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

Ashish Goyal1,2, Evgenij Fiškin1, Tony Gutschner1,2, Maria Polycarpou-Schwarz1,2, Matthias Groß1,2, Julia Neugebauer1, Minakshi Gandhi1,2, Maiwen Caudron-Herger1,2, Vladimir Benes5 and Sven Diederichs1,2,3,4,6,*

1Division of RNA Biology & Cancer, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany, 2Institute of Pathology, University Hospital Heidelberg, 69120 Heidelberg, Germany, 3Division of Cancer Research, Department of Thoracic Surgery, Medical Center-University of Freiburg, 79106 Freiburg, Germany, 4Faculty of Medicine, University of Freiburg, 79085 Freiburg, Germany, 5Genomics Core Facility, EMBL Heidelberg, 69117 Heidelberg, Germany and 6German Cancer Consortium (DKTK), 79104 Freiburg, Germany

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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, 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-

*To whom correspondence should be addressed. Tel: +49 6221 424380; Email: s.diederichs@dkfz.de

Present addresses:
Evgenij Fiškin, Institute of Biochemistry II, Goethe University School of Medicine, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany.
Tony Gutschner, Faculty of Medicine, University of Halle-Wittenberg, 06120 Halle (Saale), Germany.
entation of the IncRNA with respect to its neighboring gene (Supplementary Figure S1).

As much as 40% of all IncRNAs belong to the class of antisense transcripts making it the largest class of IncRNA molecules (6–10). Antisense IncRNAs often regulate the expression of their overlapping sense protein-coding genes through diverse mechanisms in cis through transcription or transcription-dependent mechanisms (11–14). Although they can also act in trans to regulate the expression of other genes, antisense IncRNAs 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 IncRNAs 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 IncRNAs have emerged to be major regulators of the DDR pathway like ncRNA-CCND1, ANRIL, WRAP53, IncRNA-ROR, IncRNA-p21, PANDAR, ERIC, PINT, DINO, DDSRI, LINP1 or NORAD (49–65). Given these examples of the importance of IncRNAs in the DDR, we aimed to identify novel DNA damage-induced antisense IncRNAs to study their regulatory interaction with their sense mRNA counterparts. We identify NOP14-ASI and LIPE-ASI as IncRNAs 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

LentiCas9-krAB-PURO iv sgRNA was generated as described earlier (10). LentiCas9-VPI60-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 LentiCas9-KRAB-PURO iv sgRNA to replace the KRAB domain in frame with dCas9. The resulting plasmid was named LentiCas9-VP160-PURO iv sgRNA. All sgRNAs against the NOP14-ASI, 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-EGFP Blast 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 Bovine Serum (FBS) and 1% L-glutamine at 37°C and 5% CO2 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% CO2 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% CO2 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 intrastand 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 up-regulation 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’-mG 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 ≥ 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. The 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-ASI and NOP14 are inversely co-regulated upon DNA damage. (A) NOP14-ASI genomic locus as depicted in UCSC genome browser (http://genome.ucsc.edu). The NOP14-ASI 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-ASI 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-ASI 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 Lentil 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-ASI 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-ASI and MFSD10 were transcribed from a bidirectional promoter (10).

In summary, NOP14-ASI 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-ASI and NOP14 regulation depend on p53**

As a first step to characterize the potential regulatory interaction between the inversely regulated NOP14-ASI 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). CRISPRa. Repression or induction 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-ASI 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-ASI and NOP14 expression upon DNA damage, 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 general assumptions on mechanistic links based on expression patterns could be drawn or could be misleading.

**NOP14 does not regulate NOP14-ASI 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-ASI 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-ASI 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-ASI (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-ASI levels. Moreover, we did not observe a significant further increase in NOP14-ASI 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-ASI by NOP14, an siRNA-mediated knockdown of NOP14 was performed. Loss of NOP14 mRNA and protein did not result in any NOP14-ASI 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-ASI induction upon CRISPRi-mediated knockdown of NOP14. The NOP14-ASI 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-ASI induction observed with the CRISPRi approach may be a result of in cis co-transcriptional regulation, but the DNA damage-induced regulation of NOP14-ASI was independent of NOP14 expression. Importantly, these data indicated that an independent mechanism was responsible for NOP14-ASI induction upon DNA damage.

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

Vice versa, we analyzed whether the NOP14-ASI transcript had an impact on NOP14 expression. We knocked down NOP14-ASI in NCI-H460 cells using two independent antisense LNA GapmeRs and treated them with DOXO or vehicle control (DMSO). Despite reduced NOP14-ASI 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-ASI might regulate NOP14 expression via transcriptional interference or other mechanisms of co-transcriptional regulation in cis, we additionally modulated NOP14-ASI transcription by using CRISPRi and CRISPRa. Repression or induction of NOP14-ASI transcription from its endogenous promoter using two independent sgRNAs was effective in NOP14-ASI 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-ASI had no impact on NOP14 mRNA or protein expression (Supplementary Figure S11C–F). NOP14-ASI knockdown or overexpression had no detectable impact on cell proliferation (Supplementary Figure S12). Hence, neither NOP14-ASI 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-ASI should result in transcriptional interference with NOP14. To test this model, we utilized CRISPRa to increase transcription of endogenous NOP14-ASI. Surprisingly, robust overexpression of NOP14-ASI 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 (siNOP14(1) and siNOP14(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 Blast + 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-ASI. 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-ASI and CEACAM1 were inversely regulated upon DNA damage in the microarray data and validated by RT-qPCR. While LIPE-ASI was repressed upon Cisplatin treatment in A549 cells, CEACAM1 was induced (Figure 5A and B). We generated knockdown cell lines for LIPE-ASI as well as CEACAM1 using the dCas9-KRAB CRISPRi system. Knockdown of LIPE-ASI did not impact the regulation of CEACAM1 in presence or absence of Cisplatin. Similarly, prevention of CEACAM1 up-regulation upon Cisplatin treatment using dCas9-KRAB could not reverse the Cisplatin-induced LIPE-ASI 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 mechanismically study sense-antisense loci individually and precluding the prediction of regulatory interactions based on expression correlations.

DISCUSSION

NOP14-ASI: 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-ASI, using microarray-based expression profiling. Several mRNAs involved in the DDR pathway (CDKN1A, GADD45A and PLKI) 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-ASI and discovered several new ones. The NOP14-ASI induction upon DNA damage was a transcriptional response mediated by p53 as demonstrated in genetic, RNAi- and CRISPRi-based approaches. These data add NOP14-ASI to the growing list of p53-inducible lncRNAs like IncRNA-p21, PANDAR, IncRNA-RoR and DINO (52,53,58,62).

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

The NOP14-ASI 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-ASI expression was highly significantly inversely correlated with its sense gene NOP14. The induction of NOP14-ASI, 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-ASI 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-ASI 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-ASI and NOP14 expression, we hypothesized that NOP14-ASI could regulate NOP14 expression in DNA damage or vice versa.

RNAi-mediated knockdown of NOP14 did not affect NOP14-ASI expression indicating the independence of NOP14-ASI induction from the NOP14 transcript or protein. A CRISPRi-mediated knockdown of NOP14 resulted in an induction of NOP14-ASI much less pronounced than upon DNA damage. However, this was neither rescuable by exogenous NOP14 expression, nor was there any correlation of the NOP14-ASI 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-ASI gene which was independent of the NOP14-ASI induction upon DNA damage.

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

Hence, NOP14-ASI 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-ASI, we also identified LIPE-ASI/CEACAM1 to be inversely co-regulated upon DNA damage. dCas9-KRAB-mediated knockdown of LIPE-ASI 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.
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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