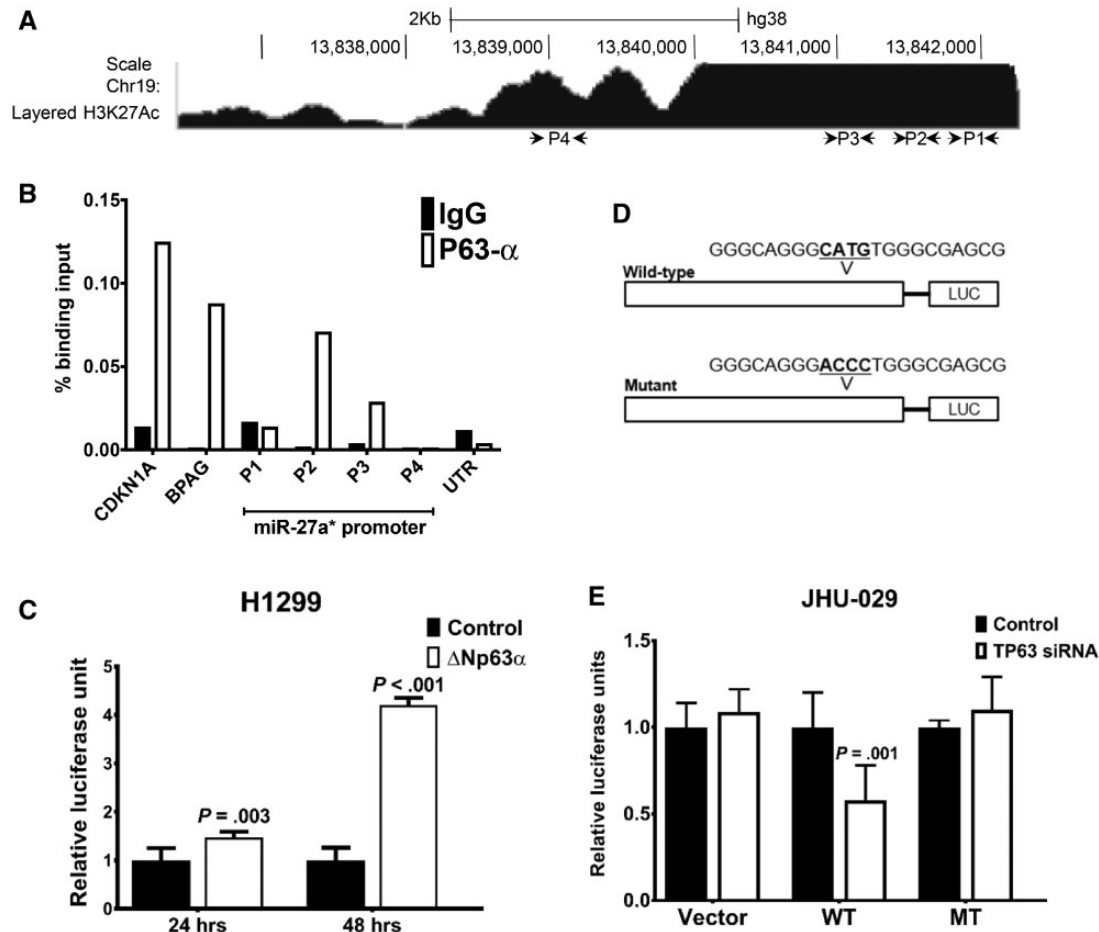


Figure 3. Transcriptional regulation of microRNA (miR)-27a* expression. (A) Schematic representation of the miR-27a* promoter with putative TP53 and TP63 binding sites (P1-P4) along with chromatin immunoprecipitation-RNA sequencing (ChIP-seq) enrichment of H3K27ac from ENCODE. (B) Binding of endogenous TP63 α in UM-SCC-22A (22A) cells to the miR-27a* promoter element was analyzed by quantitative ChIP. Representative experiment was performed in triplicate and carried out three times independently. (C) Results of luciferase assays in H1299 cells with the miR-27a* promoter luciferase (miR-27a*-luc) in the presence of vector control (control) or Δ Np63 α cDNA. (D) Schematic of wild-type (WT) and mutant (MT) miR-27a*-luc constructs with indicated alterations in the TP63 binding site. (E) Results of luciferase assays, carried out using the WT and MT miR-27a*-LUC constructs in JHU-029 cells with control or TP63 small interfering RNA (siRNA). Empty luciferase vector (vector) was transfected along with control or TP63 siRNA in JHU-029 cells as a control, and data were normalized to Renilla luciferase. Luciferase experiments represent the average of two independent experiments with error bars representing mean (SD). *P* value was calculated using a two-sided Student *t* test.

patient datasets, murine models, and in vitro studies (27,28). EAp53 stratifies tumors with TP53 mutations associated with poor outcomes—that is, high risk (HR)—from mutations with outcomes similar to patients with WT TP53—that is, low risk (LR). As has been previously published (29), we grouped the patient tumors in the HNSCC TCGA cohort into two clusters based on TP53 mutations: HR/other and LR/WT. miR-27a* expression levels are lower in the HR/other group compared with the LR/WT group in the HNSCC (WT/LR $n = 210$, HR/other $n = 258$; $P = .005$; Figure 4D), OCSCC (WT/LR $n = 120$, HR/other $n = 165$; $P = .03$; Figure 4E), and the HPV-OCSCC (WT/LR $n = 107$, HR/other $n = 161$; $P = .04$; Figure 4F) cohorts (Figure 4, D–F). To analyze the role of TP53 mutations on miR-27a* expression in SCCs in an in vivo setting, we analyzed miR-27a* expression in cutaneous SCCs that developed in mice generated by conditional activation of TP53 deletion (TP53^{-/-}) or the TP53 R172H mutation (TP53^{R172H/-}) in stratified epithelia, as previously described (30). qPCR analysis established lower levels of miR-27a* and miR-27a in tumors from TP53^{R172H/-} mice than in tumors from TP53^{-/-}

mice (Figure 5, A and B) (miR-27a* log₁₀ difference between means = -0.042, 95% CI = -0.07907 to -0.004923; two-sided Welch *t* test, $P = .03$; Figure 5A; miR-27a log₁₀ difference between means = -8.708, 95% CI = -16.6 to -0.8168; two-sided Welch *t* test, $P = .03$; Figure 5B).

Tumors generated in the TP53^{R172H/-} and TP53^{-/-} mice were either well to moderately differentiated (wSCC) or poorly differentiated SCCs (pSCC; Figure 5C), or spindle cell carcinomas in numbers as described previously (30). To examine whether levels of miR-27a* were associated with the differentiation status of the tumors, we grouped the tumors into two categories (wSCCs and pSCCs) irrespective of genotype and examined the miR-27a and miR-27a* levels in the two groups. We found that pSCCs had lower levels of miR-27a* and miR-27a than did wSCCs (Figure 5, D and E) (miR-27a* log₁₀ difference between means = -0.05, 95% CI = -0.09139 to -0.007982; two-sided Welch *t* test, $P = 0.02$; Figure 5D; miR-27a log₁₀ difference between means = -11.99, 95% CI = -19.66 to -4.32; two-sided Welch *t* test, $P = .006$; Figure 5E). Consistent with these findings,

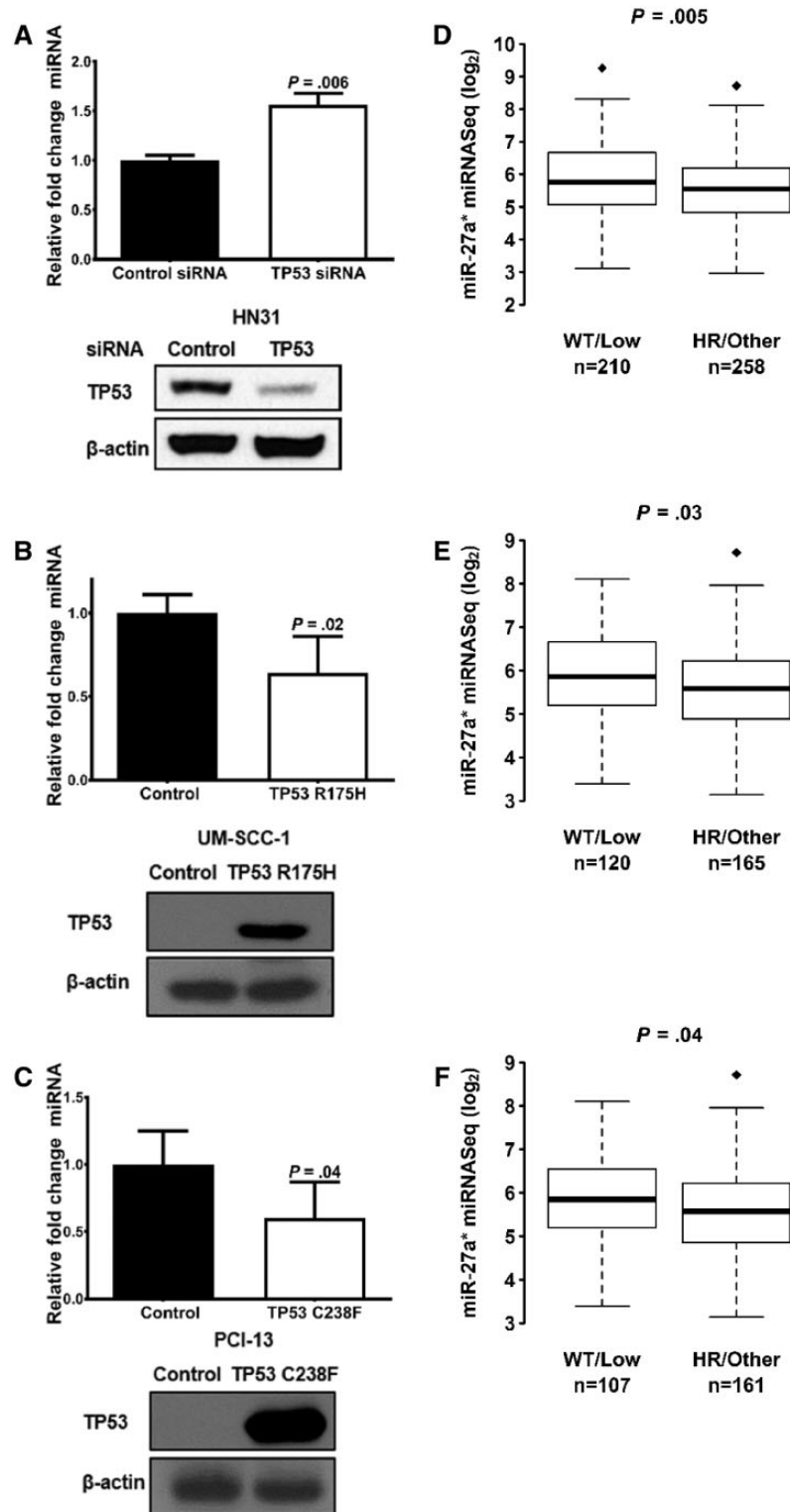


Figure 4. Effect of mutant TP53 on microRNA (miR)-27a* in head and neck squamous cell carcinoma (HNSCC). **A)** Analysis of miR-27a* and TP53 protein levels in HN31 cells transfected with control small interfering RNA (siRNA) or TP53 siRNA. miR-27a* and TP53 protein levels were examined in **(B)** UM-SCC-1 and **(C)** PCI-13 cells engineered to express TP53 R175H and TP53 C238F, respectively. Error bars represent mean (SEM). RNU6B was used as an endogenous control for miRNA analysis. Mutation status of HNSCC tumors in TCGA was classified according to the EAp53 scoring system into wild-type (WT), low-risk (LR), high-risk (HR), and other. miR-27a* expression analyzed in **(D)** HNSCC cohort, **(E)** oral cavity cohort, **(F)** and oral cavity HPV- cohort. Box-and-whisker plots are shown (box plot represents first [lower bound] and third [upper bound] quartiles; whiskers represent 1.5 times the interquartile range). P values are indicated. All statistical tests were two-sided.

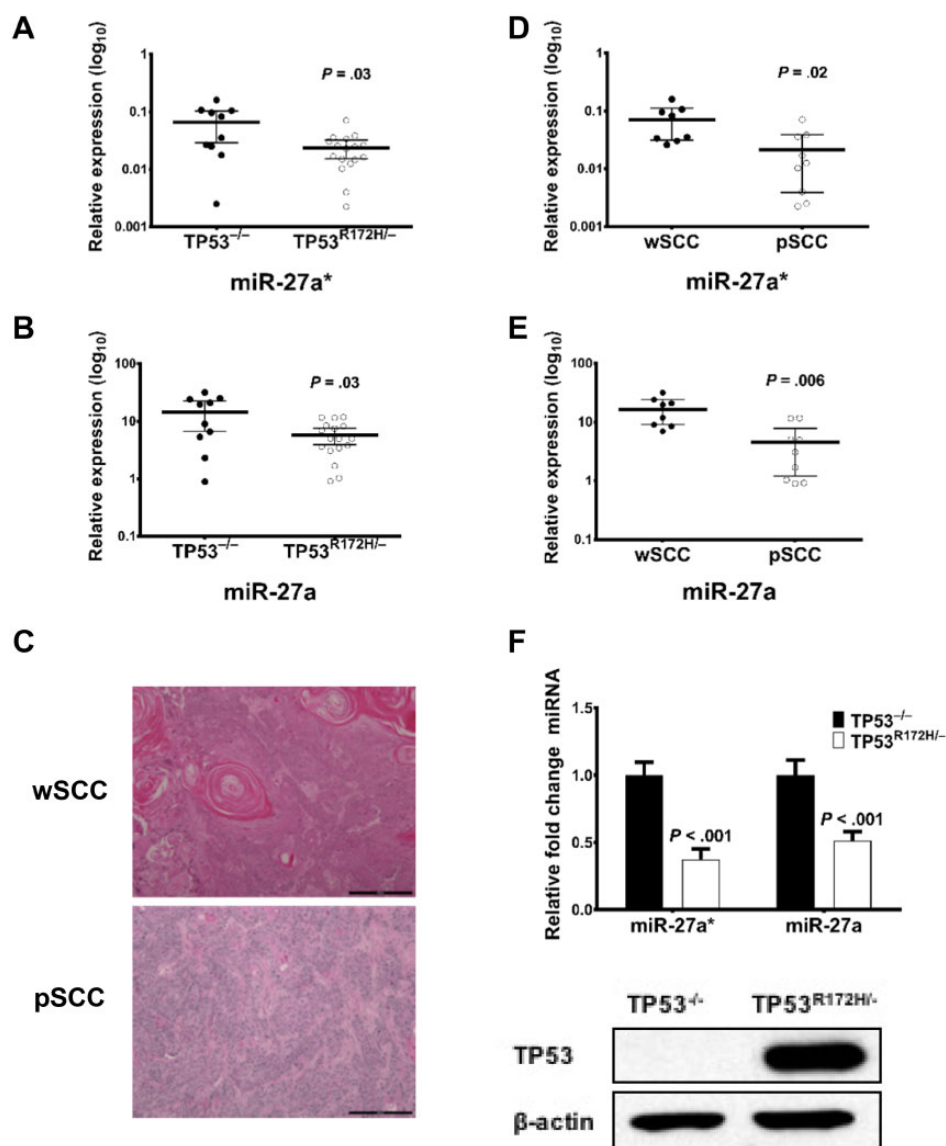


Figure 5. TP53^{R172H/-} activation in murine skin. Quantitative polymerase chain reaction (qPCR) analysis of (A) microRNA (miR)-27a* and (B) miR-27a levels in RNA extracted from tumors obtained from TP53^{-/-} and TP53^{R172H/-} mice. Error bars represent mean 95% confidence intervals. (C) Hematoxylin and eosin stain of tumor sections showing a gross appearance of well-differentiated squamous cell carcinoma (wSCC) and poorly differentiated SCC (pSCC) (scale bars = 200 μ m). Analysis of (D) miR-27a* and (E) miR-27a levels in wSCC tumors and pSCC tumors. Error bars represent 95% confidence intervals of the mean. P value determined using a two-sided Student t test. (F) Expression levels of TP53 protein and mRNA in cells generated from SCC obtained from TP53^{-/-} and TP53^{R172H/-} mice. qPCR data were normalized to Sno234. Error bars represent mean (SEM).

analysis of miR-27a* expression in cell lines generated from tumors showed that miR-27a* levels were lower in TP53^{R172H/-} cells than in TP53^{-/-} cells (Figure 5F).

Expression of miR-27a* and Survival

Viability of HNSCC cells is drastically affected by transient transfections of miR-27a* (14). To create a more controlled environment to study the biological activity of miR-27a*, we generated 22A cells with doxycycline-inducible miR-27a* (22A-miR-

27a*) construct coexpressing green fluorescent protein. Addition of doxycycline to 22A-miR-27a* cells resulted in green fluorescent protein expression and an increase in levels of miR-27a* but not miR-27a (Figure 6, A and B). Increased miR-27a* expression resulted in a decrease in cell viability as measured by MTT and clonogenic assays (Figure 6, C and D; Supplementary Figure 4, available online).

Transcription factors are often targets of the miRNAs they transcriptionally regulate, forming regulatory feedback loops (31). Additionally, Δ Np63 α has been shown to play an important role in promoting HNSCC cell survival, and lowered

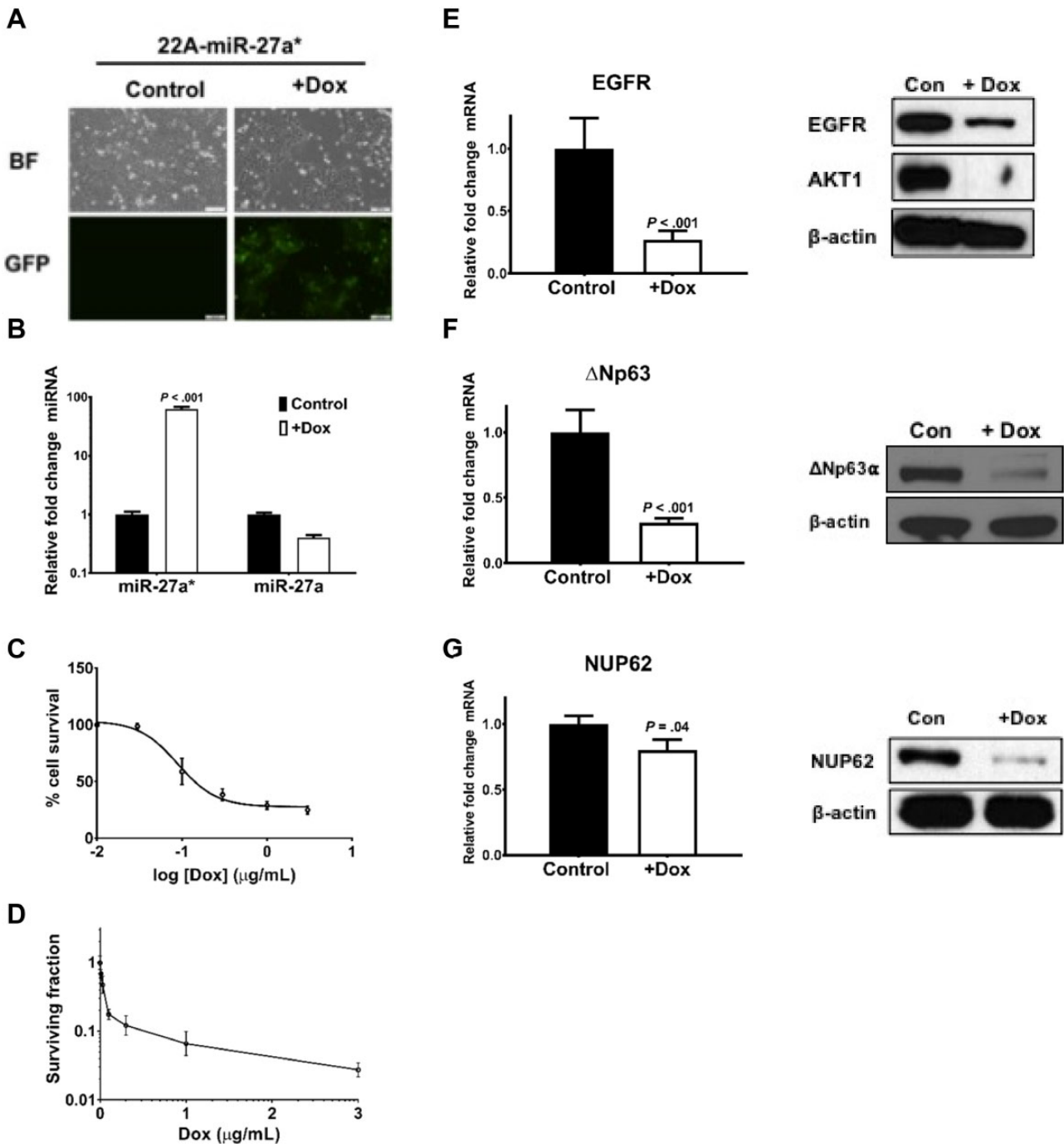


Figure 6. $\Delta\text{Np63}\alpha$ expression levels following microRNA (miR)-27a* induction. (A) Green fluorescent protein (GFP) expression in the 22A-miR-27a* cells is induced only on the addition of doxycycline (Dox) (BF: brightfield) (scale bars = 100 μm). (B) Expression of miR-27a* and miR-27a following treatment with Dox as measured by quantitative polymerase chain reaction (qPCR). Cell viability assessed using the (C) MTT assay and (D) clonogenic assay following miR-27a* induction. Error bars represent mean (SD). Quantification of mRNA and protein levels of (E) epidermal growth factor receptor (EGFR), (F) ΔNp63 , and (G) NUP62 as measured by qPCR and immunoblotting, respectively, on induction of miR-27a* in 22A-miR-27a* cells. Error bars represent mean (SEM). TATA-box binding protein (TBP) was used as endogenous control in qPCR. All statistical tests were two-sided.

levels of $\Delta\text{Np63}\alpha$ greatly affect cell viability (10). To assess the presence of a miR-27a*- $\Delta\text{Np63}\alpha$ loop, we induced miR-27a* in 22A-miR-27a* cells, which resulted in a substantial decrease in not only EGFR, a previously identified miR-27a* target (14), but also ΔNp63 mRNA and $\Delta\text{Np63}\alpha$ protein (Figure 6, E and F).

Ectopic expression of miR-27a* mimic in HN31 cells resulted in similar findings (Supplementary Figure 5, available online).

We identified a novel miR-27a* target, NUP62, which regulates $\Delta\text{Np63}\alpha$ transport to the nucleus. NUP62 promotes $\Delta\text{Np63}\alpha$ activity and is overexpressed in SCC and HNSCC

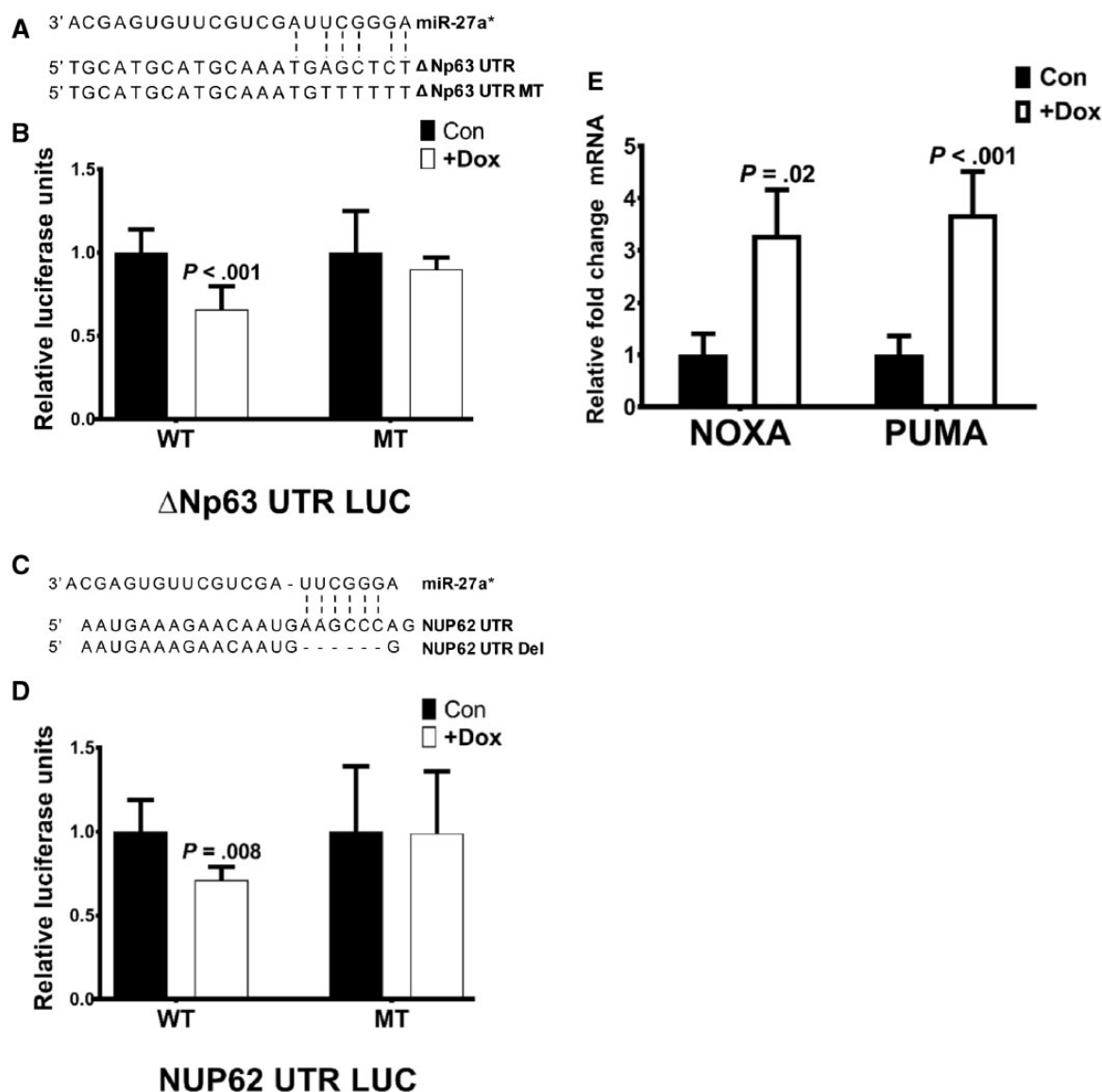


Figure 7. Interaction of microRNA (miR)-27a* with the Δ Np63 network. Schematic representation of the miR-27a* binding sites on the 3'UTR of (A) Δ Np63 and (C) NUP62 with the mutations (MT) or deletions (Del) generated in the respective miR-27a* binding site sequences. Luciferase assays with the (B) Δ Np63 and (D) NUP62 wild-type (WT) and MT UTR LUC constructs in 22A-miR-27a* cells. Renilla was used as a normalization control. Data represent the average of two independent experiments carried out in triplicate. Error bars represent mean (SD). (E) Expression levels of Δ Np63 target genes NOXA and PUMA on induction of miR-27a* in 22A-miR-27a* cells were analyzed by quantitative polymerase chain reaction. Error bars represent mean (SEM). TATA-box binding protein (TBP) was used as endogenous control in qPCR. P value calculated using two-sided tests.

(Supplementary Figure 6, available online: normal $n = 39$, tumor $n = 466$; $P < .001$) (32). Loss of NUP62 does not affect total Δ Np63 protein levels, but has been shown to affect Δ Np63 levels in the nucleus of SCC cells, thereby affecting its ability to transcriptionally regulate target genes, inhibiting proliferation of SCC cells (32). Induction of miR-27a* expression in 22A-miR-27a* cells resulted in a decrease in NUP62 mRNA and protein (Figure 6G).

Multiple Components of the Δ Np63 Network as Direct miR-27a* Targets

To validate Δ Np63 and NUP62 as direct targets of miR-27a*, we generated luciferase constructs to encompass the putative miR-27a* binding sites on target genes along with the respective

companion mutant constructs with alterations and/or deletions of the miR-27a* binding site (Figure 7, A and C). Luciferase assays with the WT Δ Np63 (Δ Np63 UTR LUC) and NUP62 (NUP62 UTR LUC) constructs in 22A-miR-27a* cells showed a decrease in luciferase activity on induction of miR-27a*. This decrease in luciferase activity was not observed with the respective mutant constructs on miR-27a* induction because of abrogation of miR-27a* binding caused by mutation or deletion of the binding site (Figure 7, B and D).

Maintenance of high levels of Δ Np63 is crucial to HNSCC survival because Δ Np63 represses the transcription of many genes involved in apoptosis and survival (10). Expression of miR-27a* not only affects Δ Np63 expression but also targets NUP62, essential to transport of Δ Np63 to the nucleus. To examine the effects of inhibition of Δ Np63 function on miR-27a* induction,

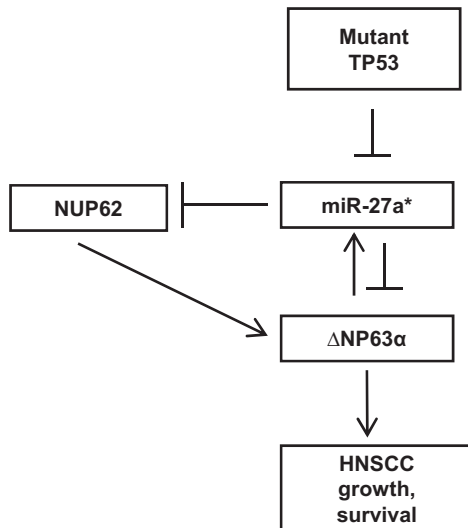


Figure 8. Schematic of microRNA (miR)-27a* regulation in head and neck squamous cell carcinoma (HNSCC). Δ Np63 α transcriptionally upregulates miR-27a*. Δ Np63 α is a direct target of miR-27a*, forming a feedback loop. NUP62 regulates Δ Np63 α protein transport into the nucleus and is a direct target of miR-27a*. Mutant TP53 disrupts the Δ Np63 α -miR-27a* autoregulatory loop by repressing miR-27a*.

we examined the expression of Δ Np63 α -regulated genes involved in HNSCC survival in 22A-miR-27a* cells. An increase in NOXA and PUMA, proapoptotic genes repressed by Δ Np63, was observed on miR-27a* induction (Figure 7E). This suggests that miR-27a* inhibits Δ Np63 α function, including its ability to bind to the promoters of genes and transcriptionally regulate them.

Discussion

In the current study, we confirmed repression of miR-27a* expression in tumors from patients with OCSCC. Furthermore, we identified two particular features of miR-27a* expression: 1) increased expression of miR-27a* was associated with survival in OPSCC and HPV+ HNSCC patients and 2) repression of miR-27a* was associated with poorly differentiated and advanced HPV+ tumors. Introduction of miR-27a* induces cell death in HNSCC (14) and prostate cancer (14,33) and sensitizes multiple myeloma cells to bortezomib (34), demonstrating the therapeutic potential for miR-27a*.

Our studies to elucidate the mechanism underlying miR-27a* expression identified Δ Np63 α as a transcriptional activator of miR-27a*. Knockdown experiments using siRNA targeting either all TP63 isoforms or only Δ Np63 isoforms suggest that the TA isoforms of TP63, present at reduced levels in HNSCC, are not involved (35). We also found that miR-27a* directly targets Δ Np63 α . This work supports a feedback loop that balances levels of the prosurvival Δ Np63 α and proapoptotic miR-27a*, maintaining cellular homeostasis (Figure 8). Additionally, miR-27a* targets NUP62, a protein essential for nuclear translocation of Δ Np63 α in SCCs and overexpressed in HNSCC (32). Thus, in addition to Δ Np63 α , miR-27a* targets NUP62, which is essential for Δ Np63 α function and might have an independent role as an oncogene in SCC progression (Figure 8) (32). The Δ Np63 α -miR-27a* balance is disrupted in HNSCCs by mutations in TP53 (Figure 8). Knockdown of mutant TP53 in HNSCC cells resulted in an increase in miR-27a* expression; similarly, levels of miR-27a* were lower in tumor cells engineered to express mutant TP53,

suggesting that mutant TP53 represses miR-27a* expression. This repressive effect is also seen in TCGA, in which disruptive TP53 mutations are shown to repress miR-27a* expression in OCSCC, including HPV-OCSCC, which has a very poor prognosis. This suggests that mutant TP53 probably disrupts Δ Np63 control over target genes as has been previously observed (36).

Furthermore, the ability of mutant TP53 to lower miR-27a* levels was also observed in the gain-of-function mutant TP53^{R172H/-} mouse model, which developed cutaneous SCCs; lower levels of miR-27a* were found in tumors from TP53^{R172H/-} mice than in tumors from TP53^{-/-} mice. An interesting finding is that regardless of genomic background, pSCCs, which were the only tumors that developed metastases, had lower levels of miR-27a* than did wSCCs. This finding complements our findings in HPV+ patient tumors in which advanced-stage tumors had reduced levels of miR-27a*. Cutaneous SCC, like HNSCC, presents with high levels of Δ Np63 α and a preponderance of TP53 mutations, suggesting that our findings might have a broader relevance to other SCCs beyond HNSCC.

A number of transcriptional and posttranscriptional programs tightly control Δ Np63 α levels in tissues (37–39). Enforced expression of Δ Np63 α in murine *in vivo* models promotes tumorigenesis, whereas deletion of Δ Np63 α results in apoptosis and regression of tumors, suggesting that survival in SCCs is dependent on Δ Np63 α (40). The current lack of Δ Np63 α -targeting molecules precludes testing this hypothesis in patients. Disruption of the miR-27a*- Δ Np63 α loop has clinical relevance because Δ Np63 α mediates cisplatin resistance via AKT1 upregulation (41) and further regulates a miRNA program that mediates cisplatin response in HNSCCs (42). There is a high overlap between mutant TP53 and TP63-binding sites (43), with studies suggesting that mutant TP53 uses Δ Np63 α as a chaperone to affect transcriptional programs and promote invasion (44). Mutant TP53 suppression of miR-27a* could represent a mechanism that allows Δ Np63 α accumulation.

Advanced HNSCC tumors can have mutations in multiple components of the same pathway, as observed in the PI3K/AKT1/mTOR pathway (6). This has led to evaluation of simultaneous targeting of EGFR and mTOR in HNSCC preclinical models with promising results (45,46). A PanCancer Atlas study across multiple tumor types in the TCGA, which aimed at identifying features distinguishing SCC, found a number of important molecular pathways relevant to SCCs (35). A notable genomic feature, typical of SCC, is increased expression of Δ Np63 α . Copy number alterations in several components of the PI3K/AKT1/mTOR axis across several chromosomes were also a characteristic feature of SCCs compared with other tumor types. Additional mutations were found in other genes that enhance oncogenic potential of the PI3K and TP63 networks. Alterations in TP63 in SCCs can affect therapeutic response and disease progression (35). Overexpression of Δ Np63 α was found to be associated with alterations in PI3KCA, SOX2, and ACTLA6 pathways, which are involved in growth, stemness, and survival (35). All of this suggests that in addition to targeting multiple components of a single pathway, targeting of a focused subset of pathways may be required to overcome adaptive and innate resistance in HNSCCs.

Outcomes for HPV-HNSCC patients with advanced disease remain poor (17). Even with the advent of EGFR-targeted therapies, cisplatin remains a mainstay in the treatment of advanced HNSCC (47). TP53 mutations can often be indicative of cisplatin resistance (8,27). As we have comprehensively demonstrated in the current study, miR-27a* targets multiple components of the Δ Np63 α pathway essential to its oncogenic role in SCC, in

addition to the $\Delta Np63\alpha$ protein itself. Our earlier work has clearly detailed that multiple components of the PI3K/AKT1/mTOR pathway (AKT1 and mTOR) and EGFR are direct targets of miR-27a*, causing tumor inhibition in murine orthotopic xenograft models (14). Although we have defined a set of miR-27a* target proteins essential to HNSCC viability, further work is needed to validate other targets that might be important in HNSCC biology.

In conclusion, although others have shown that mutant TP53 affects miRNA function in other tumor types (48,49), to our knowledge, this study for the first time provides a regulatory framework of miR-27a* expression in HNSCC. miR-27a* connects and regulates the TP63/TP53 pathways and mTOR/AKT1 networks, which are fundamental to SCC progression. The importance of miR-27a*-regulated targets necessitates repression of miR-27a* for tumor survival. Thus, our study presents not only a regulatory model for miR-27a* expression but also a new modality to disrupt multiple oncogenic pathways essential to tumor survival, potentially having a profound impact on HNSCC therapy.

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Notes

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Conception and design: SYL and NSC. Acquisition of data (provided reagent, acquired and managed patients, provided facilities, etc): NSC, AAP, AAO, JNM, MDW, XL, JL, CC, GAC. Analysis and interpretation of data (eg, statistical analysis, biostatistics, computational analysis): NSC, CI, CBP, SYL. NSC and SYL wrote the manuscript, and all authors were involved in manuscript review.

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