**LINC00261 Is an Epigenetically Regulated Tumor Suppressor Essential for Activation of the DNA Damage Response**

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**Abstract**

Lung cancer is the leading cause of cancer-related death in the United States. Long noncoding RNAs (lncRNA) are a class of regulatory molecules whose role in lung carcinogenesis is poorly understood. In this study, we profiled lncRNA expression in lung adenocarcinoma (LUAD) cell lines, compared their expression with that of purified alveolar epithelial type II cells (the purported cell of origin for LUAD), cross-referenced these with lncRNAs altered in the primary human tumors, and interrogated for lncRNAs whose expression correlated with patient survival. We identified LINC00261, a lncRNA with unknown function in LUAD, adjacent to the pioneering transcription factor FOXA2. Loss of LINC00261 was observed in multiple tumor types, including liver, breast, and gastric cancer. Reintroduction of LINC00261 into human LUAD cell lines inhibited cell migration and slowed proliferation by inducing G2–M cell-cycle arrest, while upregulating DNA damage pathway genes and inducing phosphorylation-mediated activation of components of the DNA damage pathway. FOXA2 was able to induce LINC00261 expression, and the entire locus underwent hypermethylation in LUAD, leading to loss of expression. We have thus identified an epigenetically deregulated lncRNA, whose loss of expression in LUAD promotes the malignant phenotype and blocks activation of the DNA damage machinery, predisposing lung cells to cancer development.

**Significance:** These findings identify LINC00261 as a tumor suppressor that blocks cellular proliferation by activating the DNA damage response and suggest that epigenetic therapy to inhibit DNA methylation may enhance treatment of LUAD.

**Graphical Abstract:** [Image](http://cancerres.aacrjournals.org/content/canres/79/12/3050/F1.large.jpg).

See related commentary by Davalos and Esteller, p. 3028

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Introduction
Lung cancer continues to be the leading cause of cancer-related death in the United States, with approximately 150,000 deaths reported annually (1, 2). Annual deaths attributed to lung cancer surpass colorectal, breast, and prostate cancers combined (3). Non–small-cell lung cancer (NSCLC) represents the majority of new lung cancer cases, encompassing approximately 85% of all diagnosed lung cancers (4). Among NSCLC, the most commonly occurring subtype in the United States is lung adenocarcinoma (LUAD; ref. 1). Understanding the specific occurring subtype in the United States is lung adenocarcinoma (LUAD; ref. 1). Analysis of significant driver mutations in LUAD has identified KRAS, EGFR, and EML4-ALK translocations as the most prevalent mutations, present in over half of all cases (7). Targeted therapy directed toward EGFR mutations has shown a positive response to the tyrosine kinase inhibitors, such as gefitinib and erlotinib (8). However, resistance soon arises, leading to overwhelming relapse rates (9–11). Furthermore, of LUAD cases screened, approximately 30% harbor no known oncogenic driver mutations (12, 13), emphasizing the need for a deeper understanding of the molecular mechanisms underlying carcinogenesis.

LINC00261 expression was lost in LUAD through epigenetic silencing of LINC00261, a transcription factor locus, and removal of DNA methylation silencing was able to reactivate such as genetic and epigenetic regulatory changes associated with lung carcinogenesis. Transcriptome-wide bioinformatic analysis through activation of ATM phosphorylation.

RNA- and whole-genome bisulfite-sequencing and high-dimensional analysis

For the cell lines and purified alveolar epithelial cells (AEC), 1 μg of RNA underwent RiboZero (Illumina) depletion and subsequent library preparation using the Illumina TruSeq Kit (Illumina). Samples were multiplexed and underwent paired-end 50-bp sequencing on the Illumina HiSeq2000. FASTQ files were processed to remove the bases 1–12 of all the reads and low sequence complexity elements. Filtering was performed to retain only the reads with the Quality scores >20 for 90% of the read length. Once cleaned, FASTQ files were aligned to the Lincipedia2.1 transcriptome and RefSeq58 (hg19) using Bowtie version 1.1. Transcripts were eliminated that had fewer than 10 reads across the dataset before differential analysis using EdgeR. For the transgenic H522-CMV-LINC00261 and H522-CMV-NEO controls, 1 μg of RNA underwent library preparation as above, then single-end 76-bp sequencing using the Illumina NextSeq 500 and analysis using Edge R. Raw FASTQ files were cleaned as above and aligned to the RefSeq77 (hg38) transcriptome. Whole-genome bisulfite sequencing (WGBS) processing and analysis were described previously (28).

PCR for cloning LINC00261 and promoter fragments
LINC00261 was synthesized in two segments using different methods due to high CpG and low complexity in the different fragments. The Phusion High-Fidelity DNA Polymerase kit (New England Biolabs) was used for the PCR amplification of the major exon of LINC00261. The single-stranded DNA oligonucleotide primers (Integrated DNA Technologies, IDT) used for PCR reactions are listed in Supplementary Table S1. Human genomic DNA (Promega) was used as the template for all PCR reactions. The PCR reaction consisted of 1× Phusion HF buffer, 200 μmol/L dNTPs, 0.5 μmol/L forward primer, 0.5 μmol/L reverse primer, 150 ng template genomic DNA from a human male (G147A; Promega), 3% DMSO, 0.3 μl. Phusion DNA polymerase, and water to a total volume of 30 μL. LINC00261 upstream exons 1–3 were synthesized by IDT-DNA using their gene synthesis technology and cloned along with the major exon into the pCMV6-vector backbone (PS100001, Origene).

Generation of stable cell lines expressing LINC00261
Mycoplasma-negative A549 cells underwent single-nucleotide polymorphism typing for cell-line verification and were then transfected with either the linearized LINC00261 shRNA construct or scrambled control shRNA (HC137604, Origene). A total of 7 × 10⁴ cells per well were plated into 24-well dish and puromycin (0.625 μg/mL) was used for selection. H522 LUAD cells were transfected with either the linearized LINC00261 shRNA construct or scrambled control shRNA (HC137604, Origene). A total of 7 × 10⁴ cells per well were plated into 24-well dish and puromycin (0.625 μg/mL) was used for selection. H522 LUAD cells were transfected with either the linearized LINC00261 shRNA construct or scrambled control shRNA (HC137604, Origene). A total of 7 × 10⁴ cells per well were plated into 24-well dish and puromycin (0.625 μg/mL) was used for selection. H522 LUAD cells were transfected with either the linearized LINC00261 shRNA construct or scrambled control shRNA (HC137604, Origene). A total of 7 × 10⁴ cells per well were plated into 24-well dish and puromycin (0.625 μg/mL) was used for selection. H522 LUAD cells were transfected with the linearized LINC00261 cDNA construct and cultured in the presence of puromycin to select for stable transfectants. The selected clones were used for further experiments.

Materials and Methods
Cell lines and primary cells
LUAD cell lines were obtained from the laboratory of E. Haura or the ATCC and fingerprinted to verify their identity prior to experimentation at the University of Arizona (Tucson, AZ). Cells were verified Mycoplasma-free every 2 months in the laboratory via established protocols (26). Remnant human transplant lungs were obtained in compliance with Institutional Review Board–approved protocols for the use of human source material in research (HS-07-00660) and processed within 3 days of death. Lungs were processed for primary alveolar epithelial type II cell isolation as in ref. 27.

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transfected with either the linearized full-length LINC00261 plasmid or the control C-terminal Myc-DDK–tagged pCMV-6 entry vector. Optimized transfection conditions for H522 cells consisted of 8 × 10⁴ cells per well with a G418 concentration of 166 μg/mL. Stable cell lines were assayed by qRT-PCR every third passage to verify stable knockdown was maintained.

Data access
All datasets are deposited in the public Gene Expression Omnibus database (GSE110025).

RNA isolation and qRT-PCR
RNA was harvested from cells using TRIzol reagent according to the manufacturer’s protocol (Sigma). cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s protocol.

Proliferation assay
Cell proliferation was measured by seeding 1 × 10⁶ cells per well in sets of 4 wells in a 24-well dish. One well of each set was trypsinized at 24-hour intervals over a period of 4 days and counted using a Bright-Line hemocytometer (Sigma-Aldrich). Three technical replicates were performed on three different stably transfected cell lines per assay.

Transwell migration and invasion assay
Transwell migration and invasion assays were performed as described previously (29). Briefly, cell migration was measured using 8-μm-pore-size, Corning Transwell inserts (Corning). In each well, 5 × 10⁴ cells were added to the top chamber and suspended in 100 μL of serum-free medium (RPMI 1640, 1% penicillin/streptomycin, 0.1% BSA; RMBIO). In the bottom chamber, 600 μL of the complete medium (RPMI 1640, 1% penicillin/streptomycin, 10% FBS) was added. After 24-hour incubation, the top of the membrane was dried with a cotton swab to remove any remaining nonmigrated cells. Transwell inserts were fixed with 70% ethanol for 10 minutes, dried, and stained with 0.2% crystal violet (Santa Cruz Biotechnology). Using an inverted microscope with a magnification of ×100, migrated cells were counted in three randomly selected fields and averaged. Cell invasion was measured by coating wells with 15 μL of Matrigel (Corning) diluted in 0.01 mol/L Tris (pH 8.0) and 0.7% NaCl (Promega; Amresco) and allowing them to dry for 4 hours at 37°C prior to adding cells. Three biological and technical replicate experiments were performed for each assay.

Flow cytometry
Cells were plated onto Corning 6-well tissue culture dishes (Corning). Once 70% confluent, cells were hypotonically lysed in 300 μL of the DNA staining solution consisting of 0.5 mg/mL propidium iodide (PI), 0.1% sodium citrate, 0.05% Triton X-100 (Sigma-Aldrich). Lysates were filtered using a 40-μm nylon cell strainer (BD Falcon) to remove cell membranes and debris. PI-stained nuclei were detected using a PL-2 detector with a 575-nm band-pass filter on a Beckman-Coulter FACS analyzer with laser output adjusted to 488 nm. A total of 1 × 10⁶ nuclei from the total population were analyzed per sample at a rate of 100–200 nuclei/second. The percentages of cells within the G₁, S, and G₂–M phases of the cell cycle were determined by analyzing the output histogram using FlowJo v10.1. Technical triplicates were averaged and statistics performed on three biological replicates.

Scratch assay
A total of 4 × 10⁵ cells per well were plated into a 6-well dish and the cells were grown to confluence. The plate was then vertically scratched using a P20 pipette tip, and visually inspected using an inverted phase contrast microscope. After 24 hours, the cells were washed with PBS and media were replaced, followed by visualization. The plates were then analyzed with T-Scratch software (30). The protocol was modified from ref. 31. Technical quadruplicates were averaged and statistics performed on three biological replicates.

Tumor xenografts
All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Southern California protocol # 20633. Eight-week-old female athymic nude mice were purchased from Jackson Laboratory (Jackson Laboratory). All animal studies were performed in compliance with the University of Southern California IACUC guidelines. H522 CMV-NEO and CMV-LINC00261 cells were suspended in 150 μL PBS with 50% Matrigel and subcutaneously injected in the dorsal flanks of mice (1 × 10⁵ cells per flank). Tumors were measured three times per week and their volumes (V) are calculated by the previously published formula V = hw³/2, where l and w are the larger and smaller length diameters, respectively (32). Mice were euthanized after 6 weeks by intraperitoneal injection of Euthasol at the experiment endpoint and tumors were excised and weighed.

Western blot analysis
Total protein lysates were obtained from both H522-CMV-LINC00261 and H522-CMV-NEO controls using radio-immunoprecipitation assay (RIPA) buffer containing 1 mmol/L phenylmethylsulfonyl fluoride. Protein lysates were run on 10% Tris-polyacrylamide gels and then electrochemically transferred to Immuno-Blot polyvinylidene difluoride membranes. The membranes were then incubated overnight at 4°C with antibodies from Cell Signaling Technology DNA Damage Repair Kit (CS#9947) as well as β-actin (Cell Signaling Technology #4970), BRCA2 (Origene #TA313520), and phospho-BRCA2 (Invivogen #PAS37499). The membranes were blocked for 1 hour in 5% nonfat dry milk at room temperature and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin was applied at a dilution of 1:10,000 for 2 hours at room temperature. HSP90 (GTX109753, GeneTex, 1:1,000) was used as a loading control and the blots were visualized using a...
Molecular Image ChemiDoc XRS+ (Bio-Rad). The analysis software used was ImageJ (NIH, Bethesda, MD). Ku-55933 (Sigma-Aldrich) was used as a specific inhibitor of ATM kinase phosphorylation. Representative blots of three independent experiments are shown.

Luciferase reporter assay
Constructs were transfected into A549 cells using FugeneHD (Promega) and H522 cells using Lipofectamine 2000 (Thermo Fisher Scientific). Luciferase assays were conducted as described previously (28). Technical triplicates were averaged and statistics performed on three biological replicates.

Statistical analysis
Statistics for biological experiments were calculated using Prism5. Two-sided paired t tests were used for cell line comparisons between test and controls. ANOVA was used for trend tests. Fisher Scientifi

Results
Identification of LINC00261 as a tumor suppressor in LUAD
To identify LUAD-specific candidate IncRNAs, we analyzed 16 different LUAD cell lines for their whole IncRNA transcriptome as defined by Incpedia2a.1 (Supplementary Fig. S1A). This revealed approximately 32,000 IncRNAs expressed to some level in LUAD. Differentially expressed IncRNAs between cancerous cells and normal human alveolar epithelial cells (AEC), the purported cells of origin for LUAD, were identified by comparing LUAD cell lines to previously generated whole transcriptomic profiles of purified AECs from three human donor lungs not used for transplant (27). Comparison of IncRNA expression revealed that 833 IncRNA transcripts (649 genes) were differentially expressed between LUAD cell lines and AECs (Supplementary Fig. S1B; Supplementary Table S2). To exclude IncRNAs differentially expressed due to effects of in vitro cell culture, we compared this set of IncRNAs to those differentially expressed in publicly available datasets of primary human LUAD tumors profiled by TCGA, as previously determined by Mather and colleagues (33), resulting in a narrowed list of 16 IncRNAs with potential relevance in both primary human tumors and cell line model systems.

While previous transcriptomic analyses have determined that thousands of genes are differentially expressed in cancers when compared with the adjacent normal tissues, few are “drivers” of carcinogenesis. The majority of alterations are “passive” and the result of tumorigenesis, not causal. To assess which of these IncRNAs may be driving carcinogenesis, we evaluated the survival outcomes and stage at loss of expression. Six of the candidate 16 IncRNAs had significant effects on survival, and LINC00261 emerged as the top candidate with a dramatic stage-dependent effect on expression (Fig. 1A) and survival (Fig. 1B). Using qRT-PCR, LINC00261 was significantly downregulated in the panel of LUAD cell lines compared with the purified primary AECs (Fig. 1C). To determine whether the loss of expression was LUAD-specific or more generalized phenomenon across cancer types, we extracted the expression of LINC00261 in many TCGA-profiled cancer types using IncRNAtor (34). LINC00261 loss is observed in multiple epithelial cancers (Fig. 1D), with the effects on survival and stage-dependent expression in liver and breast (Supplementary Fig. S2A and S2B) as well as previous reports in gastric cancer (35), indicating that LINC00261 is a candidate of high interest as a potential tumor suppressor across multiple epithelial cancer types. In addition, LINC00261 expression was significantly correlated with tobacco smoking history in LUAD, suggesting an environmental trigger may initiate loss of this gene in the development of cancer (Supplementary Fig. S2C).

LINC00261 expression reduces proliferation, migration, and initiates G2–M cell-cycle arrest in LUAD cells
To determine whether LINC00261 plays a functional role in LUAD carcinogenesis, we constructed an ectopic expression vector containing a CMV promoter and the full-length LINC00261 transcript. H522 LUAD cell lines were chosen for ectopic reintroduction as they lack endogenous LINC00261 expression (Supplementary Fig. S3A). Stable reintroduction of the CMV construct resulted in a level of expression equivalent to the endogenous levels of LINC00261 expression in the primary AECs (Supplementary Fig. S3B). Ectopic expression of LINC00261 resulted in a significant decrease in proliferation of H522 cells (Fig. 2A, P < 0.01) relative to CMV-NEO vector control over the course of four days. In addition to increased proliferation, one of the hallmarks of cancer is the acquisition of migratory capacity, leading to metastasis. The migratory capability of H522 CMV-LINC00261–stable cell lines was tested through a scratch assay. LINC00261 was able to decrease the migration of H522 LUAD cells compared with NEO controls (Fig. 2B). Quantification over the course of four independent experiments using TScratch revealed that LINC00261 significantly reduces migratory capacity in vitro (Supplementary Fig. S3C).

To determine how LINC00261 blocks cellular proliferation, we performed cell-cycle analysis on H522-CMV-LINC00261 and H522-CMV-NEO controls. FACS analysis demonstrated that reintroduction of LINC00261 arrests H522 cells in the G2–M phase of the cell cycle (Fig. 2C; Supplementary Fig. S3D). Quantification revealed a significant shift in the population from the G0–G1 to the G2–M phase of the cell cycle (Fig. 2D). Finally, a key hallmark of defining a gene as a tumor suppressor is the ability to inhibit tumor formation in vivo. H522-CMV-LINC00261 cells alongside CMV-NEO controls were implanted into nude mice and the growth of tumors was measured for 6 weeks. LINC00261 resulted in decreased tumor weight as compared with NEO-treated mice controls (Fig. 2E). LINC00261 was also able to inhibit tumor growth over time (Fig. 2F). LINC00261-expressing tumors also showed a decrease in vascularization relative to the empty vector controls (Fig. 2G).

While the majority of LUAD primary tumors and LUAD cell lines lack the expression of LINC00261, there are few stage 4 primary tumors and aneuploid cell lines that express measurable endogenous LINC00261, including the A549 LUAD cell line (Fig. 1C). To determine whether the ablation of LINC00261 was able to affect the hallmarks of cancer phenotypes, we generated stably knocked down LINC00261 using short hairpin RNAs (A549-shLINC00261) in A549 cells, one of the cell lines that expresses endogenous LINC00261 (Fig. 3A). Ablation of LINC00261 in A549 cells caused a significant increase in A549 cell proliferation (Fig. 3B), and also significantly increased colony formation (Fig. 3C and D) and invasion (Fig. 3E and F). Migration of A549 cells was also affected by the knockdown of LINC00261; however, this result did not reach statistical significance (Supplementary Fig. S4A). FACS analysis of A549-shLINC00261 showed a significant increase in the proportion of cells with >G2 DNA content (Fig. 3G and H), suggesting that the lack of LINC00261 results in chromosomal instability and aneuploidy. Consistent
with this, we began to observe an increase in cells with higher order DNA content as evidenced by the increase in macronucleated cells (Supplementary Fig. S4B). These cells accounted for approximately 10%–15% of the population at any given time, similar to our FACS findings.

Bioinformatic profiling and pathways analysis of LINC00261 targets

To determine the major pathways affected by LINC00261 function, we performed RNA sequencing (RNA-seq) on the H522-CMV-NEO and H522-CMV-LINC00261–stable cell lines. RNA-seq analysis indicated that 108 genes were differentially expressed upon ectopic LINC00261 reintroduction (Fig. 4A and B; Supplementary Table S3). Ingenuity Pathways Analysis (IPA) revealed that the major pathways altered were G2–M DNA damage checkpoint signaling, GADD45, and RAN signaling (Fig. 4C, blue bars). We then analyzed publicly available datasets to determine which genes and related pathways were significantly correlated to LINC00261 expression levels. To do so, we utilized the TANRIC database, which has calculated coexpression networks for differentially expressed lncRNAs in numerous cancers. A total of 342 genes were significantly correlated with the expression of LINC00261 in LUAD from TCGA RNA-seq profiling (P < 1.0E−15, normals are excluded from this analysis). Performing enrichment analysis on those correlated genes indicated that G2–M cell-cycle arrest and the DNA damage response (DDR) were the top correlated pathways (Fig. 4C, purple bars). Strikingly, LINC00261 reintroduction caused an upregulation in mRNA levels of ATM kinase, TOP2A, DNA helicase, and other critical members of the DDR pathway (Fig. 4D). Many of the same genes had expression correlated to LINC00261 levels in TCGA LUAD gene expression profiling (Supplementary Fig. S5A and S5B).
The DNA damage pathway regulation is typically measured by the activation of protein phosphorylation. To determine whether there was altered phosphorylation of key DDR members, we performed phospho-specific antibody staining on lysates from H522-CMV-LINC00261 alongside H522-CMV-NEO controls (Fig. 4E). This demonstrated that the reintroduction of LINC00261 was able to increase the amount of detectable ATM phosphorylation, as well as phosphorylation of CHK2, a
downstream target of ATM. LINC00261 reintroduction also increased the phosphorylation of BRCA2, as well as increased total levels of ATR and BRCA1 protein (Supplementary Fig. S5C). The increased activation of ATM in the presence of LINC00261 was striking, as ATM is the sensor of DNA damage within the cell. To further assess whether LINC00261 was acting upstream or downstream of ATM kinase, we treated both CMV-NEO and CMV-LINC00261 cells with Ku-55933, a specific ATM inhibitor (36). Ku-55933 was able to block ATM phosphorylation as well as subsequent downstream phosphorylation events, indicating that LINC00261 acts upstream of ATM activation to facilitate DNA damage response activation (Fig. 4E). Therefore, LINC00261 may affect the ability of ATM to sense DNA damage and direct coordinated repair of the damaged loci.

**Regulation of LINC00261 by the pioneering transcription factor FOXA2**

In searching for a mechanism by which LINC00261 expression is regulated in cancer, we discovered that LINC00261 is in a close genomic proximity to the pioneering transcription factor FOXA2. This transcription factor has critical regulatory functions in prostate, lung, liver, and overall endoderm development. FOXA2 is essential for normal differentiation of the alveolar epithelium, as FOXA2 ablation results in disrupted alveolarization (37). To determine whether FOXA2 plays a role in regulating LINC00261 expression, we performed a correlation analysis using coexpression from the TANRIC database. FOXA2 and LINC00261 expression were highly correlated in LUAD (R = 0.89; P = 3.5E–136; Fig. 5A). In addition, expression of LINC00261 was positively correlated to FOXA2 in several other epithelial cancers of endodermal origin, including hepatocellular carcinoma (R = 0.66; P = 0.3E–5), renal clear cell carcinoma (R = 0.56; P = 3.3E–38), prostate adenocarcinoma (R = 0.702; P = 4.1E–22), and lung squamous cell carcinoma (R = 0.87; P = 4.8E–71). Essentially all cancer types where LINC00261 was found to be significantly downregulated in tumor versus normal comparisons were also highly correlated in expression to FOXA2. We then asked whether this effect was due to the regulation of LINC00261 expression by FOXA2. To do this, we introduced ectopic CMV-FOXA2 into H522 cells, which lack endogenous LINC00261 and FOXA2 expression. Ectopic reintroduction of FOXA2 stimulated the expression of endogenous LINC00261 in transfected cells (Fig. 5B). However, when CMV-LINC00261 was introduced into H522 cells, we did not find a concomitant increase in FOXA2 expression (Fig. 5C), suggesting unidirectional regulation. To determine whether endogenous LINC00261 could affect FOXA2 levels as reported previously (38), we utilized A549 cells, which have endogenous expression of FOXA2 and LINC00261. shRNA-mediated ablation of FOXA2 decreased endogenous expression of LINC00261 (Fig. 5D). However, knockdown of LINC00261 did not affect FOXA2 levels (Fig. 5E).

FOXA2 is known to act as a pioneering transcription factor. We therefore utilized ENCODE FOXA2 ChIP-seq data in A549 cells to identify candidate FOXA2 transcription factor–binding sites that may affect LINC00261 expression. We identified a FOXA2-binding site directly upstream of the LINC00261 transcription start site and cloned this region into the pGL3 promoter luciferase reporter construct (Fig. 5F). Reporter cells containing the LINC00261 promoter construct and minimal promoter vector controls were transfected alongside CMV-FOXA2 and CMV-NEO plasmid controls. We observed a significant increase in the overall promoter activity when comparing pGL3 empty vector to the vector containing the LINC00261 promoter, indicating the promoter fragment was functional (Fig. 5G; P = 0.0361). In addition, CMV-FOXA2 transfection was able to stimulate LINC00261 promoter activity significantly more than the empty vector control (P = 0.0210; Fig. 5G). The level of induction seen for the LINC00261 promoter far exceeded the small increase observed for the reporter vector.

**Epigenetic regulation of the FOXA2-LINC00261 locus**

Because loss of FOXA2 and LINC00261 expression were correlated in LUAD, and they occupy the same genomic locus, we sought to identify whether a common epigenetic mechanism of regulation was disrupted in cancer that could explain their mutual downregulation. We performed WGBS on purified AEcs (27) and compared this to the WGBS profile of LUAD cell lines obtained from the Japanese database DRTIS. We observed that the FOXA2-LINC00261 locus contains a 25 kb unmethylated domain in normal AEcs and this region shows extensive hypermethylation in the majority of tested LUAD cell lines (Fig. 6A).

While the WGBS results suggested that DNA methylation may play a role in regulation of the FOXA2-LINC00261 locus in LUAD, this experiment was performed on purified cells and cell lines. To determine whether a similar correlation in DNA methylation was observed in the primary tumors, we extracted the CpG methylation state and RNA expression levels from all LUAD TCGA tumors that had both data types available. Many of the CpG representation sites surrounding the LINC00261 (supplemental Fig. S6A) promoter region showed hypermethylation in LUAD, the most statistically significant of those is shown in Fig. 6B. A significant negative correlation was observed between specific CpG methylation near the FOXA2 (Fig. 6C) and LINC00261 (Fig. 6D) promoters and their respective gene expression levels. Further examination of the DNMT family of enzymes implicates DNMT1 in the aberrant hypermethylation observed (Supplementary Fig. S6B).

Having established that hypermethylation was present at the FOXA2-LINC00261 locus, we sought to understand the functional consequences on FOXA2 and LINC00261 expression in LUAD. To test this, we extracted previously published reduced representation bisulfite sequencing information on A549 LUAD cancer cells treated for 13 days using multiple doses of 5-aza-cytidine (5-aza-Cdr; ref. 39). This drug incorporates into DNA during replication and binds irreversibly to one of the major DNA methylation transferases, DNMT1, effectively inducing global demethylation. We observed decreased methylation of the CpG island proximal to the LINC00261 transcription start site in the presence of 5-aza-Cdr (Fig. 6E). This effect was observed at both tested doses compared with the vehicle controls. To determine whether the CpG island methylation status within the FOXA2-LINC00261 locus could affect the transcriptional rate of LINC00261 and hypermethylation of FOXA2 promoter (Supplementary Fig. S7A), we subjected both LINC00261 and FOXA2 promoter constructs inserted into CpgLess vector to in vitro Sssl-mediated methylation. Vector backbone with and without Sssl methylation were used as normalization controls. Methylated and unmethylated constructs were then transfected into A549 cells, and a significant decrease in LINC00261 promoter activity was observed (Fig. 6F). Hypermethylation also reduced the activity of the
Figure 3.
Knockdown of LINC00261 results in increased proliferation and migration in lung cell lines. A, Generation of stable A549 LUAD cell lines with short hairpin knockdown of LINC00261. Stable cell lines (n = 3) were generated and verified by qRT-PCR. B, Cell proliferation was counted using Trypan blue staining. Statistical analysis was performed on doubling times between stable cell lines shown (N = 4). C, Representative field (magnification, ×40) showing colony formation assay of A549-shScrambled and A549-shLINC00261 cells after 3 weeks of growth in 0.3% agar. D, Quantification of colony formation assay from counting colonies from three random fields in three biological replicates. Colony defined as area >10,000 μm². Significance calculated using a paired t test. E, Representative field (magnification, ×100) for cell invasion assay of A549-shScrambled and A549-shLINC00261 cells. F, Quantification of invasion assay from counting colonies from five random fields in three biological replicates. Statistical differences calculated using paired t test. G, Flow cytometric analysis of DNA content of LUAD cells using propidium iodide. Population was analyzed using FlowJo v10.1. N = 3 (≥10,000 cells per sample). Representative FACS analysis shown. H, Quantification of the flow analysis in E. Three biological replicates representing isolated stable clones were quantified in the technical triplicates. * P ≤ 0.05; ** P ≤ 0.01; ns, nonsignificant.
RNA-seq analysis of H522 CMV-NEO and CMV-LINC00261 stable cell lines reveals a role for LINC00261 in the DNA damage pathway response. 

A, Unsupervised hierarchical clustering using ward method for reads per kilobase million (RPKM) of H522 CMV-NEO and CMV-LINC00261 stable cell lines. Top 5% most variant genes shown.

B, Volcano plot of the differential gene expression profiling. Red, upregulated in H522 CMV-LINC00261; green, downregulated in H522 CMV-LINC00261, as compared with H522 CMV-NEO control.

C, IPA pathway enrichment of differentially expressed genes. Purple, correlated and anticorrelated genes with LINC00261 expression in TCGA LUAD, as computed by TANRIC (45). Dark blue, differentially expressed genes between H522 CMV-NEO and CMV-LINC00261. Pathways are BH-corrected.

D, IPA network analysis of significantly differentially expressed genes in the DNA damage response pathway with altered expression in the H522 CMV-LINC00261 RNA-seq analysis.

E, Western blotting of H522 CMV-NEO and H522 CMV-LINC00261 upon exposure to multiple doses of ATM inhibitor Ku-55933.
Figure 5.
FOXA2 regulates expression of LINC00261 in LUAD. A, Correlation between FOXA2 and LINC00261 expression from the TCGA LUAD dataset as computed by TANRIC (45). $R^2 = 0.91$. B, qRT-PCR of FOXA2 and LINC00261 expression in H522 cells transiently transfected with CMV-FOXA2 or CMV-NEO control. Y-axis is (log) fold change in expression between CMV-FOXA2 and CMV-NEO control. C, IGV image of BigWig tracks from H522-NEO and H522-CMV-LINC00261 RNA-seq data. All tracks are scaled 0–100 for read depth. Each row is an independent stable cell line. D, qRT-PCR of FOXA2 and LINC00261 expression in A549-shLINC00261 and A549-shScrambled controls. E, qRT-PCR of FOXA2 and LINC00261 expression in transiently transfected A549-shFOXA2 and A549-shScrambled controls. F, IGV image of cloned LINC00261 promoter relative to the LINC00261 transcription start site (promoter fragment is black). Gray, identified peaks from FOXA2 ChIP-seq in A549 cells generated by ENCODE. G, Luciferase assay in A549 cells of LINC00261 promoter, activity in the presence or absence of transiently transfected CMV-FOXA2. Luciferase values are normalized to total protein content and corrected for background pGL3 luciferase activity. RLU, relative light units. A paired t test was used to determine the significance from four independent experiments, each with technical triplicates of plasmids derived from independent minipreps.

$^* P \leq 0.05; ^{**} P \leq 0.01; ^{***} P \leq 0.001.$
Figure 6.
The DNA hypermethylation deactivates expression at the FOXA2/LINC00261 locus in LUAD. A, IGV track showing WGBS of the primary alveolar epithelial cells from three donor lungs and reduced representation bisulfite sequencing from DBTSS-generated LUAD cell lines. Red, methylated; blue, unmethylated; gray, non CpG sequence. B, Methylation of cg15588464 (LINC00261 promoter, hg19 chr20:22,559,803, −523 from TSS) and cg07003030 (FOXA2 promoter, hg19 chr20:22,565,995, −894 from TSS) in TCGA LUAD 450K array profiling (12) compared with adjacent tumor normal controls. C, Correlation between FOXA2 and Infinium 450K, probe methylation in TCGA LUAD data for FOXA2 promoter (R² = 0.56). D, Correlation between LINC00261 expression and Infinium 450K, probe methylation in TCGA LUAD for the LINC00261 promoter (derived from cBioPortal, R² = −0.41). E, Methylation levels derived from reduced representation bisulfite sequencing of A549 cells treated with 5-azaCdr. Image shows percent methylation of multiple treatment conditions across the LINC00261 promoter region. Green, CpG islands. F, Luciferase assay for the activity of LINC00261 promoter in the presence or absence of in vitro SssI methylation. Data are normalized to CpGLess empty vector values from appropriate treatment group. A paired t test was performed on N = 3 biological replicates, each performed in technical triplicate. *, P ≤ 0.05; ns, nonsignificant.
FOX2 promoter; however, these results did not meet the threshold for statistical significance (Supplementary Fig. S7B).

These results suggest that the hypermethylation of the FOXA2-LINC00261 locus decreases the expression of both genes, resulting in a loss of downstream function.

Discussion

We have identified LINC00261 as a noncoding RNA with tumor suppressor characteristics in LUAD and describe mechanistically how LINC00261 could be responsible for the initial observations found in Liu and colleagues (40). LINC00261 expression is lost in the vast majority of the primary LUAD tumors and cell lines. Reintroduction of LINC00261 into LUAD cells was able to block proliferation, migration, and invasion capacity in vitro. Furthermore, we observed that LINC00261 is an integral part of the DNA damage response in lung cells, without which the cells are unable to effectively initiate G1-M cell-cycle arrest and the DNA damage repair signaling pathways critical for maintenance of healthy lung cells. In addition, we identified FOXA2 as a regulator of LINC00261 expression, the regulation of which is disrupted in LUAD by hypermethylation of the entire LINC00261-FOXA2 locus. Examination of RNA-seq expression levels suggest that increased expression of DMNT1 may be responsible for the observed hypermethylation; however, this would need to be further explored through detailed analysis of DMNT activity.

While our results point to the applicability of LINC00261 as a tumor suppressor in other forms of endodermally derived epithelial lung cancers where FOXA2 is a critical transcription factor during development, we have not directly tested the effect of LINC00261 on carcinogenesis in other cancer types. Even when restricted to LUAD, there is controversy as to whether FOXA2 functions as a tumor suppressor, as conflicting data exist within the literature. Our results suggest that the tumor-suppressive properties of FOXA2 may be mediated by its downstream target, LINC00261.

Many cancers exhibit global hypomethylation and localized hypermethylation in the CpG islands of gene promoters. However, many of these changes are thought to be passive events with little consequence on the overall proliferative and metastatic potential of the tumor (41). The observed epigenetic effect is interesting in the context of conflicting reports regarding the role of FOXA2 in lung cancer development. Many studies implicate FOXA2 as a tumor suppressor, equating this transcription factor as a differentiation signal that antagonizes cancer to help maintain the normal state. However, the actual mechanism by which FOXA2 exerts a tumor suppressing role is unknown, and there are also a few studies that demonstrate a pro-oncogenic role for FOXA2 in other epithelial cancers (42). Studies have evaluated the binding site affinity for FOXA2 (vs. the homolog FOXA1) and seen enrichment in lipid metabolism genes, none of which were implicated in its effect on carcinogenesis (43). Demonstration of a functional role for CpG island methylation at this locus suggests a possible causal role for DNA methylation in tumorigenesis by deactivating LINC00261. Therefore, differential epigenetic silencing of LINC00261 in LUAD in specific contexts may resolve the confusion in the literature. Whether or not epigenetic silencing of the LINC00261-FOXA2 locus plays a role in other cancer types remains to be tested. Our results also suggest that epigenetic therapies such as 5-azacytidine may be a viable alternate strategy for the treatment of LUAD and other epithelial cancers, as this could reactivate LINC00261 and restore proper DNA damage response in these tumors.

We have therefore identified a lncRNA, LINC00261, which behaves as a tumor suppressor by blocking cellular proliferation through activation of the DNA damage signaling pathway to arrest cellular division. In addition, ATM, topoisomerase 2A (TOP2A), and other members of the DNA damage repair machinery have altered expression in the presence of LINC00261. Specifically, LINC00261 has been reported to alter the efficacy of cisplatin therapy in colon cancer (44). Our study provides a mechanistic basis for this observation. In identifying a critical regulatory component of the DNA damage response, we have uncovered a new aspect of this critical pathway in carcinogenesis, one that opens up the possibility for the development of novel chemotherapeutic agents targeting this lncRNA to treat this deadly disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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