A BRCA1-interacting lncRNA regulates homologous recombination

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Abstract

Long non-coding RNAs (lncRNAs) are important players in diverse biological processes. Upon DNA damage, cells activate a complex signaling cascade referred to as the DNA damage response (DDR). Using a microarray screen, we identify here a novel lncRNA, DDSR1 (DNA damage-sensitive RNA1), which is induced upon DNA damage. DDSR1 induction is triggered in an ATM-NF-κB pathway-dependent manner by several DNA double-strand break (DSB) agents. Loss of DDSR1 impairs cell proliferation and DDR signaling and reduces DNA repair capacity by homologous recombination (HR). The HR defect in the absence of DDSR1 is marked by aberrant accumulation of BRCA1 and RAP80 at DSB sites. In line with a role in regulating HR, DDSR1 interacts with BRCA1 and hnRNPUL1, an RNA-binding protein involved in DNA end resection. Our results suggest a role for the lncRNA DDSR1 in modulating DNA repair by HR.

Keywords BRCA1; hnRNPUL1; p53; RAP80; repair

Subject Categories DNA Replication, Repair & Recombination; RNA Biology

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Introduction

Genomes continuously experience a wide variety of damages. In order to maintain genome stability, cells have evolved complex mechanisms to sense and repair DNA damage caused by intrinsic and extrinsic insults. Among the various types of DNA damage, double-strand breaks (DSB) are the most toxic and difficult to repair. DSB repair, by either non-homologous end-joining (NHEJ) or homologous recombination (HR), is mediated by proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family, particularly ATM, ATR, and DNA-PKcs [1]. The choice of repair pathway is influenced by the cell cycle phase [2]. During G1, DSBs are primarily repaired by the error-prone NHEJ, which involves direct rejoining of DNA ends [3], whereas in S/G2, HR predominates, using a homologous DNA template sequence for error-free repair [4]. For DNA repair by HR, DSBs are detected by the MRE11-RAD50-NBS1 (MRN) complex, which promotes ATM activity by autophosphorylation [5]. ATM activation in turn results in phosphorylation of various DNA repair factors such as the core histone variant H2AX, CtIP, BRCA1, and exonuclease EXO1 [4]. The 53BP1 protein prevents BRCA1 accumulation at DSB sites in the G1 phase of the cell cycle and promotes NHEJ, whereas BRCA1 promotes end resection and HR [6–8]. DSB repair also involves extensive reorganization and modification of chromatin near break sites to facilitate access for repair factors [9].

Genomewide transcriptome analysis has led to the identification of numerous long non-coding RNAs (lncRNAs), which are loosely defined as non-coding transcripts longer than 200 nucleotides [10,11]. Numerous lncRNAs have been shown to play important regulatory roles in various biological processes ranging from cell cycle control, pluripotency, and differentiation, to disease [10–14]. LncRNAs may affect the expression of genes in cis or trans via association with proteins such as chromatin modifiers or ribonucleoprotein complexes or with other lncRNAs, although the precise mode of action for individual lncRNAs is often unknown [10,11,14,15].

LncRNAs are also emerging as regulators of DNA damage-sensitive gene expression programs [16,17]. ATM modulates the expression of lncRNAs in response to DNA damage [18,19]. LncRNA expression altered in response to DNA damage appears to be modulated by transcription factor p53 [20–23]. LincRNA-p21 interacts with hnRNP-K and participates in p53-mediated gene repression [20], and the p53-induced lncRNA, PANDA, interacts with the transcription factor NF-YA to impede induction of apoptotic genes [21]. p53 also induces lincRNA Pint which interacts with the polycomb repressive complex 2 (PRC2) to mediate gene silencing [22]. LncRNAs have also been implicated in regulating DNA repair by HR. Loss of one such ATM-regulated lncRNA, ANRIL, has been shown to impair DNA repair by homologous recombination [19]. Also, PCAT-1, a prostate-specific lncRNA, regulates BRCA2 and controls homologous recombination in cancer [24].

In the current study, we examined lncRNA expression profiles across the genome in response to DNA damage treatment and...
identified a novel lncRNA, DDSR1 (DNA damage-sensitive RNA1). We show that DDSR1 is induced in an ATM-NF-κB-dependent manner upon DNA damage and negatively regulates p53 target genes, analogous to other DNA damage-induced lncRNAs. However, in addition, DDSR1 contributes to HR by a transcription-independent mode of action by interacting with BRCA1 and modulating BRCA1 and RAP80 access to DSBs sites. Our results identify lncRNA DDSR1 as an integral player in the mammalian DNA damage response.

**Results**

**Identification of DNA damage-induced lncRNAs**

We sought to identify, in an unbiased fashion and at a genome-wide scale, lncRNAs whose expression is responsive to DNA damage. We performed gene expression analysis using the NCode™ microarray of total or nuclear RNA isolated from hTert-immortalized human skin fibroblasts treated for 3 h with DNA-damaging agents nocodazinostatin (NCS, 50 ng/ml), camptothecin (4 μM), or etoposide (10 μM). Cells treated with vehicle were used as controls. Using a 1.5-fold change and P-value < 0.05 as a threshold, we identified 127 and 54 non-coding transcripts showing differential expression in screens of total or nuclear RNA, respectively (Tables EV1 and EV2). Six transcripts were common between both screens, but only one was upregulated in both screens. The lncRNA clone AK0217744 (NCBI GenBank) was found to be induced upon DNA damage both in total (2.5-fold) and in nuclear (2.6-fold) RNA extracts in response to all DNA-damaging agents, suggesting a global role in the DNA damage response. We refer to this lncRNA as DDSR1 (DNA damage-sensitive RNA1).

Using 5’ and 3’ RACE and sequencing, we identified DDSR1 as an intronless transcript of 1,616 nucleotides, located on human chromosome 12q23.3(+). (Fig 1A, Dataset EV1). DDSR1 contains no overlapping sequence with transcripts from its neighboring genes C12orf455 (5’) and ALDH1L2 (3’) and is an independent inter-genic transcript. Evaluation of the protein-coding capacity of DDSR1 using coding potential calculator software [25] confirmed a negligible protein coding potential for DDSR1. LncRNA-DDSR1 was detectable by qRT–PCR as a moderately expressed transcript in undamaged cells, but was upregulated upon DNA damage by ~2-fold in response to the various DNA-damaging treatments (Fig 1B). DDSR1 induction was dose independent in response to treatment with NCS levels above 25 ng/ml for 4 h (Fig 1C) or camptothecin (4–50 μM) (Fig EV1A). Time-course analysis identified DDSR1 as a delayed transcript with an induction of ~2-fold at 3 h of treatment, ~2.5- at 6 h, ~3-fold at 12 h, reaching a plateau thereafter (Fig 1D). Based on copy number measurement by PCR, we estimate its abundance in human fibroblast cells to be about twelve-fold lower than the common housekeeping gene mRNA TBP. DDSR1 induction upon DNA damage was not cell type specific as it was also expressed and induced upon DNA damage in PC3 (prostate), A549 (lung), U2OS (osteosarcoma), and HCT116 (colon) cells (Fig 1E). Moreover, DDSR1 induction was specific to DNA damage and was not indicative of a general stress response since no change in DDSR1 levels was observed upon heat shock (Fig EV1B). RNA fluorescence in situ hybridization (FISH) in U2OS cells confirmed DDSR1 expression at low levels in both the cytoplasm and nucleus and its induction upon DNA damage (Fig EV1C).

**Regulation of DDSR1 expression by ATM, NF-κB, and p53**

Since the ATM kinase is the primary responder to DSBs and a major regulator of the DDR signaling cascade, we tested whether induction of DDSR1 upon DNA damage is ATM dependent. Inhibition of ATM during DNA damage with its specific inhibitor KU55933 markedly reduced DDSR1 induction as compared to cells treated with NCS alone (Fig 2A). In order to identify the transcription factors involved in DDSR1 expression upon DNA damage, we analyzed the 2,000-bp putative promoter sequence upstream of the DDSR1 transcription start site for consensus transcription factor binding sites. In silico analysis using Alibaba2 revealed an NF-κB binding site in the DDSR1 promoter region ~1.1 kb upstream of the TSS. Consistent with the known role of ATM as an activator of NF-κB in response to DNA damage [26], treatment of cells with the NF-κB inhibitor BAY11-7085 for 1 h before induction of DNA damage strongly suppressed DDSR1 induction upon DNA damage (Fig 2B). No effect of BAY11-7085 was observed in the absence of DNA damage. These results suggest that induction of DDSR1 upon DNA damage is regulated by the ATM-NF-κB signaling pathway.

Another master regulator of the DNA damage response, and of numerous lncRNAs, is the tumor suppressor p53. DDSR1 induction was unaffected in the p53-null cell line H1299 upon DNA damage, suggesting p53 independence of DDSR1 induction (Fig 2C). On the other hand, expression of exogenous p53 was sufficient to induce DDSR1 in H1299 p53-null cells in the absence of DNA damage and DDSR1 transcript levels were higher in H1299 cells expressing p53 upon DNA damage as compared to p53-null H1299 cells (Fig 2C). These results suggest that p53 is not required for DDSR1 induction upon DNA damage, consistent with the absence of a p53 binding site in the promoter, but that p53 can promote DDSR1 expression, likely through other p53 targets associated with transcription or p53 binding to enhancer regions.

**Regulation of gene expression by DDSR1**

LncRNA transcripts often regulate gene expression, directly or indirectly, in cis and trans [10–12,14]. Using microarray analysis, we identified 119 genes that show differential expression upon knockdown of DDSR1 by siRNA for 72 h as compared to control siRNAs in human fibroblasts (>1.5-fold; P < 0.05) (Fig 3A, Table EV3). We verified microarray results by qRT–PCR validation of 15 select genes (Fig 3C), and similar results were obtained with another siRNA targeting a distinct sequence in DDSR1 as well as a locked nucleic acid antisense oligonucleotide (ASO) targeted to DDSR1 (Fig EV2A and B). Of the 119 mRNAs showing differential expression, 67 were upregulated and 52 were downregulated. IPA network analysis showed that they fall predominantly into the categories of cell death and survival (CDK6, E2F7, OLR1, JAG1, TNS5F18, MCM6, IFT3) and DNA replication, recombination, and repair (CENPW, MCM6, ANP32E, HLLS). Interestingly, depletion of DDSR1 led to upregulation of a subset of p53 target genes (DRAM, DHRS3, HMOX1), suggesting that DDSR1 participates in transcriptional repression
of p53 target genes. To further confirm that DDSR1 modulates p53 target gene expression, we performed qPCR analysis for additional p53-regulated mRNAs GADD45, TIGAR, p21, and PUMA, along with those identified in the microarray analysis upon DDSR1 knockdown in the presence and absence of DNA damage with camptothecin in U2OS cells. DDSR1 knockdown resulted in a significant upregulation of all p53 target mRNAs (Fig 3C). Moreover, the levels of DRAM1, GADD45, p21, and PUMA mRNAs were significantly higher in DDSR1 knockdown cells treated with camptothecin as compared to cells transfected with non-specific siRNA and treated with camptothecin (Fig 3C), suggesting that DDSR1 modulates p53 gene expression upon DNA damage.

**DDSR1 modulates cell proliferation and impairs DDR signaling**

To assess the cellular effects of DDSR1 in the regulation of cell proliferation, we first examined cell proliferation in control and DDSR1-silenced human skin fibroblast cells. DDSR1 knockdown cells showed slower proliferation rates at days 3 and 4 than cells transfected with non-specific siRNA, consistent with DDSR1 modulating genes involved in cell proliferation and survival as...
Since DDSR1 is induced upon DNA damage, we evaluated the effect of DDSR1 depletion on activation of DDR signaling molecules by Western blot analysis. Consistent with the fact the DDSR1 acts downstream of ATM, loss of DDSR1 did not alter phospho-ATM levels upon DNA damage. However, the levels of γ-H2AX, phospho-RPA, phospho-Chk1(Ser345), and phospho-p53(Ser15) were significantly reduced upon DNA damage by CPT in cells with silenced DDSR1 compared to control cells (Figs 4B and EV3B). These results suggest that DDSR1 acts downstream of ATM and modulates DDR signaling.

Loss of DDSR1 inhibits DNA repair by homologous recombination

Since DDSR1 knockdown impaired DDR signaling and its induction upon DNA damage is NF-κB dependent, combined with the fact that NF-κB is known to stimulate DNA repair by promoting homologous recombination (HR) [27], we hypothesized that DDSR1 might be involved in regulating DNA repair by HR. To evaluate this possibility, we determined HR repair efficiency upon DDSR1 knockdown using a previously characterized doxycycline-inducible I-SceI-mediated gene conversion assay in TRI-DR-U2OS cells [28], which was derived from DR-U2OS cells containing a mutant version of GFP with an internal I-SceI endonuclease restriction site, and another GFP mutant with 3' and 5' end truncations, neither of which expresses a functional protein [29]. In this assay, a gene conversion event by recombination repair of an I-SceI-induced DSb restores wild-type GFP and indicates repair by HR [29], siRNA or antisense locked nucleic acid-mediated knockdown of DDSR1 in TRI-DR-U2OS or DR-U2OS cells reduced HR repair efficiency by ~50% as compared to mock-transfected cells (Figs 5A and EV4A). This decrease in HR upon DDSR1 knockdown was not due to altered expression of common DNA repair genes as their transcript levels remained unaltered upon DDSR1 knockdown (Fig EV4B). DDSR1 knockdown also resulted in a modest, albeit statistically insignificant, increase in repair by NHEJ (Fig EV4C), but did not significantly change the cell cycle profile of cells (Fig 5B) or the proportion of cells in S/G2 phase as assessed by cyclin A staining (Fig EV4D), suggesting that HR repair defects due to loss of DDSR1 are not due to changes in the cell cycle profile of cells. Importantly, DDSR1 depletion did not markedly perturb DNA replication as revealed by BrDU incorporation analysis by FACS (Fig EV3E). To assess whether DDSR1 contributes to cell viability following genotoxic stress, we evaluated the ability of cells deficient in DDSR1 to sustain growth in clonogenic survival assays. Since cells deficient in HR are sensitive to PARP inhibitors due to synthetic lethality [30], we challenged DDSR1-depleted cells with the PARP inhibitor olaparib in clonogenic assays. Cells depleted for DDSR1 showed increased sensitivity to olaparib (Fig 5C). Similar results were obtained after challenge with neocarzinostatin (Fig EV3F). These results suggest a function for DDSR1 in supporting cell viability in response to genotoxic stress.

DDSR1 interacts with mediators of HR

LncRNAs often exert their functions via interaction with protein complexes. To identify proteins that associate with DDSR1, we used MS2-TRAP-tagged RNA affinity purification [31]. DDSR1 tagged with a multimer of viral MS2 stem loops was co-expressed with a MS2 coat protein (MCP)-GST fusion protein recognizing the
MS2 RNA hairpins, and MS2-DSRS1 RNA/protein complexes were affinity purified using GST beads (see Materials and Methods for details). Mass spectrometry analysis identified several DNA damage-related proteins including factors involved in HR, such as heterogeneous nuclear ribonucleoprotein U-like 1 (hnRNPUL1, also known as E1B-AP5), previously implicated in DNA repair, UBR2, the BRCA1-interacting partner BRIP/FANCJ, and RAD54B, which all specifically associated with DSRS1 upon DNA damage, but not with MS2 control RNA (Table EV4). Association of lncRNA DSRS1 with RNA-binding protein hnRNPUL1 in the presence and absence of DNA damage was confirmed by immunoprecipitation (IP) of ribonucleoprotein (RNP) complexes (RIP analysis) with anti-hnRNPUL1 in parallel with control immunoglobulin G (IgG) IP. DSRS1 was enriched ~1.8-fold in hnRNPUL1 IP samples (P < 0.05) compared with IgG IP samples, and this association was found to be further increased upon DNA damage (Fig 5D). Since hnRNPUL1 depletion has previously been shown to inhibit DNA repair by regulating DNA end resection [32], we evaluated resection upon DSRS1 depletion by GFP-RPA recruitment to laser-induced DSBs and by detection of ssDNA formation by native BrDU staining upon irradiation in S/G2 cells. DSRS1 depletion led to significantly reduced GFP-RPA recruitment to DSBs (Fig 5E; P < 0.02) and also reduced ssDNA formation in S/G2 cells (Fig 5F; P < 0.001), demonstrating reduced resection efficiency in the absence of DSRS1. These observations are consistent with reduced pRPA and pChk1 levels observed upon DNA damage in DSRS1-depleted cells.

Figure 3. DSRS1 modulates gene expression.

A Heatmap representing the relative abundance of differentially expressed genes (DEG, ≥ 1.5-fold, P < 0.05) in human fibroblasts upon treatment for 72 h with siDDSR1 or siNS.

B Gene ontology analysis of DSRS1-sensitive mRNAs.

C Validation of DSRS2-regulated mRNAs. Human fibroblasts were transfected with siNS or siDDSR1, and 72 h later, the transcript levels of the genes indicated were measured by qRT–PCR. RNA samples were analyzed by quantitative RT–PCR, and error bars represent the mean ± SEM from three independent experiments. *Significant change compared to cells transfected with siNS (P < 0.05).

D DSRS2 modulates p53 target gene expression during DNA damage. U2OS cells transfected with siNS or siDDSR1#1 were treated with or without 2 μM with camptothecin for 1 h followed by 3-h release, and transcript levels of indicated genes were measured. RNA samples were analyzed by quantitative RT–PCR, and error bars represent the mean ± SEM from three independent experiments. #Significant change compared to cells transfected with siNS and treated with camptothecin (P < 0.05).

Data information: Statistical comparisons were made using Student’s t-test.
Having established a role for DDDSR1 in modulating DNA repair by HR, we next asked how the depletion of DDDSR1 accounts for the observed HR defects. Since BRCA1 is a key regulator of DNA repair [33], we probed the effect of DDDSR1 depletion on BRCA1 recruitment to laser-induced DSBs by live cell imaging in a previously described BRCA1-GFP stable cell line [34]. siRNA-mediated depletion of DDDSR1 significantly increased BRCA1 accumulation at laser-induced DSBs (Fig 6A). At 4 min after damage, BRCA1 levels were 1.25-fold higher in the absence of DDDSR1 compared to control cells (P < 0.05) and the difference was maintained over the duration of the measurement with about 1.4-fold more BRCA1 at in DDDSR1 knockdown cells as compared to control cells at 10 min post-damage (Fig 6B; P < 0.05, see heatmap in figure).

Although BRCA1 recruitment at DSBs primarily promotes DNA repair by HR [35,36], BRCA1 interacts with the ubiquitin-binding protein RAP80, which promotes its recruitment to DSBs [37] and the aberrant activity of this BRCA1-RAP80 complex limits HR by inhibiting DSB end resection [38–40]. Hence, we probed the effect of DDDSR1 depletion on RAP80 recruitment to laser-induced DSBs. As expected, DDDSR1 knockdown resulted in an increase in RAP80 recruitment to sites of damage as compared to cells transfected with non-specific siRNA (Fig 6B). While initial recruitment of RAP80 during the first 90 s was unaffected by DDDSR1 loss, the subsequent rate of accumulation of RAP80 was significantly increased in the absence of DDDSR1 (Fig 6B; P < 0.05, see heatmap in figure). Knockdown of DDDSR1 by siRNA in U2OS cells did not result in any significant changes in accumulation of γ-H2AX and 53BP1 upon DNA damage by camptothecin compared to cells transfected with non-specific siRNA at early stages of DNA damage (Fig 6C). However, 1 h post-DNA damage, 53BP1 recruitment was modestly increased and γ-H2AX levels concomitantly decreased in DDDSR1 knockdown cells as compared to cells transfected with non-specific siRNA (Fig 6D). These results suggest that loss of DDDSR1 leads to increased recruitment of BRCA1 and RAP80 and in this way limits HR.

**DDDSR1 regulates BRCA1 recruitment to DSBs**

Since DDDSR1 interacts with hnRNPU1 and alters BRCA1 recruitment to laser-induced DSBs, we next examined whether hnRNPU1 plays any role in BRCA1 recruitment to DSBs. Depletion of hnRNPU1 led to a significant increase in BRCA1 recruitment to laser-induced DSBs similar to the effect of DDDSR1 depletion on BRCA1 recruitment (Fig 7A). We then evaluated...
the effect of hnRNPUL1 depletion on RAP80 recruitment to laser-induced DSBs. Consistent with an increase in BRCA1 recruitment upon hnRNPUL1 depletion, RAP80 recruitment to laser-induced DSBs was also elevated upon hnRNPUL1 depletion as compared to cells transfected with non-specific siRNA (Fig 7B). These changes in BRCA1 and RAP80 recruitment upon hnRNPUL1 knockdown were not due to altered DDSR1 expression, since DDSR1 transcript levels remain unaltered upon hnRNPUL1 depletion (Fig 7C). These results point to a functional interaction between DDSR1 and hnRNPUL1 which likely modulates HR by regulating access of BRCA1 and RAP80 to DSBs.

**BRCA1 interacts with DDSR1**

We did not pull down BRCA1 in our RNA affinity mass spectrometry screen, since DDSR1 knockdown altered recruitment of BRCA1 to
DSBs sites and DDSR1 interacts with BRCA1-interacting proteins such as FANCJ and MSH6 (Table EV4). We hypothesized that DDSR1 binds to BRCA1 to modulate its access to DNA repair sites. To test this hypothesis, we performed formaldehyde-cross-linked RIP with BRCA1. We detect significant BRCA1 binding to DDSR1 (7-fold enrichment compared to IgG IP; P < 0.05) and this association was decreased by ~30% upon DNA damage with camptothecin (Fig 7D). These results are in line with our observation in imaging
experiments, suggesting a role of DDSR1 in controlling the access of BRCA1 to sites of DNA damage.

Discussion

We describe here a lncRNA involved in the DNA damage response and characterize its role in homologous recombination. DDSR1 is a 1.6-kb transcript induced with intermediate kinetics by the ATM-NF-kB pathway in response to DNA damage by several agents. DDSR1 interacts with several proteins involved in promoting HR including BRCA1 and hnRNPUL1. DDSR1 loss impairs DDR signaling, alters recruitment of HR repair factors BRCA1 and RAP80 to DSBs, and affects DNA repair by HR. Based on these observations, we suggest that DDSR1 is a regulatory factor in DDR and plays an important role in maintaining genome stability.
The activation of the cellular DDR results in a complex signaling cascade leading to cell cycle arrest so as to allow cells to repair damage or, if the damage is irreparable, to eliminate them via apoptosis or entry into senescence. Both miRNAs and lncRNAs are emerging as important contributors to regulation of the cell cycle and of apoptosis and are as such likely key players in the DDR [16]. DROSHA- and DICER-dependent small RNAs called DDRNAs, generated from regions near the DSB site, contribute to DNA repair foci formation [41]. Moreover, two RNA-binding proteins, RBMX and hnRNPUL1, are recruited to the site of DSBs and promote HR [32,42]. It is likely that additional RNA species interacting with these and other repair proteins may be involved in DNA repair by HR. Two lncRNAs are known to be involved in promoting HR. Depletion of lncRNA ANRIL inhibits DNA repair by HR [19], although the molecular mechanisms of ANRIL-mediated HR are unknown. Interestingly, just like DDSR1, ANRIL modulates both gene expression and DNA repair by HR [19,43]. A second lncRNA known to be involved in regulating HR is prostate-specific lncRNA PCAT1, whose expression is not responsive to DNA damage, but it inhibits HR in prostate tissue by causing posttranscriptional repression of BRCA2 [24].

ATM-mediated DNA damage signaling regulates the expression of lncRNAs and micro-RNA biogenesis [18,44]. Similar to the DNA damage-responsive lncRNA Jade1 [18], DDSR1 induction upon DNA damage is regulated by the ATM-NF-kB pathway. DDSR1 induction upon DNA damage is p53 independent. On the other hand, we find p53 is sufficient to induce DDSR1 expression, although we did not find any p53-responsive elements on the putative promoter region of DDSR1, suggesting that induction of DDSR1 by p53 is not by direct binding at its promoter, but likely through other p53 targets associated with its transcription or p53 binding to enhancer regions.

Expression of several lncRNAs has been linked to p53, and they participate in the p53 network [20–22]. Interestingly, loss of DDSR1 leads to upregulation of p53 target genes, suggesting that DDSR1 negatively regulates p53-mediated gene expression. This is not surprising as several p53-induced lncRNAs, such as TUG1, lincRNA-p21, and PINT1, are involved in negatively regulating p53 targets by various mechanisms [20,22,45]. In addition, knockdown of DDSR1 leads to mis-regulation of numerous genes involved in DNA damage and repair such as CENPW, MCM6, ANP32E, HELLS, HIST1H2A, and HIST1H2B. Considering that many lncRNAs appear to act in trans on target genes, it is possible that some of these effects are direct gene regulatory events rather than secondary events. Based on these observations, it is tempting to speculate that DDSR1 interacts with protein complexes to transcriptionally or epigenetically repress DNA damage response genes, including some in the p53 pathway.

DDSR1 is induced relatively late upon DNA damage (~3 h), at a time when recruitment of repair factors to DSBs and initial phases of DNA repair have already occurred. Yet, we find that loss of DDSR1 acutely reduces DNA repair efficiency by HR, indicating that the basal levels of DDSR1 are important for DNA repair. In line with this observation, while DDSR1 is induced about 2- to 3-fold over several hours in response to DNA damage, it is also expressed in undamaged cells at moderate basal levels. Intriguingly, the decrease in repair efficiency by HR upon DDSR1 knockdown is accompanied by increased recruitment of BRCA1 and its recruitment factor, the ubiquitin-binding protein RAP80 [37], to the sites of DNA damage. These observations, coupled with the fact that DDSR1 interacts with BRCA1 and this interaction is reduced upon DNA damage, suggest that DDSR1 sequesters the BRCA1-RAP80 complex and prevents it from promiscuous DNA binding. This model is consistent with our finding that we cannot detect DDSR1 at sites of laser-induced DSBs, although we cannot strictly exclude the possibility that low levels of endogenous DDSR1 are present but undetectable at DSB sites. One possibility the cells require a mechanism to carefully control the amount of BRCA1-RAP80 cells binding to DSBs is because of the ability of this complex to restrict DNA end resection, thereby limiting DNA repair by HR [38,39]. This interpretation agrees well with our finding that DDSR1 knockdown results in reduced DNA end resection. It is possible that that low basal levels of DDSR1 regulate BRCA1 recruitment and access to DSB sites. However, upon persistent DNA damage, when DDSR1 levels become limited, its induction is triggered, via the ATM and NF-kB signaling pathways, to maintain the correct levels of BRCA1 at sites of DNA damage so as to ensure efficient repair or to remove BRCA1 from the DSB sites once repair is complete. Our findings suggest that DDSR1 functions in DDR repair progression and BRCA1 turnover at DNA repair sites.

In line with a role of DDSR1 in HR, we also observed that DDSR1 interacts with hnRNPUL1, a protein which is transiently recruited to DSBs sites and is required for effective DNA end resection and DSB repair by HR [32]. Recruitment of hnRNPUL1 to DSBs is dependent on the MRN complex [32] and poly(ADP-ribose)lation mediated by PARP1 [46], but is RNA independent [32]. Hence, DDSR1 may not be involved in recruitment of hnRNPUL1 to DSBs. Depletion of hnRNPUL1 is thought to limit DNA repair by inhibiting recruitment of the BLM helicase to DSB, but the resection defect upon hnRNPUL1 depletion cells is not overcome by BLM overexpression [32]. This suggests the existence of additional defects, which lead to DNA repair inhibition upon hnRNPUL1 depletion. In contrast to the earlier finding that hnRNPUL1 knockdown has no effect on BRCA1 recruitment at DSBs [32], we observe that knockdown of hnRNPUL1 leads to increased recruitment of BRCA1 at laser-induced DSBs similar to the effect observed upon DDSR1 depletion. This discrepancy is likely due to different technical and experimental conditions used for measurement of BRCA1 recruitment (BRCA1 foci formation in fixed cells after IR and camptothecin versus BRCA1-GFP recruitment in living cells to laser-induced DSBs). Similar to what is seen in DDSR1-depleted cells, BRCA1 recruitment at DSBs in hnRNPUL1-depleted cells was accompanied by enhanced recruitment of RAP80. These results allow for the possibility that the DNA end resection and HR defects observed upon hnRNPUL1 depletion are, at least in part, due to increased BRCA1 and RAP 80 recruitment. Taken together, these results suggest that the interaction of hnRNPUL1 with DDSR1 plays a role in regulating BRCA1 and RAP80 access to DSBs in modulating HR. Intriguingly, hnRNPUL1 is also implicated in regulation of transcription and p53-mediated gene expression [47,48] and it is plausible that the interaction of DDSR1 with hnRNPUL1 may also contribute to its role in transcription regulation.

In sum, in this study we have identified and characterized the lncRNA DDSR1 as a regulator of DNA repair by HR. The involvement
of IncRNAs in DNA repair pathways expands the number and diversity of molecular components in the DDR and suggests that the DDR is even more complex than currently appreciated.

Materials and Methods

Cell culture and treatments

Human hTert-immortalized fibroblast cells [49] were grown in MEM containing high glucose supplemented with glutamine and 15% fetal bovine serum (FBS) (Atlanta Biologicals). U2OS, A549, PC3, H1299 (p53-null) were obtained from ATCC and were grown in DMEM containing high glucose, supplemented with glutamine and 10% FBS. To induce DNA damage, cells were treated with DNA-damaging agents neocarzinostatin (NCS) (*N9162), camptothecin (CPT) (*C9911), etoposide (*1383), or bleomycin (*2434) all from Sigma-Aldrich for indicated doses and times. To examine the role of ATM and NF-κB in IncRNA DDSR1 induction, cells were pretreated with 20 μM KU55933 (ATMi, Tocris) or 10 μM NF-κB inhibitor BAY11-7085 (Tocris) 1 h prior to DNA damage treatment and treatment was continued throughout the course of the experiment along with DNA damage-inducing agent. For Western blotting, cells were treated with 2 μM camptothecin (CPT) for 1 h, washed, and kept for 3 h before protein isolation. For I-SceI induction, cells were treated with 5 μg/ml doxycycline for the indicated time periods. For laser micro irradiation-induced DSBR generation, cells were presensitized with Hoechst 33342 (0.1 μg/ml) for 60 min. For double-thymidine block, cells were treated with 2.5 mM thymidine (Sigma) for 18 h, followed by a 9-h release and a second 17-h thymidine treatment. For clonogenic survival assays, cells were transfected with siRNAs and were treated overnight with the indicated doses of olaparib (AZD-2281, Selleckchem).

Cell proliferation assays

For cell proliferation assay, cells were seeded at a concentration of 2,500–5,000 cells/well in 96-well plates and transfected with siRNAs at 100 nM. Cell numbers were quantified using reagent WST-1 (Cat#. 05015944001, Roche) at the indicated time according to the manufacturer’s instructions.

Colony formation assay

For clonogenic assays, U2OS cells were seeded into 60-mm dishes and treated with or without olaparib or neocarzinostatin at indicated doses 72 h after transfection with 100 nM siRNA against IncRNA-DDSR1 or non-specific siRNA. Colonies, which formed between 14 and 16 days after plating, were stained with a crystal violet solution and counted. Assays were done in triplicate.

Flow cytometric analysis of DNA content and BrDU incorporation in dsDNA

U2OS cells 72 h post-transfection with siNS or siDDSR1#1 were fixed in 70% ethanol and stored at −20°C. The fixed cells were washed in PBS, resuspended in propidium iodide solution (BD Biosciences) for 20 min at room temperature, and analyzed on FACSCalibur (Becton Dickinson). BrDU incorporation in dsDNA (replicating cells) of U2OS cells upon DDSR1 knockdown for 72 h was determined using FITC BrdU Flow Kit (BD Biosciences San Jose, CA) as per manufacturer’s instructions. Analysis was carried out using FlowJo software package.

RNA FISH

RNA FISH for DDSR1 detection was performed using a pool of 20 fluorescent probes purchased from Stellaris Biosearch Technologies, following the manufacturer’s protocol.

qRT–PCR

Nuclear RNA was isolated using the Ambion PARIS system (Life Technologies). Total RNA was isolated using the RNaseasy plus mini kit (Qiagen). cDNA synthesis was carried out with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed in triplicates with the iQ SYBR Green supermix (Bio-Rad) on a Bio-Rad CFX-96 real-time PCR system. The comparative Ct method was employed to quantify transcripts. Normalization was performed to TBP expression. Primers used in this study are provided in Table EV5.

5′–3′ RACE

Total RNA extracted from human fibroblast cells was used to perform RACE to identify the full-length sequence of IncRNA DDSR1. 3′ RACE was performed using the RLM RACE kit (Life Technologies) following the manufacturer’s protocol. cDNA ends were amplified with universal primers and gene-specific primers. The first PCR product was used to perform a “nested” PCR with the nested universal primer and the nested gene-specific primers. 5′ RACE-PCR experiments were performed using 5′/3′ RACE kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Primer sequences were as follows (5′ to 3′):

- Reverse transcription for 5′ RACE: TCTCTGATAATGGAGTT-GACCC
- GP3 (3′ RACE): AAGAGTGGAAATCTCCTCCCCA
- GP5 (5′ RACE): TGTCTATTTGATCATTTTCTCCCA

Nestd GP2 (3′ RACE): TGCAACAATGCTGTCATTACT
Nestd GP2 (5′ RACE): CCCAGATTATTCTCTTGTAG

The resulting DNA fragments were cloned by TA cloning and sequenced (Geneviz). Sequence data of the full-length human IncRNA-DDSR1 can be found in Dataset EV1. Using the sequence information from the two ends, we cloned the full-length DDSR1 sequence and tagged it with MS2 hairpins.

siRNA and plasmid transfections

Transfections of siRNAs or antisense locked nucleic acid gapmers were done using DharmaFect 1 as per manufacturer’s instructions. Fibroblasts were transfected once with 100 nM duplex siRNA. U2OS or its derivative cell lines were transfected twice with 75 nM duplex siRNA on successive days or once with 50 nM antisense locked nucleic acid oligonucleotides. The siRNA duplexes (Dharmacon) used in this study are as follows siRNA #1 for DDSR1: sense 5′CUAGUGUGUUGCAAAAUAUU-3′, antisense 5′P-UUAAUUUGC
AACACUAGUU-3'; siRNA #2 for DDSR1: sense 5'-GCUACUAG UCUAGAGUU-AAU-3', antisense 5'-UAUCUCUAGACGUUA GCUU-3'; siRNA against hnRNPU1: sense 5'-GCAUGGGAACCA GUAAUUU-3', antisense 5'-AUAGUCUGUUUCCGCUU-3'.

ON-TARGET plus non-targeting pool (# D-001810-10) was used as a control. The sequence of antisense oligonucleotide against DDSR1 and negative control purchased from Exiqon is as follows: 5’-TTCGATGCGCAATTAA-3’ and 5’-AACAGTCTATAGC-3’, respectively. The pS3 overexpression construct was a gift from Dr. Bert Vogelstein and is described in [50]. MS2 coat protein (MCP)-GST construct is described in [51]. GFP-RAP80 was a gift from Marc Wold [53]. Plasmids were transfected using X-tremeGENE HP

DNA repair assays

HR assays upon DDSR1 knockdown were done in TRI-DR-U2OS cells expressing inducible I-SceI cells or after transient I-SceI transfection in DR-GFP U2OS cells as described previously [28]. NHEJ assay after transient I-SceI transfection was done in stable, NHEJ-U2OS reporter cells generated as described [28] using the pEJ5 construct [54]. HR or NHEJ efficiencies were analyzed by fluorescence-activated cell sorting and are shown as % GFP + cells.

Immunofluorescence microscopy

After siRNA knockdowns, cells were treated with camptothecin for indicated time and fixed with 4% buffered paraformaldehyde in PBS for 15 min at room temperature followed by permeabilization with 0.5% Triton X-100 in PBS. Following permeabilization cells were blocked in 5% BSA in 0.05% PBS Tween-20 for 1 h. Blocked cells were stained with antibodies for cyclin A (1:100, Neomarkers # Ab6), cyclin B (1:50; Santacruz #sc-751) diluted in 2.5% BSA in PBS and primary antibodies for BrDU (1:500; BD Biosciences #347580) and cyclin A (1:50; Santacruz # sc-751) diluted in 2.5% BSA in PBS solution for 2 h at room temperature. Cells were then washed 3× with PBS (0.05% Tween-20) solution and incubated with goat anti-mouse Alexa 568 (1:500) and goat anti-rabbit Alexa (1:500) for 1 h. The cells were then washed 4× with PBS (0.05% Tween-20) solution, and mounted in vectashield mounting medium containing DAPI from Vector Laboratories. Images of the cells were captured with a CCD camera on a Zeiss LSM 780 Meta confocal microscope using a 40× objective. The BrDU intensity of cyclin A-positive cells (S/G2) was analyzed using a custom-built macro plugin for Image J.

Immunoblotting

Cells lysates were prepared using RIPA buffer supplemented with protease and phosphate inhibitors. Protein samples were run on a 4–12% bis-tris gel and transferred to a PVDF membrane. Primary antibodies against pATM (1:5,000; Abcam # ab81292), pp53 (1:1,000, Cell Signalling #9284), p53 (Santa Cruz # sc126), pChk1 (1:1,000; Cell Signalling #2341), pRPA (1:2,000, Bethyl #A300-245), RPA (1:5,000; Abcam # ab2175), Chk1 (1:1,000; Santa Cruz # sc-8408), H2AX (1:2,000; Novus #NB100-638), hnRNPU1 (Novus #NB110-40586), and γ-H2AX (1:2,000; Millipore) were incubated overnight at 4°C. HRP-anti-rabbit or mouse immunoglobulins (1:20,000; Santa Cruz) were incubated for 1 h at room temperature and ECL-plus kit (Amersham) was used for blot development. Anti-Hsc70 antibody (1:20,000; Abcam #ab19136) was used for normalization. All membrane images were acquired using a ChemiDocTM MP imaging system (Bio-Rad).

Microarray

RNA labeling and hybridization were carried out by the Laboratory of Molecular Technology, SAIC-Frederick. To identify ncRNAs induced upon DNA damage, total RNA or nuclear RNA was reverse-transcribed, labeled, and hybridized to the NCode™ Human array following the Agilent’s Quick Amp Labeling Kit protocol. Fold changes relative to control were calculated by averaging fold change observed with each drug. To identify genes regulated by DDSR1, total RNA was reverse-transcribed, labeled, and hybridized using GeneChip Human Gene 2.0 ST array following the Ambion 4WT Expression Kit Protocol. Data were analyzed using Partek Genomics Suite (Partek Incorporated), and differentially expressed genes (DEG) were defined as genes having ≥ 1.5-fold change of expression in pairwise comparisons, with a P-value < 0.05.

Native RNA immunoprecipitation

Native RIP was performed as described [55], with minor modifications. Briefly, control and DNA-damaged cells were suspended in ice-cold cell disruption buffer (20 mM Tris pH 7.5, 10 mM KCl, and 1.5 mM MgCl2, 0.1% Triton X-100) supplemented with protease, phosphatase, and RNase inhibitors for 10 min on ice and centrifuged at 1,500 × g for 5 min at 4°C to isolate the nuclei. To isolate RNA-containing nuclear extracts, nuclei were resuspended in polysome lysis buffer (10 mM HEPES pH 7.0, 100 mM KCl, 5 mM MgCl2, 0.5% NP-40, 10 mM DTT) supplemented with protease, phosphatase, and RNase inhibitors for 10 min on ice and centrifuged at 10,000 × g for 15 min at 4°C. The supernatants were incubated with antibodies against hnRNPU1 (Novus #NB110-40586) and control IgG (Cell Signalling #2729) over night at 4°C. Antibody-bound protein and RNA was collected using 40 μl protein G magnetic beads (Pierce). The beads were then washed 4× for 5 min with NT2 buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM MgCl2, and 0.05% NP-40) supplemented with protease, phosphatase, and RNase inhibitors. To isolate RNA, beads were resuspended in TRIzol followed by isolation of RNA from the aqueous fraction using the RNeasy Micro Kit (Qiagen). RNPs isolated from the IP materials were further analyzed by
qRT-PCR. Normalization of RIP results was carried out by quantifying in parallel the relative levels of GAPDH mRNA in each IP sample.

**Formaldehyde-cross-linked RIP**

BRCA1 RIP was performed as described [56], with minor modifications. Briefly, 30 million cells treated with or without 10 μM camptothecin for 30 min were fixed with 1% formaldehyde in PBS to cross-link protein-bound RNA/DNA complexes, and formaldehyde was quenched by the addition of glycine to a concentration of 0.125 M. Cells were then washed with PBS and incubated in 1 ml collection buffer [100 mM Tris–HCl (pH 9.4)] and 100 mM DTT, protease inhibitor, RNaseOUT] on ice for 15 min. Nuclei were isolated by resuspension in 1 ml buffer A (10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, and 200 mM NaCl) to produce DNA/RNA fragments of about 500 nucleotides. The sonicated DNA/RNA was supplemented with 2 μg proteinase K (NEB) and made up to a total volume of 170 μl of elution buffer (10 mM Tris pH 8, 1 mM EDTA, 1% SDS, RNaseOUT), 50 μl of the Dynabeads and 4 μg BRCA1 (Ab-1; Calbiochem) or IgG (12-371; Millipore) antibodies, and incubated overnight at 4°C. The beads-bound RNA/DNA complexes were washed six times with RIPA buffer (50 mM Hepes pH 8, 1 mM EDTA, 1%NP-40, 0.7% DOC, 0.5 M LiCl, and protease inhibitor mix, RNaseOUT), and once with TE buffer. The DNA/RNA was extracted from the beads by the addition of 100 μl of elution buffer (10 mM Tris pH 8, 1 mM EDTA, 1% SDS, RNaseOUT) and incubated at 65°C with gentle shaking for 15 min. For reversing the cross-linking, elution buffer containing RNA/DNA was supplemented with 2 μl of 5 M NaCl and 2 μl proteinase K (NEB) and made up to a total volume of 170 μl and incubated for 5 h at 70°C. After reverse cross-linking, 750 μl of TRIzol was added per sample and RNA extracted as per manufacturer’s instructions. DNase I (Roche) treatment was performed as per manufacturer’s protocol, and cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed in triplicates with 2 μl cDNA using the iQ™ SYBR® Green supermix (Bio-Rad) on a Bio-Rad CFX-96 real-time PCR system. The primers used for DDSRI are forward 5’-AAGAGTTGAAAAATTCTATCCCCA-3’ and reverse 5’-TGTCATGTTCAGATATT TTTCCTCCA-3’. The results are expressed as fold enrichment over input and normalized to IgG.

**Laser microirradiation and imaging**

Laser microirradiation was performed using a Zeiss LSM780 confocal microscope. Before imaging, cells were incubated with 0.1 μg/ml Hoechst 33342 for 1 h and then switched to FluoroBrite phenol red-free media containing 10% fetal bovine serum and glutamine, antibiotics without Hoechst and 5 mM HEPES. The cells were damaged with a 355-nm (10 μJ) laser beam, with total UV laser output set to 20%, ten iterations, and laser scan speed set to 7 (pixel dwell time 3.15 μs) as described previously [57]. The cells were laser-microirradiated in a rectangle and images taken every 10 s for 10 min, maintaining cells at 37°C and 5% CO₂. To measure the recruitment of GFP-BRCA1 or GFP-RAP80 in laser-microirradiated cells, MIPAVER software (v.5.1) was used. For measurements, the regions of interest were drawn within the laser-microirradiated regions, within a non-laser-microirradiated regions in the nucleus and a region outside the cells [28]. The mean fluorescence intensity within the region of interest was measured for each time point (10 s) within the time series. The fluorescence intensity values were background subtracted, and the ratio of intensity within the microirradiated nuclear area to non-micro-irradiated area was calculated. The ratios were normalized to prelased time point.

**MS2-TRAP for tagged RNA affinity purification**

Human fibroblasts in 10-cm dishes were co-transfected with 6 μg of plasmid containing DDSRI tagged with MS2 stem loop RNA or MS2 stem loop RNA alone as a control together with 3 μg MS2 coat protein (MCP)-GST construct using X-tremeGENE (Roche) according to the manufacturer’s instructions. Twelve hours post-transfection, cells were treated with NCS (50 ng/ml), or not treated as a control for 24 h. Cells were collected and lysed in lysis buffer (20 mM Tris–HCl at pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.3% IGEPAL CA-630) supplemented with protease (Sigma), phosphatase (Calbiochem), and RNase inhibitors (Promega), and 10 mM DTT for 30 min on ice. This was followed by two freeze-thaw cycles on dry ice to help nuclear lysis. Cell lysates were cleared of debris by centrifugation at 10,000 g for 15 min at 4°C. For isolation of MCP-GST-bound RNPs, cleared lysates were incubated at 4°C for 3 h with glutathione magnetic beads (Thermo Scientific # 88821) and washed as per manufacturer’s instructions. After washing, glutathione beads were suspended in 25 mM ammonium bicarbonate pH 8.4 and processed for mass spectrometry.

**Statistical analysis**

Results are presented as mean ± SEM, unless otherwise stated. We used paired Student’s t-test for comparisons between two experimental groups. Additional statistical tests information is described in the figure legends. P < 0.05 was considered statistically significant.

**Accession numbers**

The lncRNA DDSRI sequence has been deposited to GenBank with accession number KT318134. The NCBI Gene Expression Omnibus accession number for the microarray data is GSE72358.

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Author contributions
VS and TM designed the study; VS performed majority of the experiments and data analysis; NK aided in the execution and analysis of high-throughput imaging; SK aided in execution and analysis of HR experiments and PO advised on the HR experiments; KA and MG did DOSR1-MS2 cloning and advised on MS2 pull-down and native RIP experiments. VS and TM wrote the manuscript. All the authors read and approved the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References