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Long non-coding RNA ANRIL (CDKN2B-AS) is induced by the ATM-E2F1 signaling pathway

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Abstract

The maintenance of genome integrity is essential for the proper function and survival of all organisms. Human cells have evolved prompt and efficient DNA damage response to eliminate the detrimental effects of DNA lesions. The DNA damage response involves a complex network of processes that detect and repair DNA damage, in which long non-coding (lncRNAs), a new class of regulatory RNAs, may play important roles. Recent studies have identified a large number of lncRNAs in mammalian transcriptomes. However, little is known about the regulation and function of lncRNAs in the DNA damage response. In the present study, we demonstrate that one specific lncRNA, ANRIL, is transcriptionally up-regulated by the transcription factor E2F1 in ATM-dependent manner following DNA damage, and elevated levels of ANRIL suppress the expression of INK4a, INK4b and ARF at the late-stage of DNA damage response, allowing the cell to return to normal at the completion of the DNA repair.

Keywords

Long non-coding RNA; ANRIL; CDKN2B-AS; ATM; E2F1; DNA damage response

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1. Introduction

Long non-coding RNAs (lncRNAs) are a new class of regulatory RNAs that are defined as transcribed RNA molecules ranging in length from 200 to 100,000 nucleotides and lacking protein-coding capacity. Recent high-throughput transcriptome analyses have identified a large number of lncRNAs in mammalian genomes. While only 1.5% of the genome is responsible for protein coding genes, a vast majority of non-coding regulatory elements are transcribed to noncoding RNAs, among which lncRNAs have been recognized for their essential role in controlling every level of the gene expression program in various physiological processes including development, differentiation and metabolism [1, 2]. Current reports have suggested that lncRNAs regulate gene expression by executing as signals, decoys, guides and scaffolds and act as a repressor or activator to modulate the process of gene transcription and translation [3]. For instance, lncRNA PTENP1, previously considered as a biologically nonfunctional RNA, acts as a decoy molecule that sequesters miRNAs to abrogate their functions in regulating target gene expression. In particular, the 3' UTR of PTENP1 binds the same set of regulatory miRNA sequences that target the tumor suppressor PTEN. As a result, the miRNA inhibition of PTEN expression is attenuated and PTEN levels are increased [4]. Similar to normal protein-coding genes, transcription of lncRNAs is mediated by RNA polymerase II complex. However, it has not been well studied about how lncRNA transcription is regulated. A recent study by Huarte et al. identified a number of lncRNAs that are transcriptionally regulated by the central tumor suppressor p53. One of the p53-transactivated lncRNAs, lincRNA-p21 (long intergenic non-coding RNA-p21) serves as a key repressor in p53-dependent transcriptional responses by physically associating with hnRNP-K and modulating its localization [5]. Further study also revealed that lincRNA-p21 functions as a posttranscriptional inhibitor of translation by selectively binding JUNB and CTNNB1 mRNAs, thus repressing their translation [6]. In recent years, lncRNAs have been emerging as a critical layer in regulation of gene transcription program. However, it is yet to understand the function of lncRNAs in context of various physiological conditions.

The DNA damage response (DDR) is an important anti-cancer barrier to maintain genome integrity against intrinsic and extrinsic genotoxic stresses including ultraviolet light (UV), ionizing radiation (IR), chemo- and radio- therapeutic agents, oncogenic insults, and reactive oxygen species. The DDR involves a number of networks connecting tumor suppressor genes to DNA repair pathways, damage tolerance processes, cell-cycle checkpoints and apoptosis [7, 8]. The DDR is predominantly initiated by PI3K family proteins, ATM (ataxia-telangiectasia mutated), ATR (ATM and Rad3-related) and DNA-PKcs (DNA-dependent protein kinase, catalytic subunit), which control the activity and localization of various downstream proteins and orchestrate many events at transcriptional, post-transcriptional and post-translational levels [9, 10]. The ATM kinase is a key sensor in the DDR pathway that responds in particular to double-strand DNA breaks, the most severe genomic lesions. The ATM-mediated phosphorylation of downstream target proteins triggers a cascade of signals to activate cell cycle checkpoints and DNA repair [9]. In addition to canonical DNA damage signaling pathways, epigenetic alterations, such as altered DNA methylation status, histone modification patterns, chromatin remodeling, and non-coding RNA (in particular, microRNA and lncRNA) regulation have been shown to contribute as novel layers of regulation to the complexity of the DDR signaling network [11-14].

Given the large quantity of lncRNAs in genomes, it is assumed that expression of lncRNAs may be regulated in the DDR and provide feedback effects on the DDR. Aberrant expression of individual lncRNAs has been reported in tumors of various tissue origins [15] and recent data revealed that lncRNA transcripts can modulate gene activity in response to DNA damage [16]. A long non-coding RNA, ANRIL (antisense non-coding RNA in the INK4

locus) was found to be involved in the repression the INK4B-ARF-INK4A locus [17-19]. The INK4B-ARF-INK4A locus spans around 35 kilobases on human chromosome 9p21 that includes three intimately linked tumor suppressor genes (INK4a, INK4b, and ARF) that trigger the anti-proliferative activities of both RB and p53. Loss of the INK4B-ARF-INK4A locus is the most frequent copy number alteration across tumors and cancer cell lines [20, 21]. ANRIL is transcribed in anti-sense direction with respect to the primary INK4 and ARF transcripts [22]. ANRIL was shown to be involved in epigenetic regulation of the INK4B-ARF-INK4A locus by direct binding to the INK4b transcript and recruiting the Polycomb Repressor Complex (PRC) to repress the transcription of genes at this locus [18, 19]. However, how ANRIL is regulated in response to genotoxic stress is largely unknown. In the present study, we investigated the expression of ANRIL and its roles in the DDR. We show that ANRIL is induced by E2F1 transcription factor in an ATM-dependent manner after DNA damage, and that elevated ANRIL suppresses the expression of INK4B-ARF-INK4A at the late-stage of DDR, forming a negative feedback loop to the DDR.

2. Materials and Methods

2.1. Cell lines and Plasmids

GM0637 cell line is a human fibroblast line that expresses wildtype ATM. It was obtained from Coriell Cell Repositories. U2OS cell line (human osteosarcoma line) was obtained from the American Type Culture Collection (ATCC). HCT116 p53^{+/+} and HCT116 p53^{-/-} cell lines were obtained from the Vogelstein laboratory at Johns Hopkins University. Cells were cultured following standard protocols described previously [23].

Human ANRIL cDNA clone L6ChoCKO-2-E10 with functional region was provided by the Functional Genomics Research Center, Korean Research Institute of Biosciences and Biotechnology. ANRIL-Luciferase vector was kindly provided by Dr. Kenichi Yoshida at the University of Meiji, Japan. Retrovirus-based shRNA construct for ANRIL was a kind gift from Dr. Yojiro Kotake, Hamamatsu University, Japan. The ANRIL shRNA target sequence GGTCATCTCATTGCTCTATCC was also cloned into pSIH-H1-copGFP shRNA Expression Lentivector (#SI501A-1, System Biosciences). E2F1 construct was a gift from the Helinski Lab (Addgene plasmid # 24225), while E2F1 shRNA, ATM shRNA and p53 shRNA were purchased from the shRNA core at the University of Texas MD Anderson Cancer Center.

2.2. DNA damaging agents and ATM inhibitor

Cells were treated with neocarzinostatin (500 ng/ml) (NCS, #N9162, Sigma-Aldrich), etoposide (50 μ M) or bleomycin (20 μ g/ml) (#2434, Sigma-Aldrich) and harvested at indicated time points after treatment for the analyses of RNAs and proteins. To examine the role of ATM, cells were pretreated with 10 μ M ATM kinase inhibitor CGK733 (#118501, Calbiochem) 2 h prior to DNA damage treatment.

2.3. Gene expression analysis by RT-PCR

Total RNA (500 ng) was used for cDNA synthesis by Superscript III first strand synthesis (Invitrogen, USA). SYBR Premix Taq (BioRad, USA) was used for real-time PCR assays. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (β -actin) were detected and their mRNA levels were used as endogenous controls. Primer pairs used for quantitative RT-PCR were ordered from Sigma-Aldrich and their sequences (5'-3') are: ANRIL: TTGTGAAGCCCAAGTACTGC, TTCACTGTGGAGACGTTGGT; p21: GCAGACCAGCATGACAGATTT, GGATTAGGGCTTCCTCTTGGA; p15INK4b: GGGAGGGTAATGAAGCTGAG, GGCCGTAACTTAACGACACT; p16INK4a: GGGTCCCAGTCTGCAGTTA, GGAGGGTCACCAAGAACCT; p14ARF:

TCCTCAGTAGCATCAGCACGAG, AGAACATGGTGCAGGTTCTTG; GAPDH:
AGAAGGCTGGGGCTCATTTG, AGGGGCCATCCACAGTCTTC; β -actin:
CTGGGACGACATGGAGAAAA, AAGGAAGGCTGGAAGAGTGC.

2.4. Luciferase reporter assay

2×10^4 cells were transfected with FuGene6 (Roche, Basel, Switzerland) according to the manufacturer's instruction. Briefly, 200 ng of plasmid DNA for the gene of interest, 200 ng of firefly luciferase reporter plasmid DNA and 0.6 ng of *Renilla* luciferase reporter plasmid pRL-TK (Promega) per 24-well dish were used for each transfection. Cells were lysed 24 h after transfection by adding 100 μ l passive lysis buffer from the Dual Luciferase Reporter Assay kit (Promega) into each well. 20 μ l of cell lysate was used for the luciferase reporter assay using the same kit. Luminescence was quantified in a GloMax 20/20n Luminometer (Promega). Experiments were performed at least in triplicates. The firefly luciferase activity values were normalized to the *Renilla* luciferase activity values that reflect transfection efficiency. Data are presented as mean values ($\pm$ SD). Statistical differences were analyzed using two-tailed Student's t-tests. A value of $P < 0.05$ was considered as statistically significant difference.

2.5. Western Blot assays and antibodies

Western blot assays were performed by standard methods described previously [23]. Anti-p15INK4b (#612), Anti- β -actin (#1616), anti-p53 (#126), HRP-anti-goat IgG (#2020), HRP-anti-rabbit IgG (#2054), HRP-anti-mouse IgG (#2055) were purchased from Santa Cruz; Anti-ATM (#300-299A), Anti-p16INK4a (#301-267A), Anti-ARF (#300-340A) and Anti-E2F1 (#300-766A) antibodies were purchased from Bethyl Laboratories. Anti-p53 (pSer15) (#9286) was purchased from Cell Signaling.

2.6. RNA stability assay

To measure half-life of RNA, 10 μ g/ml Actinomycin D (#A1410, Sigma-Aldrich) was added to cell culture to block transcription (nascent RNA synthesis). Cells were pre-treated with Actinomycin D for 1 h followed by treatment with DNA damaging agent, NCS. Total RNAs were extracted from cell samples harvested at indicated time points. ANRIL expression was quantified by real-time RT-PCR. The half-life of ANRIL RNA was calculated from two independent experiments.

2.7. DNA-ChIP analyses

DNA-ChIP (chromosome immunoprecipitation) assays were performed as previously described [24]. Briefly, cells were cross-linked with 1% formaldehyde for 20 min, and cell pellets were resuspended in buffer B (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], $1 \times$ protease inhibitor, 50 U/ml RNase inhibitor). The pellets were incubated 10 min in ice and disrupted by sonication, and the lysates were cleared and subjected to immunoprecipitation with anti-E2F1 or control antibody. The precipitates were then subject to stringent washing, elution, and reversal of crosslinking. The DNA was resuspended in 20 μ l of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and incubated with DNase-free RNase for 30 min at 37°C to remove any remaining RNA. The DNA was purified using PCR clean kit (Invitrogen, USA) and eluted in 20 μ l of nuclease-free water. Quantitative PCR reactions were then performed on real-time PCR machine (Realplex2, Eppendorf) with ChIP primers: AAGATCTCGGAACGGCTCT, TCAGGTGACGGATGTAGCTA.

2.8. Cell proliferation, viability and apoptosis assays and cell cycle analysis

In cell proliferation assays, cells were seeded ~ 1000 cells per well in flat bottom 96-well microplates. Cell proliferation was measured at indicated times using WST-1 reagent

(#05015944001, Roche), according to the manufacturer's instructions. In cell viability assay, cells were seeded into 96-well plates at 3000 cells/well and incubated for 24 h. Each DNA damaging drug was then added at various concentrations to quadruplicate wells in a final volume of 100 μ l of medium. After 24 h of incubation, cells were cultured for 2 days and cell viability was measured using WST-1 reagent.

In cell cycle analysis, flow cytometric measurements of cellular DNA content were performed for 70% ethanol-fixed cells using the intercalating DNA fluorochrome, propidium iodide (PI). Fixed cells were washed with phosphate buffered saline (PBS), treated with 200 μ g/ml RNase-A (Sigma, USA) for 30 min at 37°C, and stained with 50 μ g/ml propidium iodide (Sigma, USA) in PBS. Measurements were made with a laser-based (488 nm) flow cytometer (Guava EasyCyte HT; Millipore, USA), and data was analyzed using Incyte software (Millipore, USA).

In apoptosis assays, Annexin-V conjugated to FITC along with 7AAD was used to detect apoptotic cells (Annexin-V positive, 7AAD positive) cells. Trypsinized cells were washed twice with PBS and resuspended in binding buffer containing Annexin-V-FITC and 7-AAD cocktail (Millipore, USA). The cells were incubated at room temperature for 15 min in the dark. Samples were analyzed using flow cytometer (Guava EasyCyte HT; Millipore, USA).

2.9. Homologous recombination repair analysis

DR-U2OS cell line containing a single copy of the homologous recombination (HR) repair reporter substrate DR-GFP in a random locus were generated and previously described [25]. DR-U2OS cells were infected with retroviruses expressing control or ANRIL shRNA. Cells were transfected with control or I-SceI-expressing plasmid. Flow cytometric analysis was performed to detect GFP positive cells on Guava EasyCyte™ Flow Cytometer (Millipore, USA) and HR frequencies were calculated.

2.10. Statistical Analysis

Statistical differences were determined by analysis of variance using one-way ANOVA and Turkey's Multiple Comparison Test on GraphPad Prism 5 software.

3. Results

3.1. ANRIL is induced in the DNA damage response

To examine the regulation of lncRNAs in the DDR, we assessed the genome-wide lncRNA expression profiles in human fibroblast GM0637 cells that have functional DDR [23]. The cells were treated with a radiomimetic drug, neocarzinostatin (NCS) that generates double strands breaks, and harvested at various time points (0, 4 and 8 h). A total of 10602 human lncRNAs were examined using a specific lncRNA microarray containing oligo probes for these lncRNAs. As many as 1903 specific lncRNAs, representing about one-fifth of total identified human lncRNAs, were shown to be significantly (2-fold) induced in the NCS-treated GM0637 cells, representing the DNA damage-induced lncRNAs. We assessed the expression of 15 previously studied candidate lncRNAs (HOTAIR, TUG1, ANRIL, MALAT1, NEAT1, GAS5, MEG3, UCA1, CCND1, HULC, H19, PTENP1, KCNQ1OT1, XIST and HOTAIRM1) in the DDR, and found only TUG1, ANRIL and CCND1 lncRNAs were induced after DNA damage (Fig. 1A). The ANRIL gene, also designated as CDKN2BAS, contains 19 exons with 3,857 nucleotides in length and locates in the INK4B-ARF-INK4A locus with opposite direction that produces anti-sense transcript (Fig. 1B). We confirmed the level of ANRIL following DNA damage by real-time PCR analysis in human U2OS cells. It was noted that a significant induction of ANRIL occurred at the late-stage (8-24 h post-damage) of DNA damage response (Fig. 1C). p53 and p53-transactivated p21,

both induced after DNA damage, were used as positive controls indicating the DDR functionality. To exclude the possibility that the induction of ANRIL is specific for certain type of DNA damaging agent, we measured the induced levels of ANRIL in the cells treated with different types of DNA damaging agents, including neocarzinostatin, etoposide and bleomycin. ANRIL was induced in response to each type of DNA damage although the intensity of induction varied in these different DNA damaging agents, suggesting the induction of ANRIL is independent of DNA lesions (Fig. 1E).

3.2. Induction of ANRIL is dependent on ATM

We postulated that the induction of ANRIL may be a part of canonical DNA damage signaling. Because the ATM-p53 signaling is a major DNA damage response pathway, we tested whether the induction of ANRIL is dependent on ATM or p53. We first measured the induction of ANRIL in control and ATM-silenced cells in response to NCS treatment. In both HCT116 p53^{+/+} and U2OS cells, the level of ANRIL was robustly increased after NCS treatment, but this induction was almost completely abolished in the cells expressing specific ATM shRNA (Fig. 2A and 2B). ATM shRNA knocked down the expression level of ATM over 80% in both of the cell lines. These results suggest that ANRIL is induced in an ATM-dependent manner. Because p53 is a central downstream player in the ATM-initiated DNA damage signaling pathway, we next examined whether p53 is responsible for the increased ANRIL expression. ANRIL levels were measured in a pair of isogenic HCT116 cells (p53-proficient or p53-null) treated with NCS (Fig. 2C and 2D). We observe that ANRIL was induced in both HCT116 p53^{+/+} and HCT116 p53^{-/-} cells, and the induction of ANRIL was not significantly affected by p53 depletion or restoring wild-type p53 in the HCT116 p53^{-/-} cells (Fig. 2C and 2D), suggesting the expression of ANRIL is not associated with p53 levels.

3.3. Transcriptional up-regulation by E2F1 is responsible for ANRIL induction

To determine whether the induction of ANRIL is due to post-transcriptional regulation, we examined the stability of the ANRIL RNA in the presence or absence of DNA damage. We treated the cells with Actinomycin D to block nascent RNA synthesis prior to DNA damage treatment. The stability of RNA was not significantly altered in the U2OS cells treated with or without NCS (Fig. 3A), suggesting that transcriptional regulation is a major mechanism that contributes to the induction of ANRIL in the DDR. To test this hypothesis, we analyzed the promoter region of the *ANRIL* gene and found 1 putative E2F1-binding element in the promoter (Fig. 3B). To determine whether E2F1 transactivates ANRIL in the DDR, we measured the promoter activity of ANRIL in HCT116 p53^{+/+} cells by luciferase assays. The promoter activity of ANRIL was markedly increased during DNA damage, but knockdown of E2F1 depleted this increase (Fig. 3B). To verify the direct interaction between E2F1 and the ANRIL promoter, DNA Chromatin-Immunoprecipitation (ChIP) assay was performed to measure the enrichment of E2F1 to the putative E2F1-binding DNA regions. Much higher levels of this DNA fragment was detected in the E2F1 immunoprecipitate than in the control IgG immunoprecipitate, suggesting a specific binding of E2F1 with the ANRIL promoter. Following DNA damage, E2F1-bound DNA was dramatically increased, indicating elevated recruitment of E2F1 transcription factor to the ANRIL promoter (Fig. 3C). This effect was abrogated by the specific ATM inhibitor, suggesting the E2F1-mediated transactivation is ATM-dependent in the DDR (Fig. 3C). Previous study showed that ATM-mediated phosphorylation leads to increased levels of E2F1 [26]. Consistent with this study, we observed that the level of E2F1 protein was increased and the increase is dependent on the ATM activity (Fig. 3D). These results demonstrate that ATM-induced E2F1 transcriptionally activates ANRIL in the DDR.

3.4. Genes in the INK4B-ARF-INK4A locus are regulated by ANRIL in the DDR

ANRIL gene is transcribed in the antisense orientation of the INK4B-ARF-INK4A gene cluster. Previous studies have shown that ANRIL interacts with both Polycomb Repressive Complex-1 (PRC1) and -2 (PRC-2) to form heterochromatin surrounding the INK4B-ARF-INK4A locus and repress its expression [18, 19]. We investigated the role of ANRIL in the INK4B-ARF-INK4A expression in the DDR. To knock down ANRIL, we used a lentiviral vector encoding a shRNA that specifically targets the exon1 region of ANRIL. Stable HCT116 p53^{+/+} cells with ANRIL overexpression or knockdown were generated by infection with lentiviral vectors expressing ANRIL or its shRNA and single colony screen and verification (Fig. 4A). In the control and ANRIL-altered cells, we measured the expression levels of the three genes in the INK4B-ARF-INK4A locus: p15 (INK4b), p16 (INK4a) and p14 (ARF or INK4c). In the ANRIL-silenced cells, the levels of p15 and p16 transcripts were dramatically increased while the level of p14 transcripts had mild increase. In contrast, the levels of p15, p16 and p14 transcripts were reduced in the ANRIL-overexpressing cells (Fig. 4B). We further measured both the RNA and protein levels of p15, p16 and p14 throughout the DNA damage response (0-24h) (Fig. 4C and 4D). While the three proteins function as cyclin-dependent kinase inhibitors that contribute to cell cycle arrest and associated cell responses to DNA damage, they need to be suppressed at the late-stage of the DDR when cells are returning to normal. We observed that the level of p15 started to decrease gradually from 8h after DNA damage. However, knockdown of ANRIL induced p15 and remained it at very high levels throughout the DNA damage response. When ANRIL was overexpressed in cells, p15 RNA and protein were reduced to extremely low levels (Fig. 4C and 4D). Similar results were also shown in the expression of p14 and p16. ANRIL repression of p15, p16 and p14 suggests its critical role of ANRIL in regulating the DDR.

3.5. ANRIL regulates cell cycle progression and apoptosis

To assess the effect of ANRIL in the regulation of cell activities in the DDR, we first examined cell proliferation in control, ANRIL-overexpressed and -silenced HCT116 p53^{+/+} cells. The results showed that cell proliferation was substantially retarded in the ANRIL-knockdown cells compared to the control cells, while the cells overexpressing ANRIL exhibited accelerated proliferation (Fig. 5A). To examine whether ANRIL impacts the DNA damage-induced cell-cycle checkpoints, we performed cell-cycle profiling analyses in HCT116 p53^{+/+} cells with altered levels of ANRIL. Cells were treated with NCS to activate cell cycle checkpoints. In untreated HCT116 p53^{+/+} cells, overexpression of ANRIL appeared to promote DNA synthesis and cell proliferation evidenced by the higher percentage of S-phase cells (22.9% in the ANRIL-overexpressing cells versus 11.6% in control cells) (Fig. 5B). G1/S and G2/M checkpoints were intensified in the control cells 8 h after DNA damage and a majority of cells were arrested in G1 and G2/M phases 24 h post-damage. In contrast, only 15% of cells arrested at G1 phase in the ANRIL-overexpressing cells, whereas up to 35% of cells were in G1 phase in ANRIL-depleted cells at 24 h post-damage (Fig. 5B). These results suggested that ANRIL inhibits cell cycle checkpoints and promotes cell cycle progression in the DDR. We next examined the effect of ANRIL on the DNA damage-induced cell apoptosis. Apoptotic cells were quantified and analyzed by Annexin V-7AAD staining and flow cytometry. ANRIL-depleted HCT116 p53^{+/+} cells demonstrated much increased apoptosis to NCS treatment in comparison to normal cells. In the ANRIL-knockdown cells, the percentage of apoptotic cells was increased to 23.4% in comparison to 15.7% in control cells, whereas in the ANRIL-overexpressing cells, only 9.38% of apoptotic cells were detected (Fig. 5C). Consistent with the results from the apoptosis assays, depletion of ANRIL resulted in an increase in the sensitivity of HCT116 p53^{+/+} cells to the treatment with NCS (Fig. 5D), confirming that lowered levels of ANRIL in cells led to elevated apoptosis in the DDR. Homologous recombination (HR) frequencies

are a key indicator for genomic stability in cells. Previous studies have shown that DNA damage-induced p53 suppresses HR activity in order to maintain genome integrity [27]. We assessed HR frequencies in control or ANRIL-silenced human U2OS cells with a stable insert containing two defective GFP copies (Fig. 6A). This inserted sequence does not normally express GFP but successful HR can generate a functional GFP gene for assaying. Compared to the control cells, ANRIL-depleted cells suppressed homologous recombination by ~50% (Fig. 6B), suggesting that ANRIL is required for the functionality of homologous recombination.

4. Discussion

Recent genome sequencing and transcriptome analyses demonstrate that transcription is not limited to the protein-coding genes. As a matter of fact, a vast majority of transcripts are produced from those “junk” DNA regions. In addition to well-studied microRNAs, ribosomal RNAs, small nuclear RNAs, thousands of lncRNAs have been identified and this number has been increasing [1, 3]. While these lncRNAs have little or no protein-coding capacity, a major question needs to be addressed: how do they function and coordinate with the protein-coding genes in regulating cellular and organismal activities? A small portion of lncRNAs (less than 1% of total) have been shown to have distinctive biological functions [28]. In these cases, lncRNAs act as key molecules in the regulation of processes such as chromatin remodeling, transcription, and post-transcriptional processing. As examples, the lncRNA NEAT1 functions as an essential scaffold for the organization of paraspeckle structure [29]. Xist lncRNA recruit the polycomb complex to the X chromosome, trigger heterochromatin formation, repress gene expression and inactivates the X chromosome [30, 31]. Although lncRNAs are a largely unexplored field, they appear to form a new layer of gene regulation and contribute to the complexity of gene expression programs.

Only a few of lncRNAs are currently known to be associated with human diseases, including metastasis-associated lung adenocarcinoma transcript 1 (MALAT1, also designated as Neat2), HOX antisense intergenic RNA (HOTAIR), and antisense non-coding RNA in the INK4 locus (ANRIL), and lincRNA-p21 [28]. In particular, ANRIL is one of the most frequently altered lncRNA genes in human cancer. It locates in a chromosomal region that is often homozygously deleted or transcriptionally silenced in about 40% of human cancers [32]. The same locus encodes cyclin-dependent kinase inhibitors p15INK4B and p16INK4A and a positive p53 regulator, p14ARF that inhibits Mdm2-p53 interaction [33]. Current opinion suggests that ANRIL, transcribed as an antisense RNA transcripts to INK4b, acts to inhibit INK4b (via direct targeting) and INK4a and ARF (via transcriptional suppression). Accumulating evidence has shown ANRIL as a risk locus for several cancers, including breast cancer, nasopharyngeal carcinoma, basal cell carcinoma and gliomas [34-37]. However, it is still unknown about the biological functions of ANRIL under various physiological contexts and the mechanism by which ANRIL influences tumor initiation or progression.

The DDR is composed of a series of distinct, but functionally interwoven pathways. Oncogene-induced cell hyperproliferation is a major source of DNA damage in premalignant lesions or hyperplasia [8]. Recent reports from basic and clinical studies have suggested that the DNA damage response is one of the primary anti-cancer barriers during tumor progression. In support of it, highly activated DDR and its biomarker molecules have been shown in the early stage of many types of human cancer [38]. Tumor progression is often accompanied with the inactivation of the DDR. Identification of the DDR regulators is critical to understand the homeostatic and aberrant regulation of the DDR in normal and tumor cells. Among all the regulators, those proteins that counteract with the activation of DDR are often considered as oncogenic proteins. For instance, wildtype p53-induced

phosphatase 1 (Wip1, or PPM1D) is master inhibitor that dephosphorylates several key players in the DDR, including p53, CHK1, CHK2, Mdm2, and ATM [23, 39]. Amplification and overexpression of the Wip1 gene has been reported in human breast cancer, pancreatic cancer, medulloblastoma and prostate cancer. In recent years, microRNAs, a type of small non-coding RNAs, have emerged as an important regulator in tumorigenesis. A number of microRNAs have been identified as oncogenes (oncomirs) or tumor suppressor [40]. Recent studies from us and colleagues have also shown that microRNAs are regulated at transcriptional and post-transcriptional levels [14, 41-44]. A recent study from the Rinn group first reported that lincRNA-p21 is induced by the DNA damage-induced p53 and in turn regulates the transcriptional activity of p53 [5]. In the current study, we aimed to identify DNA damage-induced lncRNAs by a genome-wide approach. A radiomimetic drug, NCS, is used to produce double-stranded DNA breaks that are most lethal lesions on DNA. ANRIL stands out as one of the lncRNAs that are significantly induced following NCS treatment. The induction of ANRIL is dependent on ATM as ATM knockdown completely abolished this effect. However, expression of ANRIL was regulated by a p53-independent mechanism. We observed the similar scenarios in both p53-proficient and deficient human cells.

ANRIL contributes to the maintenance of DDR via its regulation of cell cycle checkpoints, apoptosis and DNA repair. We provide a molecular mechanism by which ANRIL is transcriptionally activated by E2F1 and this activation is mediated by ATM-induced E2F1 activation. In normal cells, ANRIL may function as a homeostatic regulator. It inhibits those CDK inhibitors and p53 and thus brings down the DDR activity at the late stage of the DDR. This is likely important for cells to return to pre-damage state at the completion of DDR. In precancerous lesions, aberrant expression of ANRIL would disrupt the delicate control of the DDR and cause a premature blockage of this important anti-cancer barrier, leading to malignant tumor progression. It is well known that tumor cells share a common feature: genomic instability that is the combined effect of DNA damage, DNA repair defects, and dysfunctional cell cycle checkpoints. Therefore, our findings on the regulation and role of ANRIL have potential clinical implications.

As a primary kinase to initiate the DDR, ATM is an important tumor suppressor. Homozygous mutations in the ATM gene lead to ataxia telangiectasia (AT), a syndrome characterized by acute sensitivity to ionizing radiation and predisposition to cancer. However, only a small population (1%) carries ATM mutations, genes in the ATM signaling pathways are much more frequently mutated in spontaneous human cancers [9, 45]. For instance, CHK2 is a major kinase that is activated by ATM and is responsible for the G2/M cell cycle checkpoint. Inactivation of CHK2 by mutations and deletion is often associated with an increased risk of breast cancer [46]. As a down-stream effector of ATM, ANRIL seems to have substantial effects on the DDR. However, further studies will still be required to reveal important molecular and biological functions of ANRIL, including the following: (1) sequence requirement for ANRIL-E2F1 interaction, (2) the correlation between E2F1 activity and ANRIL levels in human tumors, (3) INK4a/b/ARF-independent functions of ANRIL, and (4) physiological functions of ANRIL.

In summary, we have demonstrated here that ATM-E2F1 signaling regulates the expression of an important lncRNA ANRIL. ATM-induced ANRIL regulates cell cycle checkpoints, apoptosis and affect DNA repair efficiency. Our findings provide novel mechanistic insights into the DDR and a novel layer of regulation in gene expression program.

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Abbreviation

DDR	DNA damage response
hnRNP-K	Heterogeneous nuclear ribonucleoprotein K
PTEN	Phosphatase and tensin homolog
PTENP1	PTEN pseudogene 1
PI3K	Phosphatidylinositol 3-kinases
CTNNB1	cadherin-associated protein β 1
INK4	Inhibitors of CDK4

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Highlights

- A large number of lncRNAs are induced after DNA damage.
- LncRNA ANRIL is induced after DNA damage in an ATM-dependent manner.
- Induction of ANRIL is independent of p53.
- E2F1 transcriptionally activates ANRIL.
- ANRIL inhibits the expression of INK4a, INK4b and ARF in the DNA damage response.

Figure 1. ANRIL is induced after DNA damage

(A) Relative expression levels of 15 representative lncRNAs after DNA damage. GM0637 cells were treated with NCS (500 ng/ml), and cells were harvested at indicated time points for microarray analysis. Green or red color on the heat map indicates a decrease or increase of lncRNA level, and numbers correspond to fold change comparing to the control samples with mock treatment. (B) Schematic diagram showing the ANRIL gene and its transcript in the INK4B-ARF-INK4A locus. (C) ANRIL is induced in the DDR in human U2OS cells. Level of p21 was used as a positive control for the activation of the DDR. RNA samples were analyzed by quantitative RT-PCR, and error bars represent the mean $\pm$ SD. (D) Protein

levels of p53 and p21 in the DDR. E) Induction of ANRIL is independent of DNA damage types. U2OS cells were treated with neocarzinostatin, etoposide and bleomycin respectively.

Figure 2. Induction of ANRIL is dependent on ATM, but not p53

ANRIL is induced in an ATM-dependent manner in human U2OS cells (A) and HCT116 $p53^{+/+}$ cells (B) after DNA damage. The cells were infected by lentiviruses expressing control shRNA or ATM-shRNA. ATM knockdown efficiency was confirmed by western blot. (C) Silence of p53 has no significant effect on the DNA damage-induced ANRIL. HCT116 $p53^{+/+}$ cells were infected by lentiviruses expressing control shRNA or p53 shRNA. p53 knockdown efficiency was confirmed by western blot. (D) Reintroduction of p53 into HCT116 $p53^{-/-}$ cells has no effect on the DNA damage-induced ANRIL. Expression of p53 was confirmed by western blot.

Figure 3. E2F1 transcriptionally activates ANRIL in the DDR

(A) RNA stability of ANRIL is not affected by DNA damage. U2OS Cells were pretreated with Actomycin D prior to NCS treatment. Levels of ANRIL were measured by quantitative RT-PCR. (B) Schematic representation of the ANRIL promoter. One putative E2F1 binding element is shown in the indicated regions. Arrows stands for DNA-ChIP primers. (C) Activity of the ANRIL promoter is up-regulated by E2F1 after DNA damage. HCT116 p53^{+/+} cells were infected by lentiviruses expressing control or E2F1 shRNA. 72 h after infection, the cells were transfected with pGL3-basic vector or ANRIL promoter-driven firefly luciferase expression vector, and *Renilla* luciferase expression vector. The cells were treated with NCS (500 ng/ml) 24 h after transfection and then harvested 16 h after treatment. Firefly luciferase activity was measured and normalized to the activity of *Renilla* luciferase. (D) Enrichment of E2F1 in the ANRIL promoter after DNA damage. Cells were pretreated with DMSO or ATM inhibitor for 2 h prior to NCS treatment. E2F1 was immunoprecipitated and the promoter region containing E2F1-binding sequences were quantified by RT-PCR. Control IgG immunoprecipitation was used as controls. (E) Induction of E2F1 after DNA damage is dependent on ATM. Cells were pretreated with

DMSO or ATM inhibitor prior to NCS treatment. Phosphorylated p53 at Ser15 was used as indicator for the ATM activity to validate the inhibitory effects by ATM inhibitor.

Figure 4. Expression of genes in the INK4B-ARF-INK4A locus is regulated by ANRIL
 (A) Generation of stable HCT116 p53^{+/+} cells with ANRIL overexpression (OE) or knockdown (KD). Cells were infected with lentiviruses expressing ANRIL or its shRNA. Single colonies were selected and verified. RNA levels were measured by quantitative RT-PCR. (B) RNA levels of p15, p16 and p14 in HCT116 p53^{+/+} cells with altered levels of ANRIL. (C) RNA levels of p15, p16 and p14 in the DNA damage response. Cells were treated with 500 ng/ml NCS, harvested at indicated time points, subject to RNA analysis using quantitative RT-PCR. (D) Protein levels of p15, p16 and p14 in the DNA damage response. Cells were treated and harvested as above and proteins were analyzed by western blot.

Figure 5. Effects of ANRIL on cell proliferation, apoptosis and drug sensitivity

(A) ANRIL promotes cell proliferation. HCT116 p53^{+/+} cells with altered levels of ANRIL were seeded at 1,000 cells/well, cultured up to six days, and counted at indicated time points. (B) Cell cycle analysis of the above ANRIL-silenced and -overexpressing cells. Cells were treated with NCS (500 ng/ml) to induce cell cycle checkpoints. Cells were harvested at 0, 4, 8 and 24 h after treatment, fixed and stained with propidium iodide, and analyzed by flow cytometry. (C) Apoptosis analysis of the ANRIL-silenced or -overexpressing cells. Cells were harvested at indicated time points after DNA damage. Apoptotic cells were quantified and analyzed by AnnexinV-7ADD staining and flow cytometry. (D) Viability analysis of the ANRIL-silenced or -overexpressing cells. Cells were treated with NCS for one day and cell viability was measured at indicated time points after treatment.

Figure 6. ANRIL promotes homologous recombination in cells

A) Schematic of the DR-GFP construct. The DR-GFP reporter for measuring HR contains two defective GFP gene (SceGFP is interrupted by an I-SceI site and iGFP has an early stop codon). HR repair of the double-strand breaks generated by I-SceI results in a functional GFP gene. (B) ANRIL knockdown inhibits HR frequencies. (C) Working model of ANRIL in the DNA damage response. Upon DNA damage, ATM-initiated signaling pathway is activated to induce p53 that promotes cell cycle arrest, apoptosis and senescence. ATM-induced E2F1 transcriptionally activates ANRIL, which consequently represses the expression of INK4 family members, and thus alleviate p53 and pRB signaling pathways at the late-stage of the DDR. The cell will eventually return to normal at the completion of the DDR.