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A cautionary tale of sense-antisense gene pairs: independent regulation despite inverse correlation of expression

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been proven to play important roles in diverse cellular processes including the DNA damage response. Nearly 40% of annotated lncRNAs are transcribed in antisense direction to other genes and have often been implicated in their regulation via transcript- or transcription-dependent mechanisms. However, it remains unclear whether inverse correlation of gene expression would generally point toward a regulatory interaction between the genes. Here, we profiled lncRNA and mRNA expression in lung and liver cancer cells after exposure to DNA damage. Our analysis revealed two pairs of mRNA-lncRNA sense-antisense transcripts being inversely expressed upon DNA damage. The lncRNA *NOP14-AS1* was strongly upregulated upon DNA damage, while the mRNA for *NOP14* was downregulated, both in a p53-dependent manner. For another pair, the lncRNA *LIPE-AS1* was downregulated, while its antisense mRNA *CEACAM1* was upregulated. To test whether as expected the antisense genes would regulate each other resulting in this highly significant inverse correlation, we employed antisense oligonucleotides and RNAi to study transcript-dependent effects as well as dCas9-based transcriptional modulation by CRISPRi/CRISPRa for transcription-dependent effects. Surprisingly, despite the strong stimulus-dependent inverse correlation, our data indicate that neither transcript- nor transcription-dependent mechanisms explain the inverse regulation of *NOP14-AS1:NOP14* or *LIPE-AS1:CEACAM1* expression. Hence, sense-antisense pairs whose expression is strongly—positively or negatively—correlated can be nonetheless regulated independently. This highlights the requirement of individual experimental studies for each antisense pair and prohibits drawing conclusions on regulatory mechanisms from expression correlations.

INTRODUCTION

Whole transcriptome analysis of the human genome has revealed that the majority of the human genome is transcribed. Long non-coding RNAs (lncRNAs) ranging from 200 nt to >100 000 nt represent a large heterogeneous ncRNA subgroup that plays important roles in diverse cellular processes such as development, cell cycle regulation and diseases such as cancer (1–3). The function of lncRNA loci can either be mediated by their transcription or by the transcript itself. The latter often involves lncRNA-containing ribonucleoprotein complexes (RNPs), which mediate gene expression control both at the transcriptional or post-transcriptional level as well as alternative functions in the nucleus or cytoplasm (4,5).

LncRNAs are transcribed from either intergenic regions (these are then called long intergenic RNAs or lincRNAs), or from intragenic regions overlapping with other protein- or non-protein-coding genes. Intragenic lncRNAs can be further classified as sense or antisense, depending on the ori-

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entation of the lncRNA with respect to its neighboring gene (Supplementary Figure S1).

As much as 40% of all lncRNAs belong to the class of antisense transcripts making it the largest class of lncRNA molecules (6–10). Antisense lncRNAs often regulate the expression of their overlapping sense protein-coding genes through diverse mechanisms in *cis* through transcript- or transcription-dependent mechanisms (11–14). Although they can also act in *trans* to regulate the expression of other genes, antisense lncRNAs are suggested to act more frequently in *cis* rather than *in trans* due to the proximity to their overlapping sense genes (13,15). At the level of transcription, they can induce promoter methylation (16–18), recruit histone modifying enzymes (19–25), directly interfere and block the transcriptional machinery via transcriptional interference (26–29) or regulate sense mRNA splicing (30–32). Post-transcriptionally, they can bind to their sense mRNA and increase its stability by masking miRNA binding sites (33) or enhance its translation by recruiting additional factors (34) or generate endogenous siRNAs from double-stranded sense-antisense-hybrids (35,36). Genome-wide expression analysis of sense-antisense pairs has indicated that these are generally positively correlated (37). A pan-cancer analysis of sense-antisense pairs of mRNAs and lncRNAs also found an overall positive correlation between them (9). However, other sense-antisense pairs exhibit reciprocal expression (38,39).

As listed above, several examples mechanistically link antisense RNAs to the regulation of their sense genes. However, it remains unclear whether positive or negative correlation of expression between a sense-antisense pair may generally imply a regulatory mechanism between the two transcripts. Nonetheless, a tissue- or stimulus-specific expression correlation is frequently interpreted as an indicator of a regulatory mechanism between the sense-antisense pair (38,40–46) also critically reviewed in (47).

The DNA damage response (DDR) pathway is a coordinated cellular response to prevent detrimental genomic instability, altered protein production or loss of genetic material after genotoxic stress. The detection of genomic insults, such as modified bases or strand breaks, leads to the activation of DNA damage checkpoints, which mediate cell cycle arrest and allow for the repair of DNA lesions. Upon failure to correctly resolve DNA damage, apoptosis is triggered to ensure the removal of aberrant cells and to prevent the accumulation of mutations (48). Both, cell cycle arrest and apoptosis, are driven by the transcription factor and important tumor suppressor gene *TP53* (p53).

Recently, several lncRNAs have emerged to be major regulators of the DDR pathway like *ncRNA-CCND1, ANRIL, WRAP53, lncRNA-ROR, lincRNA-p21, PANDAR, ERIC, PINT, DINO, DDSR1, LINP1* or *NORAD* (49–65). Given these examples of the importance of lncRNAs in the DDR, we aimed to identify novel DNA damage-induced antisense lncRNAs to study their regulatory interaction with their sense mRNA counterparts. We identify *NOP14-AS1* and *LIPE-AS1* as lncRNAs strongly regulated by DNA damaging agents and significantly anti-correlated with their sense genes *NOP14* and *CEACAM1*. Despite this inverse correlation of expression between these sense-antisense pairs, we did not find any evidence for a regulatory interaction between the sense and antisense transcripts.

MATERIALS AND METHODS

Construction of plasmids

LentidCas9-KRAB-PURO iv sgRNA was generated as described earlier (10). LentidCas9-VP160-PURO iv sgRNA: The activation domain VP160 (10 tandem repeats of VP16) was polymerase chain reaction (PCR) amplified from pAC154-dual-dCas9VP160-sgExpression vector (66) using XbaI VP160 F and BamHI VP160 R primers (Supplementary Table S1). This was cloned into XbaI–BamHI restriction sites of the LentidCas9-KRAB-PURO iv sgRNA to replace the KRAB domain in frame with dCas9. The resulting plasmid was named LentidCas9-VP160-PURO iv sgRNA. All sgRNAs against the *NOP14-AS1*, *NOP14* and *TP53* loci were designed and cloned into these vectors as described earlier (10,67).

Lenti EGFP Blast: the *EGFP* open reading frame (ORF) was PCR amplified from PX458 (67) using AgeI EGFP F and BamHI EGFP R primers (Supplementary Table S1). This was cloned into AgeI–BamHI restriction sites of the lentiCas9-Blast (68) to replace the *Cas9* ORF in frame with the downstream *Blasticidin* ORF. The resulting plasmid was named Lenti EGFP Blast.

Lenti NOP14 Blast: the *NOP14* ORF was PCR amplified from cDNA generated from NCI-H460 cells first using NOP14 cDNA F and NOP14 cDNA R primers and then using AgeI NOP14 F and BamHI NOP14 R primers (Supplementary Table S1). This was cloned into AgeI–BamHI restriction sites of the lentiCas9-Blast to replace the *Cas9* ORF in frame with the downstream *Blasticidin* ORF. The resulting plasmid was named Lenti NOP14 Blast.

Cell culture

NCI-H460 cells were cultured in RPMI medium (Sigma-Aldrich) supplemented with 10% Fetal Bovie Serum (FBS) and 1% L-glutamine at 37°C and 5% CO_2 in a humidified chamber. HCT116 TP53(+/+) and HCT116 TP53(−/−) (69) cells were cultured in McCoy's 5A Modified Medium (Sigma-Aldrich) supplemented with 10% FBS and 1% L-glutamine at 37°C and 5% CO_2 in a humidified chamber. A549, HEK293T, MCF7 and HepG2 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS and 1% L-glutamine at 37°C and 5% CO_2 in a humidified chamber.

Drug treatments

Etoposide (Topoisomerase II inhibitor, induces double-strand breaks in genomic DNA) (33419–42-0, Cayman Chemical), Cisplatin (forms intrastrand cross-links with purine bases in genomic DNA) (CAS 15663–27-1, Merck Millipore), Bleomycin (catalyses single-strand as well as double-strand breaks in genomic DNA) (CAS 9041–93-4, Merck Millipore), Doxorubicin (DOXO) (Topoisomerase II inhibitor, induces double-strand breaks in genomic DNA) CAS 25316–40-9, Merck Millipore), Carboplatin

(forms intrastrand cross-links with purine bases in genomic DNA) (CAS 41575–94-4, Merck Millipore), Nutlin-3 (MDM2 antagonist, stabilizes p53) (CAS 548472–68-0, Sigma-Aldrich) and Actinomycin D (Intercalates with genomic DNA, inhibits transcription) (CAS 50–76-0, Sigma-Aldrich) were dissolved in DMSO (CAS 67–68-5, AppliChem GmbH) to prepare stock solutions. These were diluted in cell culture media to achieve the indicated final drug concentrations. At the indicated time point post drug treatments, cells were lysed in TRI reagent for RNA extraction or RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate supplemented with protease and phosphatase inhibitors) for protein extraction.

RESULTS

An antisense transcript induced upon DNA damage

To study the stimulus-dependent regulation of antisense transcription, we chose DNA damage as a model system given its profound impact on gene expression patterns. To identify antisense lncRNAs regulated by genotoxic stress, we treated A549 lung carcinoma and HepG2 hepatocellular carcinoma cells with selected DNA damaging drugs (Etoposide, Cisplatin and Bleomycin) and profiled the expression of lncRNAs and mRNAs by microarray analysis. The known p53-stimulated genes *CDKN1A* (*p21*) and *GADD45A* (70,71) were found induced upon genotoxic drug treatment while *PLK1* (72) was decreased, all three confirming the induction of a functional DDR under these conditions and the validity of our analysis (Figure 1A). As the only consistently regulated lncRNA, this screen reproducibly identified *NOP14-AS1* antisense lncRNA to be induced in both cell lines upon different drug treatments (Figure 1A and Supplementary Table S2). We validated these microarray results using RT-qPCR which confirmed the upregulation of *NOP14-AS1* lncRNA in multiple cell lines as well as by multiple drugs (Figure 1B and Supplementary Figure S2A).

NOP14-AS1 isoforms and localization

Since the annotation of many lncRNAs is incomplete and to determine the precise length of *NOP14-AS1* as well as its overlap with genes in sense orientation, we established the full-length sequence of *NOP14-AS1*. We performed Rapid Amplification of cDNA Ends (RACE) and detected most of the predicted *NOP14-AS1* splice isoforms as well as several novel variants (Supplementary Figure S2B, Supplementary sequences). Moreover, these isoforms were 5′-m^{7}G capped and 3′-polyadenylated as the applied RACE protocol exclusively detects such RNAs. Notably, all detected isoforms overlapped in a tail-to-tail orientation with the coding gene *NOP14*. This was corroborated by publically available CAGE and polyA+-RNA-Seq data (73,74). For all subsequent experiments, we decided to use an RT-qPCR amplicon which detected all the variants of *NOP14-AS1*. Cellular fractionation revealed that *NOP14-AS1* was primarily cytoplasmic and its localization was largely unperturbed upon DNA damage (Supplementary Figure S2C and D). The copy number per cell was determined in NCI-H460 cells, which expressed 1.3 copies per cell untreated and 17.3 copies per cell after 16 h DOXO treatment.

NOP14-AS1 is transcriptionally induced dependent on the p53 pathway

To further characterize the stimulus-dependent regulation of the antisense lncRNA *NOP14-AS1*, we aimed at determining the mechanism and pathway of its regulation upon drug treatments. To analyze whether the elevated abundance of *NOP14-AS1* upon DNA damage was the result of increased transcription or increased transcript stability, we inhibited cellular transcription with Actinomycin D and treated the cells with DOXO. Transcriptional inhibition abrogated *NOP14-AS1* induction, indicating that a transcriptional response gave rise to elevated *NOP14-AS1* and, as expected, *CDKN1A* levels (Supplementary Figure S3A and B).

We hypothesized that *NOP14-AS1* could be a p53 target gene, since p53 is a major transcription factor activated by genotoxic stress and triggers the transcription of several other lncRNAs involved in the DDR (75–78). To test this hypothesis, we monitored *NOP14-AS1* levels upon p53 activation using the MDM2 antagonist Nutlin-3 (79). Nutlin-3 treatment resulted in *NOP14-AS1* induction in the p53-wild-type A549, NCI-H460, and HepG2 cells (Figure 1C and Supplementary Figure S3C). Furthermore, *NOP14-AS1* was upregulated in *TP53* (+/+) HCT-116 cells upon treatment with Etoposide, DOXO or Nutlin-3, but not in in *TP53* (−/−) HCT-116 cells (Figure 1D and Supplementary Figure S3D). Consistently, *TP53* knockdown abrogated the *NOP14-AS1* induction upon DOXO treatment in NCI-H460 cells (Figure 1E; Supplementary Figure S3E and F), suggesting that *NOP14-AS1* upregulation upon DNA damage was p53-dependent. Since the *NOP14-AS1* promoter did not harbor any p53-binding sites (57) and *TP53* knockdown alone did not result in a reduction in *NOP14-AS1* expression in contrast to *CDKN1A* (Figure 1E and Supplementary Figure S3F), *NOP14-AS1* was likely not a direct p53-target gene.

NOP14-AS1 and *NOP14* are inversely correlated upon DNA damage

To test whether the validated stimulus-dependent regulation of *NOP14-AS1* could serve as a model to study sense-antisense gene regulation, we next analyzed the expression and regulation patterns of its neighboring sense genes. The *NOP14-AS1* antisense gene shares its genomic locus with two different coding genes in sense orientation as verified in our RACE experiment: *MFSD10* in head-to-head orientation sharing a bidirectional promoter and *NOP14* overlapping in tail-to-tail orientation (Figure 2A). During a time course of DOXO treatment of NCI-H460 cells, we uncovered a reciprocal change in *NOP14-AS1* and *NOP14* expression upon genotoxic stress: as *NOP14-AS1* lncRNA transcript abundance increased, *NOP14* mRNA and protein expression concomitantly decreased (Figure 2B; Supplementary Figure S4A and B). Similar results of inverse correlation were obtained in Etoposide- and Cisplatin-treated NCI-H460 cells (Figure 2C and D) as well as in

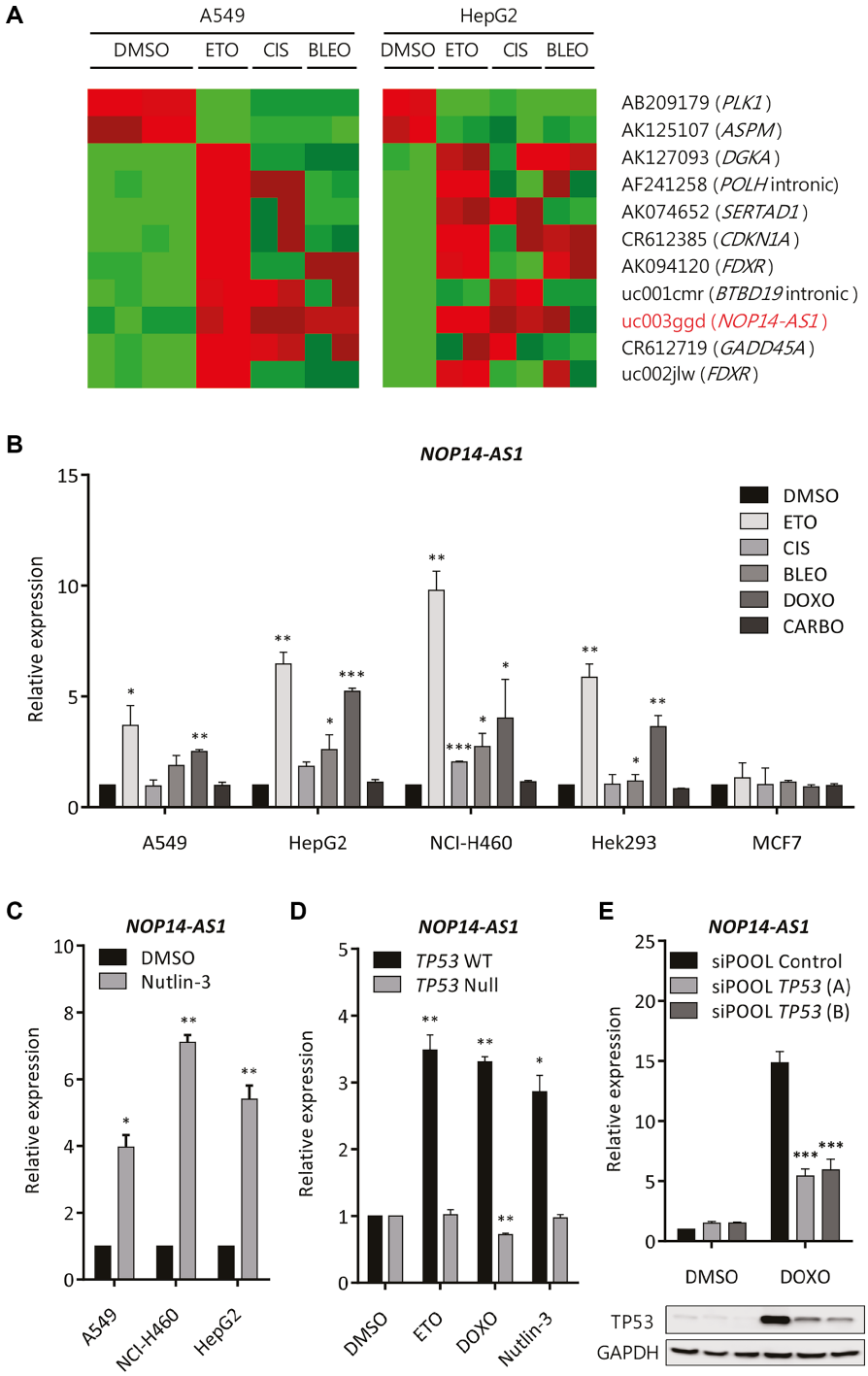

Figure 1. *NOP14-AS1*: an antisense transcript induced upon DNA damage. (**A**) Microarray analysis heat map identifying lncRNAs and mRNAs differentially expressed in A549 (left panel) and HepG2 (right panel) cells treated with 50 μM Etoposide (ETO)/50 μM Cisplatin (CIS)/20 μM Bleomycin (BLEO) or vehicle control DMSO for 8 h. (**B**): A549/HepG2/NCI-H460/HEK293/MCF7 cells were treated with 50 μM Etoposide (ETO)/50 μM Cisplatin (CIS)/20 μM Bleomycin (BLEO)/1 μM DOXO/50 μM Carboplatin (CARBO) or vehicle control DMSO for 8 h. RT-qPCR results for *NOP14-AS1* normalized to *Cyclophilin A* and DMSO control. Error bars represent SEM ($n \geq 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to DMSO control, unpaired two-sided *t*-test. (**C**) A549/NCI-H460/HepG2 cells were treated with either 10 μM Nutlin-3 or vehicle control DMSO for 24 h. RT-qPCR results for *NOP14-AS1* normalized to *Cyclophilin A* and DMSO controls. Error bars represent SEM ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to DMSO controls, unpaired two-sided *t*-test. (**D**) HCT116 TP53 WT/TP53 Null cells were treated with 50 μM Etoposide (ETO)/1 μM DOXO/10 μM Nutlin-3 or vehicle control DMSO for 12 h. RT-qPCR results for *NOP14-AS1* normalized to *Cyclophilin A* and DMSO controls. Error bars represent SEM ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to DMSO controls, unpaired two-sided *t*-test. (**E**) NCI-H460 cells were transfected with either one of the two indicated siPOOLs against *TP53* or siPOOL Control. These were then treated with either 1 μM DOXO or vehicle control DMSO for 24 h. Upper panel: RT-qPCR results for *NOP14-AS1* normalized to *Cyclophilin A* and siPOOL Control + DMSO control. Error bars represent SEM ($n = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to siPOOL Control, unpaired two-sided *t*-test. Lower panel: western blot results for TP53. GAPDH was used as a loading control.

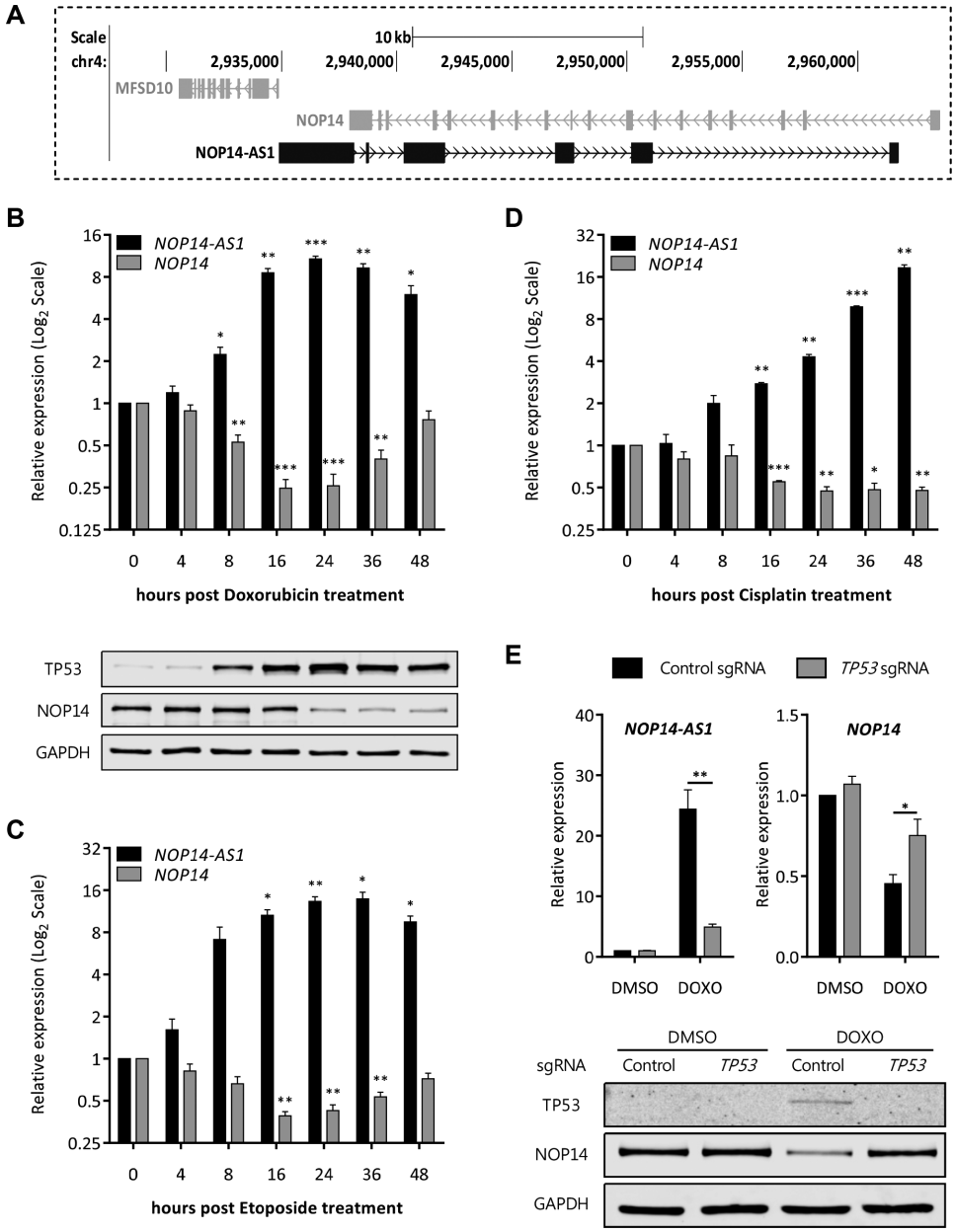

Figure 2. *NOP14-AS1* and *NOP14* are inversely co-regulated upon DNA damage. (**A**) *NOP14-AS1* genomic locus as depicted in UCSC genome browser (http://genome.ucsc.edu). The *NOP14-AS1* gene is divergently expressed from its upstream neighbor, the *MFSD10* gene and overlapping with the tail-to-tail antisense *NOP14* gene. (**B**) NCI-H460 cells were treated with 1 μM DOXO for the indicated time points. Upper panel: RT-qPCR results for *NOP14-AS1* and *NOP14* normalized to *Cyclophilin A* and untreated control. Error bars represent SEM ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to untreated control, unpaired two-sided *t*-test. Lower panel: western blot results for NOP14. GAPDH was used as a loading control. (**C** and **D**) NCI-H460 cells were treated with either (C) 50 μM Etoposide or (D) 50 μM Cisplatin for the indicated time points. RT-qPCR results for *NOP14-AS1* and *NOP14* normalized to *Cyclophilin A* and untreated control. Error bars represent SEM ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to untreated control, unpaired two-sided *t*-test. (**E**) NCI-H460 cells were transduced with Lenti dCas9-KRAB-PURO iv sgRNA containing either a control sgRNA or an sgRNA targeting the *TP53* promoter. These were then treated with either 1 μM DOXO or vehicle control DMSO for 24 h. Upper panel: RT-qPCR results for *NOP14-AS1* and *NOP14* normalized to *Cyclophilin A* and control sgRNA + DMSO controls. Error bars represent SEM ($n = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to control sgRNA, unpaired two-sided *t*-test. Lower panel: western blot results for TP53 and NOP14. GAPDH was used as a loading control.

HepG2 and A549 cells treated with Etoposide or DOXO (Supplementary Figure S4C–F). However, we did not observe any significant changes in *MFSD10* expression upon DNA damage (Supplementary Figure S5). This was remarkable given that *NOP14-AS1* and *MFSD10* were transcribed from a bidirectional promoter (10).

In summary, *NOP14-AS1* and *NOP14* presented themselves as a prime example to study the stimulus-dependent regulation of a sense-antisense gene pair with robust and highly significant inverse expression patterns.

NOP14-AS1 and *NOP14* regulation depend on p53

As a first step to characterize the potential regulatory interaction between the inversely regulated *NOP14-AS1* lncRNA and the *NOP14* mRNA, we aimed to elucidate whether these would be dependent on the same pathway. To test whether p53 was also necessary for the regulation of *NOP14* mRNA, we employed CRISPR-based approaches. Recently, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) have emerged as tools for transcriptional modulation (80–82). CRISPRi/a involves sgRNA-mediated recruitment of nuclease-inactive Cas9 (dCas9) fused to transcriptional regulator domains dCas9-KRAB/dCas9-VP160 to the vicinity of the promoter of the target gene, thereby repressing/activating its transcription, respectively (Supplementary Figure S6). Using sgRNAs against the promoter region of *TP53* in combination with dCas9-KRAB (CRISPRi), we achieved efficient knockdown of *TP53* in NCI-H460 cells (Figure 2E and Supplementary Figure S7A). *TP53* silencing resulted in decreased expression of the p53 target gene *CDKN1A* (Supplementary Figure S7B) as well as a loss of *NOP14-AS1* induction and a partial rescue of *NOP14* expression upon genotoxic stress (Figure 2E; Supplementary Figure S7C and D). These findings indicated an inverse correlation upon p53 inactivation between these two genes.

The strong negative correlation between *NOP14-AS1* and *NOP14* expression upon DNA damage induction, their co-dependence on p53 activation and their overlapping sense-antisense orientation defined this sense-antisense pair as a strong candidate to test whether inverse correlation of expression could indicate a regulatory mechanism between them and whether generally assumptions on mechanistic links based on expression patterns could be drawn or could be misleading.

NOP14 does not regulate *NOP14-AS1* expression upon DNA damage

First, we established the impact of the sense gene on the antisense lncRNA. To analyze the impact of *NOP14* transcription on *NOP14-AS1* expression, we knocked down *NOP14* in NCI-H460 cells using CRISPRi. Repression of *NOP14* using two independent sgRNAs resulted in a slight (4-fold) induction of *NOP14-AS1* steady-state expression (Figure 3A and B; Supplementary Figure S8A and B). However, this induction was much weaker than the DOXO-mediated increase of *NOP14-AS1* (22-fold) in these cells. Importantly, the decrease of *NOP14* by CRISPRi and DNA damage led to comparable *NOP14* levels, but to vastly different *NOP14-AS1* levels. Moreover, we did not observe a significant further increase in *NOP14-AS1* levels upon *NOP14* knockdown in the DOXO-treated cells. *NOP14* knockdown also resulted in increased *CDKN1A* expression and reduced cell proliferation (Supplementary Figure S9A–C).

To further corroborate the lack of regulation of *NOP14-AS1* by *NOP14*, an siRNA-mediated knockdown of *NOP14* was performed. Loss of *NOP14* mRNA and protein did not result in any *NOP14-AS1* induction (Figure 3C and D; Supplementary Figure S8C and D). In line with this finding, exogenous overexpression of *NOP14* from a lentiviral plasmid did not reverse the *NOP14-AS1* induction upon CRISPRi-mediated knockdown of *NOP14*. The *NOP14-AS1* regulation pattern remained identical to the loss of *NOP14* alone without ectopic rescue (Figure 3E and F; Supplementary Figure S8E and F). Hence, the moderate *NOP14-AS1* induction observed with the CRISPRi approach may be a result of *in cis* co-transcriptional regulation, but the DNA damage-induced regulation of *NOP14-AS1* was independent of *NOP14* expression. Importantly, these data indicated that an independent mechanism was responsible for *NOP14-AS1* induction upon DNA damage.

NOP14-AS1 does not regulate *NOP14* expression upon DNA damage

Vice versa, we analyzed whether the *NOP14-AS1* transcript had an impact on *NOP14* expression. We knocked down *NOP14-AS1* in NCI-H460 cells using two independent antisense LNA GapmeRs and treated them with DOXO or vehicle control (DMSO). Despite reduced *NOP14-AS1* expression, we did not observe any significant increase in *NOP14* mRNA or protein expression (Figure 4A–C; Supplementary Figure S10A and B). To rule out the possibility that *NOP14-AS1* might regulate *NOP14* expression via transcriptional interference or other mechanisms of co-transcriptional regulation in *cis*, we additionally modulated *NOP14-AS1* transcription by using CRISPRi and CRISPRa. Repression or induction of *NOP14-AS1* transcription from its endogenous promoter using two independent sgRNAs was effective in *NOP14-AS1* regulation but did not result in any change in *NOP14* mRNA or protein expression. This was confirmed in the presence or absence of the DNA-damaging agent DOXO (Figure 4D–F; Supplementary Figure S11A and B). We obtained similar results in A549 cells confirming that *NOP14-AS1* had no impact on *NOP14* mRNA or protein expression (Supplementary Figure S11C–F). *NOP14-AS1* knockdown or overexpression had no detectable impact on cell proliferation (Supplementary Figure S12). Hence, neither *NOP14-AS1* transcription nor the non-coding transcript regulated *NOP14*.

Based on the widely accepted model that sense-antisense transcripts with inverse expression patterns can have a regulatory relationship, increased transcription of *NOP14-AS1* should result in transcriptional interference with *NOP14*. To test this model, we utilized CRISPRa to increase transcription of endogenous *NOP14-AS1*. Surprisingly, robust overexpression of *NOP14-AS1* had no effect on *NOP14* expression levels. This result clearly demonstrated that transcription of an antisense transcript did not inherently interfere with transcription of its sense transcript partner.

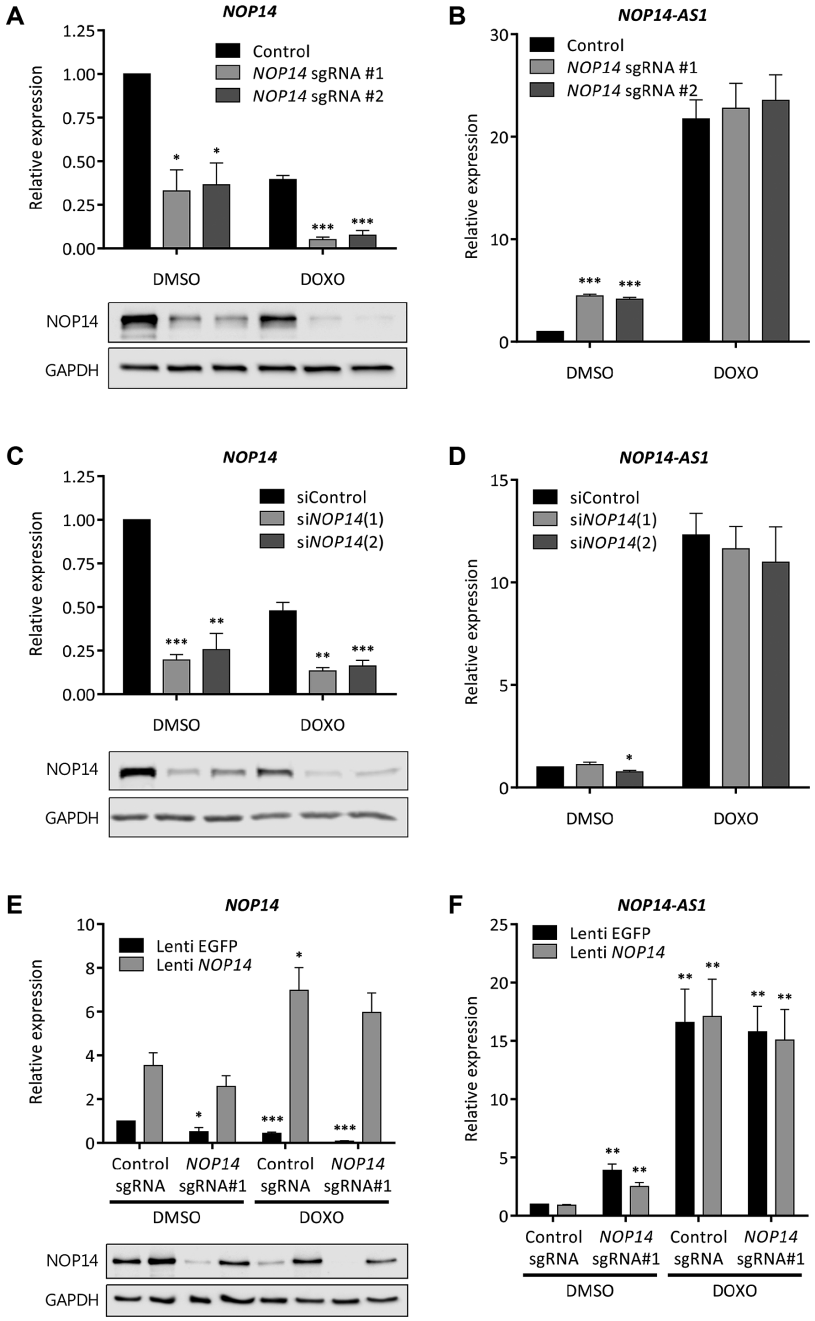

Figure 3. *NOP14* does not regulate *NOP14-AS1* expression upon DNA damage. (**A** and **B**) NCI-H460 cells were transduced with Lenti dCas9-KRAB-PURO iv sgRNA containing either a control sgRNA or two independent sgRNAs targeting the *NOP14* promoter. These were then treated with either 1 μM DOXO or vehicle control DMSO for 24 h. (A) Upper panel: RT-qPCR results for *NOP14* normalized to *Cyclophilin A* and control sgRNA + DMSO control. Error bars represent SEM ($n = 4$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control sgRNA, unpaired two-sided *t*-test. Lower panel: western blot results for NOP14. GAPDH was used as a loading control. (B) RT-qPCR results for *NOP14-AS1* normalized to *Cyclophilin A* and control sgRNA + DMSO control. Error bars represent SEM ($n = 4$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control sgRNA, unpaired two-sided *t*-test. (**C** and **D**) NCI-H460 cells were transfected with either a control siRNA (siControl) or two independent siRNAs (si*NOP14*(1) and si*NOP14*(2)) targeting the *NOP14* mRNA. These were then treated with either 1 μM DOXO or vehicle control DMSO for 24 h. (C) Upper panel: RT-qPCR results for *NOP14* normalized to *Cyclophilin A* and siControl + DMSO control. Error bars represent SEM ($n = 5$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control siRNA, unpaired two-sided *t*-test. Lower panel: western blot results for NOP14. GAPDH was used as a loading control. (D) RT-qPCR results for *NOP14-AS1* normalized to *Cyclophilin A* and siControl + DMSO control. Error bars represent SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control siRNA, unpaired two-sided *t*-test. (**E** and **F**) NCI-H460 cells were transduced with Lenti dCas9-KRAB-PURO iv sgRNA containing either a control sgRNA or an sgRNA targeting the *NOP14* promoter in combination with either Lenti EGFP Blast or Lenti NOP14 Blast. These were then treated with either 1 μM DOXO or vehicle control DMSO for 24 h. (E) Upper panel: RT-qPCR results for *NOP14* normalized to *Cyclophilin A* and control sgRNA + Lenti EGFP + DMSO control. Error bars represent SEM ($n = 6$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control sgRNA + DMSO controls, unpaired two-sided *t*-test. Lower panel: western blot results for NOP14. GAPDH was used as a loading control. (F) RT-qPCR results for *NOP14-AS1* normalized to *Cyclophilin A* and control sgRNA + Lenti EGFP + DMSO control. Error bars represent SEM ($n = 6$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control sgRNA + DMSO controls, unpaired two-sided *t*-test.

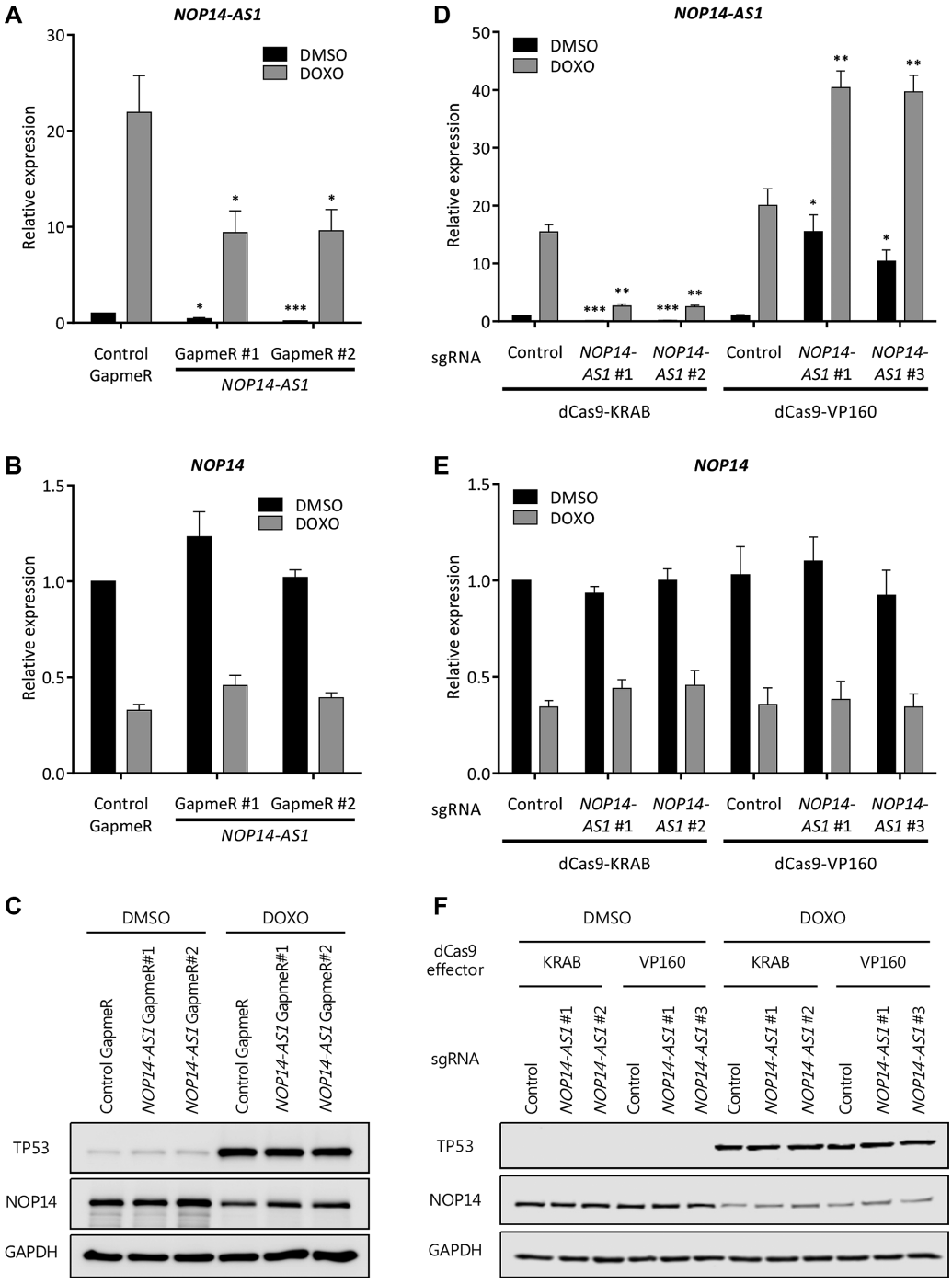

Figure 4. *NOP14-AS1* does not regulate *NOP14* expression upon DNA damage. (**A**–**C**) NCI-H460 cells were transfected with either a control GapmeR or two independent GapmeRs (GapmeR#1 and GapmeR#2) targeting the *NOP14-AS1* lncRNA. These were then treated with either 1 μM DOXO or vehicle control DMSO for 24 h. RT-qPCR results for (A) *NOP14-AS1* and (B) *NOP14* normalized to *Cyclophilin A* and control GapmeR + DMSO control. Error bars represent SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control GapmeR, unpaired two-sided *t*-test. (C) Western blot results for TP53 and NOP14. GAPDH was used as a loading control. (**D**–**F**) NCI-H460 cells were transduced with Lenti dCas9-KRAB-PURO iv sgRNA or Lenti dCas9-VP160-PURO iv sgRNA, respectively, containing either a control sgRNA or two independent sgRNAs targeting the *NOP14-AS1* promoter. These were then treated with either 1 μM DOXO or vehicle control DMSO for 24 h. RT-qPCR results for (D) *NOP14-AS1* and (E) *NOP14* normalized to *Cyclophilin A* and control sgRNA + DMSO controls. Error bars represent SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control sgRNA, unpaired two-sided *t*-test. (F) Western blot results for TP53 and NOP14. GAPDH was used as a loading control.

While transcriptional interference by sense-antisense transcript pairs had been described in the literature, establishing this relationship requires experimental validation and cannot be inferred from expression patterns alone. In consequence, this sense-antisense pair served as a notable example that even stimulus-dependent inverse regulation mediated by the same pathway did not allow an assumption of a regulatory link between the two neighboring genes.

Identification of an additional sense-antisense pair with inverse but independent regulation upon DNA damage

Lastly, we set out to extend this cautionary tale of a sense-antisense pair being inversely yet independently regulated beyond *NOP14* and *NOP14-AS1*. We performed a reanalysis of our microarray data with a focus on inversely regulated sense-antisense pairs. Even this limited dataset and with the same stimulus-dependent regulation, we found another example: *LIPE-AS1* and *CEACAM1* were inversely regulated upon DNA damage in the microarray data and validated by RT-qPCR. While *LIPE-AS1* was repressed upon Cisplatin treatment in A549 cells, *CEACAM1* was induced (Figure 5A and B). We generated knockdown cell lines for *LIPE-AS1* as well as *CEACAM1* using the dCas9-KRAB CRISPRi system. Knockdown of *LIPE-AS1* did not impact the regulation of *CEACAM1* in presence or absence of Cisplatin. Similarly, prevention of *CEACAM1* upregulation upon Cisplatin treatment using dCas9-KRAB could not reverse the Cisplatin-induced *LIPE-AS1* repression (Figure 5C and D).

Again, these data experimentally clearly proved independent regulation despite inverse expression patterns of sense-antisense pairs demonstrating the strong need to mechanistically study sense-antisense loci individually and precluding the prediction of regulatory interactions based on expression correlations.

DISCUSSION

NOP14-AS1: an antisense lncRNA induced by DNA damage

Several lncRNAs have emerged as major regulators of the DDR pathway (23,49–63,83–87). Here, we report the discovery of a novel DNA damage-inducible lncRNA, *NOP14-AS1*, using microarray-based expression profiling. Several mRNAs involved in the DDR pathway (*CDKN1A*, *GADD45A* and *PLK1*) were also differentially regulated in these analyses validating the results and indicating the usefulness of microarrays for the identification of differentially expressed lncRNAs. RACE experimentally validated several predicted splice isoforms of *NOP14-AS1* and discovered several new ones. The *NOP14-AS1* induction upon DNA damage was a transcriptional response mediated by p53 as demonstrated in genetic, RNAi- and CRISPRi-based approaches. These data add *NOP14-AS1* to the growing list of p53-inducible lncRNAs like *lncRNA-p21*, *PANDAR*, *lncRNA-RoR* and *DINO* (52,53,58,62).

NOP14: its sense mRNA is decreased upon DNA damage and affects cell proliferation

The *NOP14-AS1* gene overlaps in a tail-to-tail antisense orientation with the protein-coding gene *NOP14*. In time course expression analyses in NCI-H460, A549 and HepG2 cells treated with several DNA damaging drugs, *NOP14-AS1* expression was highly significantly inversely correlated with its sense gene *NOP14*. The induction of *NOP14-AS1*, as well as the repression of NOP14 upon DNA damage, were both indirectly p53-dependent. The absence of p53 binding sites in the promoters of these genes indicates that they are not direct targets of p53. The promoters of both *NOP14* as well as *NOP14-AS1* harbor several transcription factor binding sites, as observed from the publically available transcription factor ChIP-Seq data from the ENCODE consortium (73). We found that several of these transcription factors are known to be regulated upon DNA damage in a p53-dependent manner (88–95). Further studies will elucidate whether *NOP14-AS1* and *NOP14* are regulated by p53 indirectly through these transcription factors.

NOP14 is a nucleolar protein involved in the small ribosomal subunit biogenesis and has been implicated in pancreatic and breast cancer as a regulator of cell proliferation (96–99). The detected downregulation of *NOP14* could correspond to the inhibition of ribosome biogenesis upon DNA damage (100,101). *NOP14* knockdown resulted in a *CDKN1A* induction and reduced cell proliferation potentially indicating a functional link to the DDR.

Inverse but independent regulation of sense-antisense pairs

Given the strong inverse correlation and p53-co-dependence of *NOP14-AS1* and *NOP14* expression, we hypothesized that *NOP14-AS1* could regulate *NOP14* expression in DNA damage or *vice versa*.

RNAi-mediated knockdown of *NOP14* did not affect *NOP14-AS1* expression indicating the independence of *NOP14-AS1* induction from the *NOP14* transcript or protein. A CRISPRi-mediated knockdown of *NOP14* resulted in an induction of *NOP14-AS1* much less pronounced than upon DNA damage. However, this was neither rescuable by exogenous *NOP14* expression, nor was there any correlation of the *NOP14-AS1* levels upon similar *NOP14* loss induced by CRISPRi or DNA damage. Hence, it was most likely due to a reduced transcriptional interference of the highly transcribed *NOP14* gene into the *NOP14-AS1* gene which was independent of the *NOP14-AS1* induction upon DNA damage.

In turn, using three different approaches—LNA gapmeRs to target the *NOP14-AS1* lncRNA transcript as well as CRISPRi/a to target *NOP14-AS1* transcription—we could not find any impact of *NOP14-AS1* induction on the observed *NOP14* repression upon DNA damage.

Hence, *NOP14-AS1* lncRNA and *NOP14* mRNA are both altered upon DNA damage, inversely correlated and targeted by p53, but their regulation is independent of each other.

In addition to the sense-antisense pair of *NOP14* / *NOP14-AS1*, we also identified *LIPE-AS1*/*CEACAM1* to be inversely co-regulated upon DNA damage. dCas9-KRAB-mediated knockdown of LIPE-AS1 or CEACAM1 had no impact on their reciprocal regulation upon DNA damage, thus demonstrating that this phenomenon of inverse but independent regulation of a sense-antisense pair was not restricted to a single locus.

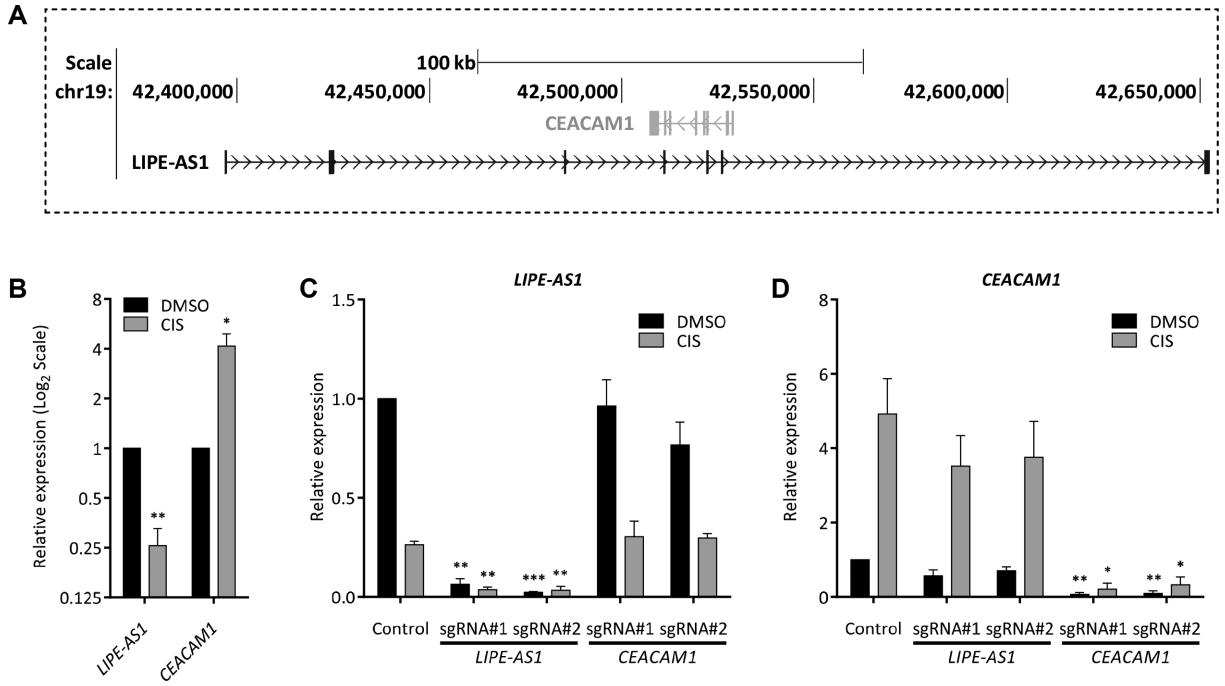

Figure 5. Identification of an additional sense-antisense pair with inverse but independent regulation upon DNA damage. (**A**) *LIPE-AS1* / *CEACAM1* genomic locus as depicted in UCSC genome browser. (**B**) A549 cells were treated with 50 μM Cisplatin (CIS) or vehicle control DMSO for 8 h. RT-qPCR results for *LIPE-AS1* and *CEACAM1* normalized to *Cyclophilin A* and DMSO control. Error bars represent SEM ($n = 4$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to DMSO control, unpaired two-sided *t*-test. (**C** and **D**) A549 cells were transduced with Lenti dCas9-KRAB-PURO iv sgRNA containing either a control sgRNA or two independent sgRNAs targeting either the *LIPE-AS1* or the *CEACAM1* promoter. These were then treated with either 50 μM Cisplatin (CIS) or vehicle control DMSO for 8 h. RT-qPCR results for (C) *LIPE-AS1* and (D) *CEACAM1* normalized to *Cyclophilin A* and control sgRNA + DMSO control. Error bars represent SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control sgRNA, unpaired two-sided *t*-test.

On a broader scale, our study documents that the correlation of expression between lncRNA-mRNA sense-antisense pairs must not be generally interpreted as an indicator of a regulatory mechanistic link between the two. These results generally call for caution when functional interpretations are drawn based on expression and correlation studies. Recent data in yeast confirm this conclusion (102). Each individual locus needs to be analyzed in well-controlled loss-of-function and gain-of-function experiments. Additionally, our data show that dCas9-based artificial transcription factors can be effectively used to modulate transcription of the antisense gene from its endogenous promoter and provide a tool to study *in cis* co-transcriptional effects on the expression of their sense counterparts. The combination of CRISPRi and RNAi can distinguish between transcript-based and transcription-based effects of a given locus and should hence be the method of choice. In summary, our study adds another layer of complexity to the science of the many thousands of lncRNA-mRNA sense-antisense pairs in the genome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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