A Role for DEAD Box 1 at DNA Double-Strand Breaks

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DEAD box proteins are a family of putative RNA helicases associated with all aspects of cellular metabolism involving the modification of RNA secondary structure. DDX1 is a member of the DEAD box protein family that is overexpressed in a subset of retinoblastoma and neuroblastoma cell lines and tumors. DDX1 is found primarily in the nucleus, where it forms two to four large aggregates called DDX1 bodies. Here, we report a rapid redistribution of DDX1 in cells exposed to ionizing radiation, resulting in the formation of numerous foci that colocalize with γ-H2AX and phosphorylated ATM foci at sites of DNA double-strand breaks (DSBs). The formation of DDX1 ionizing-radiation-induced foci (IRIF) is dependent on ATM, which was shown to phosphorylate DDX1 both in vitro and in vivo. The treatment of cells with RNase II prevented the formation of DDX1 IRIF, suggesting that DDX1 is recruited to sites of DNA damage containing RNA-DNA structures. We have shown that DDX1 has RNAse activity toward single-stranded RNA, as well as ADP-dependent RNA-DNA- and RNA-RNA-unwinding activities. We propose that DDX1 plays an RNA clearance role at DSB sites, thereby facilitating the template-guided repair of transcriptionally active regions of the genome.

DEAD box proteins, classically defined as putative RNA helicases, have been implicated in all aspects of RNA metabolism involving the modulation of RNA secondary structure (38, 47). These proteins share nine conserved motifs (including the D-E-A-D motif) required for RNA binding, RNA-dependent ATP binding/hydrolysis, and ATP-dependent RNA unwinding. Although >35 DEAD box proteins in higher eukaryotes have been identified, we still have a poor understanding of their biological roles (1). The best-characterized mammalian DEAD box protein is the translation initiation factor eukaryotic initiation factor 4A (eIF4A), which unwinds RNA-RNA and RNA-DNA duplexes in vitro. eIF4A is believed to facilitate translation initiation by removing secondary structures from the 5′ ends of transcripts (24).

Analyses of DEAD box proteins in lower eukaryotes and prokaryotes suggest roles in RNA processing, RNA stability, RNA transport, and RNA remodeling. DEAD box proteins (and related DEAH box proteins) have recently been implicated in the DNA damage response, with Saccharomyces cerevisiae DHH1 playing a role in G_{1}/S DNA damage checkpoint recovery (10) and yeast MPH1 proposed to function in a branch of homologous recombination (HR) involved in error-free bypassing of DNA lesions (52). With an estimated >20,000 DNA lesions per cell each day, the effective repair of genomic DNA is critical to the survival of the cell. Of all DNA lesions, double-strand breaks (DSBs) are the most serious threat to the genome, as they can lead to the loss of genetic information, chromosome abnormalities, and cell death. DNA DSBs can be caused by exogenous agents, such as ionizing radiation (IR), or endogenous agents, such as reactive oxygen species (30). DNA DSBs trigger a sequence of events which include DNA damage sensing, the amplification of damage signals, and the recruitment of the repair machinery, followed by DNA repair and the restoration of normal chromatin structure.

A key player in the DNA DSB response is ATM (ataxia telangiectasia mutated), a member of the phosphatidylinositol 3-kinase protein kinase family. Cells from ATM-defective ataxia telangiectasia (A-T) patients show increased chromosome instability and are profoundly defective in their response to DSBs (34). In undamaged cells, ATM exists as a catalytically inactive dimer or higher-order multimer; upon DNA DSB formation, ATM undergoes autophosphorylation, resulting in the formation of catalytically active monomers (3, 32). The Mre11-Rad50-Nbs1 (MRN) complex is required for the recruitment of activated ATM to DNA DSB sites (21, 35), where ATM in turn amplifies and sustains the DNA damage signaling cascade through the recruitment and phosphorylation of additional proteins involved in signal transduction and DNA repair (54). Substrates of activated ATM include p53, H2AX, Nbs1, BRCAl, 53BP1, and ATM itself.

The DEAD box 1 gene (DDX1) is a widely expressed gene that is amplified in a subset of retinoblastoma and neuroblastoma cell lines and tumors (25, 26). In addition to the nine motifs characteristic of DEAD box proteins, the DDX1 protein contains a 130-amino-acid SPRY domain in its N-terminal region (25, 43). DDX1 is found primarily in the nucleus, where it has a punctate distribution pattern. DDX1 also forms distinct foci, called DDX1 bodies, in the nucleus (11). A close spatial relationship among DDX1 bodies, cleavage bodies, and Cajal bodies (CBs) has been observed previously, with DDX1 bodies and cleavage bodies colocalizing and residing adjacent to CBs (37). Cleavage bodies and CBs are enriched with proteins associated with RNA metabolism and have been proposed to serve as storage/assembly sites for proteins involved in...
transcription, splicing, 3′-end processing of pre-mRNAs, and RNA degradation (22).

Upon the treatment of cells with IR, we observed a rapid redistribution of DDX1 protein into multiple foci (IR-induced foci [IRIF]) within the nucleus. A coimmunofluorescence analysis revealed the extensive colocalization of DDX1 foci with activated γ-H2AX and phosphorylated ATM (pATM) at sites of DNA DSBs. However, whereas virtually every DDX1 IRIF colocalized with γ-H2AX and pATM IRIF, only a subset of γ-H2AX and pATM IRIF colocalized with DDX1, suggesting a specialized role for DDX1-containing IRIF. The inactivation of ATM resulted in a loss of IR-induced foci, directly linking DDX1 to the ATM signaling pathway. The data presented in this paper support a role for DDX1 in the repair of transcriptionally active regions of the genome.

**MATERIALS AND METHODS**

**Cell culture and drug treatment.** The following human cell lines were used: HeLa, cervical carcinoma cells; GM38, normal lung fibroblasts; RB522A, retinoblastoma cells with amplified DDX1; RB5059, retinoblastoma cells with no amplification of DDX1; U2OS and Saos2, osteosarcoma cells; U251, malignant glioma cells; M059J, DNA-PKcs-negative malignant glioma cells; M059K, DNA-PKcs-positive malignant glioma cells; AT2BE and AT5BI, ATM-deficient primary fibroblasts established from A-T patients; pEBST (EBS) and pEBST-YZ5 (YZ5), simian virus 40-transformed cells from the same A-T patient, with YZ5 cells expressing full-length ATM and EBS cells transfected with a control vector; and BT and L3, ATM-positive normal lymphoblastoid cells and ATM-negative A-T patient lymphoblastoid cells, respectively.

Cells were irradiated using a 137Cs irradiator (Shepherd, San Fernando, CA). Recovery was at 37°C for the lengths of time indicated below. Cells were treated with the following chemicals: 20 μM wortmannin (Sigma) for 1 h prior to irradiation, and 80 μg/ml bleomycin (Mayne Pharma Pty Ltd., Australia) for 2 h prior to fixation. To enrich for cells in mitosis, HeLa cells were blocked in 2.5 mM thymidine for 24 h. Cells were washed twice in lysis buffer, three times in 100 mM Tris-HCl (pH 7.5)–1.0 M LiCl, twice in prekinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 200 nM microcystin-LR, and 1 mM NaF), and once in kinase buffer (prekinase supplement with 10 mM MgCl2). Immunoprecipitates were resuspended in 30 μl of kinase buffer and incubated with a mixture of 2 μg of recombinant DDX1 and 10 μCi of [γ-32P]ATP (3,000 Ci/mmol; GE Healthcare) at 30°C for 20 min. Where indicated, 2 μM wortmannin was included in the kinase reaction. Proteins were electrophoresed in SDS-polyacrylamide gels by electrophoresis (PAGE) gels and transferred onto nitrocellulose membranes by electroblotting. Phosphorylated DDX1 was visualized by autoradiography.

**Metallic labeling with [32P]P.** HeLa cells at ~80% confluence were labeled with [32P]P (PBS13; GE Healthcare) in phosphate-free Dulbecco’s modified Eagle’s medium for 10 min before treatment with 5 Gy of IR. One hour later, cells were lysed in lysis buffer at 4°C. Where indicated, cells were pretreated with 100 μM wortmannin for 30 min before exposure to IR. One milligram of prechilled 32P-labeled whole-cell extract was incubated with 15 μg of DDX1 anti-DDX1 antibody mixture overnight at 4°C. Immunoprecipitates were washed twice in lysis buffer and subjected to PAGE. The 32P-labeled proteins were visualized by autoradiography. To visualize DDX1, the same blots were immunostained with anti-DDX1 antibody. DNase and RNase treatment. Cells were exposed to 5 Gy of IR and incubated at 37°C for 1 h. The cells were then permeabilized (in a solution of 2% Triton X-100; Roche), RNase A (0.1 mg; USB Corporation), or RNase H (5 U; USB Corporation) in 100 μl of PBS containing 5 mM MgCl2 per coverslip for 15 min at room temperature. Cells were fixed and immunostained as described above.

**Unwinding assays.** Two RNA oligonucleotides and four DNA oligonucleotides were used to generate the various RNA-DNA, RNA-RNA, and DNA-DNA heteroduplexes used for the unwinding assays. R41(+) (top strand) RNA was prepared by the transcription of a BamHI-digested pGEM3 plasmid with SP6 RNA polymerase, followed by PAGE purification as described previously (41). R29(−) (bottom strand) RNA was synthesized and PAGE purified by Dharamacon. D29(+), D29(−), D41(+), and D41(−) DNA oligonucleotides were synthesized by Invitrogen. The sequences of RNA and DNA oligonucleotides are given in Table 1.

**TABLE 1. RNA and DNA oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence (5′→3′)</th>
</tr>
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<tbody>
<tr>
<td>R41(+)......GAAUACAAAGUCUGAUUGCUCGACGACUAGAGANC...</td>
<td>R29(−)....GAUUCAAAGUGUGACUGACUCGAGCAGUGUCAC...</td>
</tr>
<tr>
<td>D41(+)....GAACTACAGGCAGCGCTAGCGCTAGCCCTGTC...</td>
<td>D29(−)....GAACCTTCAGATGCGATCGGACGCTGTC...</td>
</tr>
<tr>
<td>D41(+)....GACTCTGCAATGCCTGACCCGGAATCTGAGAGC...</td>
<td>D29(−)....GAACCTTCAGATGCGATCGGACGCTGTC...</td>
</tr>
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*The complementary sequences of the 41-mer and the 29-mer are underlined, and an asterisk (*) indicates an opposite strand. Note that the sequences of D41(+) and D29(−) are the exact DNA counterparts of the sequences of R41(+) and R29(−), respectively. R41(+) and R29(−) are complementary to D41(−) and D29(+), respectively.*
nature for 2 h to allow duplex formation. This method resulted in the incorporation of ≥80% of the labeled strand into the annealed duplex.

A construct comprising the glutathione S-transferase gene fused to the entire coding region of the DDX1 gene was bacterially expressed, and the fusion protein was purified with glutathione-Sepharose 4B. Glutathione S-transferase was cleaved with thrombin. Unwinding assays were carried out in a 20-μl reaction volume using 50 fmol of radiolabeled DNA and/or RNA substrates and 0.3 μg (3.6 pmol) of recombinant DDX1 protein in a mixture of 20 mM Tris-HCl, pH 7.5, 70 mM KCl, 2 mM magnesium acetate, 1.5 mM dithiothreitol, and 10 U of RNAguard (GE Healthcare). Where indicated, ATP, ATP-S, ADP, or GTP was added to a final concentration of 1 mM. For reactions without Mg2+, 10 mM EDTA, pH 7.5, was also included. Reaction mixtures were incubated at 37°C for 20 min, and reactions were quenched with ice. Five microliters of loading buffer (50 mM EDTA, 40% glycerol) was added to each sample, and the samples were electrophoresed through a 12% native polyacrylamide gel (acrylamide/bisacrylamide, 29:1) in 1× Tris-borate-EDTA buffer. Gels were dried and exposed to X-ray film.

**RESULTS**

**Formation of DDX1 irradiation-induced foci upon exposure to IR.** We previously demonstrated an association between DDX1 bodies, cleavage bodies, and CBs during the S phase of the cell cycle, with DDX1 bodies and cleavage bodies colocalizing and residing adjacent to CBs (37). We further demonstrated that the inhibition of DNA replication by using aphidicolin and hydroxyurea leads to the disassembly of CstF64-containing cleavage bodies. As DNA damage also inhibits DNA synthesis (7), we pursued these observations by studying the effect of IR on nuclear bodies. HeLa cells were exposed to 5 Gy of IR and allowed to recover for 1 h. Nuclear bodies were then analyzed by immunofluorescence. Whereas the numbers of cleavage bodies (detected with anti-CstF64 antibody) and CBs (detected with anti-Sm antibody) remained largely unchanged after exposure to IR, the number of DDX1 bodies increased from an average of 2 to 4 per cell pre-IR to 30 to 40 per cell post-IR (Fig. 1A to F). Other agents, such as UV light and cisplatin, did not lead to the immediate formation of DDX1 IRIF (see Fig. S1 in the supplemental material). In contrast, DDX1 IRIF were observed in 90% of cells treated with bleomycin, a radiomimetic drug that causes DSBs (Fig. 2C, left panel). The formation of DDX1 IRIF appeared to be mediated through DDX1 protein relocalization, as no change in pre-IR DDX1 protein levels was observed post-IR (Fig. 1G).

PML nuclear bodies have been shown previously to increase in number upon IR, from a mean of 17 (pre-IR) to a mean of 24 upon exposure to 10 Gy of IR (20). As we had previously observed the occasional adjacent localization of DDX1 bodies and PML bodies in nonirradiated HeLa cells (11) (Fig. 1C), we examined the association of DDX1 and PML bodies after IR. No increase in either the adjacent localization or the colocal-
formation of DDX1 IRIF, therefore, represents an early response to IR exposure.

The examination of GM38 (normal human fibroblasts), U2OS and Saos2 (osteosarcoma cells), RB522A and RB805 (retinoblastoma cells with and without the amplification of DDX1), and U251 (malignant glioma cells) revealed DDX1 IRIF in all these cell types upon exposure to IR. The presence of DDX1 IRIF in GM38 cells indicates that these foci are not restricted to transformed cells (Fig. 2D, left panel).

Colocalization of DDX1 IRIF and γ-H2AX foci. γ-H2AX is a histone 2A variant containing a Ser139 residue that is rapidly phosphorylated upon the exposure of cells to IR, resulting in the formation of γ-H2AX foci at sites of DNA DSBs (48). There is good correlation between the number of γ-H2AX IRIF and the estimated number of DNA DSBs upon exposure to IR, with 1 Gy producing 20 to 30 DNA DSBs and approximately the same number of γ-H2AX foci (50).

To investigate a possible relationship between DDX1 IRIF and DNA DSBs, we studied the subcellular localization patterns of DDX1 foci and γ-H2AX foci after IR treatment. As shown in Fig. 2A, there was no association between DDX1 bodies and γ-H2AX foci before irradiation. However, the vast majority of DDX1 foci and γ-H2AX foci had colocalized at 1 h post-IR in cells treated with 5 Gy (Fig. 2B, far-right panel). Colocalization was also observed at earlier time points after irradiation (data not shown). To ensure that the signal detected with anti-DDX1 antibody at DNA DSBs was indeed due to DDX1, we carried out competition experiments with a recombinant DDX1 peptide (amino acids 1 to 186) as a competitor for 4 h at room temperature. Double immunostaining of irradiated HeLa cells was then carried out as described in the legend to panel D. Bars, 10 μm.

FIG. 2. Colocalization of DDX1 IRIF with γ-H2AX foci. (A and B) Control HeLa cells (A) and HeLa cells treated with 5 Gy of IR (B) were examined 1 h after IR. Arrowheads indicate γ-H2AX foci that have no DDX1. (C) HeLa cells were treated with 80 μg/ml bleomycin for 2 h and subjected to immunostaining. (D) Normal GM38 fibroblasts were fixed 60 min after exposure to 5 Gy of IR and double stained with anti-DDX1 and anti-γ-H2AX antibodies. (E) An anti-DDX1 and anti-γ-H2AX antibody mixture was incubated with 1.5 μg/ml of a recombinant DDX1 peptide (amino acids 1 to 186) as a competitor for 4 h at room temperature. Double immunostaining of irradiated HeLa cells was then carried out as described in the legend to panel D. Bars, 10 μm.

ization of DDX1 IRIF and PML bodies was observed upon the exposure of HeLa cells to 5 Gy of IR (Fig. 1F). These data suggest that DDX1 and PML act in different cellular pathways in response to DNA DSBs.

To determine whether the induction of DDX1 IRIF is dose dependent, HeLa cells were exposed to 1, 2, 5, and 10 Gy of IR and immunostained 1 h later. A direct correlation between the DDX1 IRIF number and the IR dose was observed, with the number of DDX1 foci increasing from an average of 5 at 1 Gy to 40 at 10 Gy (Fig. 1H). Furthermore, DDX1 IRIF could be visualized as early as 5 min after exposure to 5 Gy of IR, with 43% of cells being positive for these foci. More than 90% of cells were positive for DDX1 IRIF after 1 h (Fig. 1I). The
number of DDX1 IRIF, suggesting that ATM and/or DNA-PKcs is required for DDX1 IRIF formation.

To address a possible role for DNA-PKcs in DDX1 IRIF formation, we examined the paired cell lines M059K and M059J, proficient and deficient in DNA-PKcs, respectively (36). M059K and M059J cells showed normal numbers of DDX1 nuclear bodies prior to irradiation (data not shown). Exposure to IR resulted in the formation of DDX1 and γ-H2AX IRIF in both cell lines (M059J cells are shown in Fig. 3B). Thus, DNA-PKcs is dispensable for DNA DSB-induced DDX1 focus formation.

Next, we examined DDX1 IRIF formation in three ATM-deficient fibroblast lines: EBS (63), AT2BE (6), and AT5B1 (55). While DDX1 nuclear bodies were present in all three cell cultures prior to IR, there was no change in the numbers and appearance of these nuclear bodies after IR (EBS cells are shown in Fig. 3C). In contrast, γ-H2AX foci were induced in all three cultures after exposure to IR. The expression of functional ATM in cells of the YZS line, an isogenic derivative of the EBS line, restored the formation of DDX1 IRIF, albeit at lower numbers than those observed in cells that naturally express ATM (Fig. 3D). These results indicate a role for ATM in the response of DDX1 to IR.

When cells are exposed to IR, pATM accumulates at DNA DSBs (3). The coimmunostaining of irradiated HeLa cells with anti-DDX1 antibody and anti-pATM Ser1981 antibody revealed extensive colocalization of DDX1 and pATM IRIF (Fig. 3E). The analysis of three-dimensionally reconstructed z-stack images showed that 91% of DDX1 foci colocalized with pATM foci in irradiated cells, whereas 46% of pATM foci colocalized with DDX1 foci, in support of the conclusion that DDX1 foci accumulate at a subset of DNA DSBs.

Coimmunoprecipitation of DDX1, ATM, and the MRN complex. The colocalization of DDX1 and pATM IRIF suggests that DDX1 and pATM may reside in the same cellular complex. To test this possibility, we carried out reciprocal coimmunoprecipitations of DDX1 and ATM. Using anti-DDX1 antibody, we were able to immunoprecipitate endogenous ATM (Fig. 4A, top panel). Similarly, DDX1 coimmunoprecipitated with endogenous ATM when anti-ATM antibody was used (Fig. 4B). The amounts of coimmunoprecipitated DDX1 and ATM in control and irradiated cells were similar, suggesting that DDX1 and ATM can exist in the same complex in the absence of DNA damage. The proportion of ATM that coimmunoprecipitated with DDX1 (and vice versa) was small (~1%), indicating either a weak interaction between the two proteins or an association limited to a subset of DDX1 and ATM proteins. These results are similar to those reported by others upon the coimmunoprecipitation of ATM with known binding partners of ATM, such as Nbs1 and BRCA1 (18). ATR was not detected in the DDX1 immunocomplex, providing further evidence that ATR is not involved in IR-induced DDX1 relocalization (Fig. 4A).

In vitro and in vivo phosphorylation of DDX1. The colocalization and coimmunoprecipitation of DDX1 and ATM raise the possibility that DDX1 is a substrate of the ATM kinase. Sequence analysis of DDX1 reveals five consensus S/TQ motifs that may be phosphorylated by ATM (31). We therefore tested whether ATM was able to phosphorylate DDX1 in vitro. Endogenous ATM was immunoprecipitated from HeLa cells and incubated with recombinant DDX1 in the presence of [γ-32P]ATP. As shown in Fig. 4C, DDX1 was phosphorylated by ATM in vitro. DDX1 phosphorylation was strictly dependent on Mn2+ and significantly inhibited by wortmannin (Fig. 4C), consistent with wortmannin’s inhibition of ATM kinase activity.
activity and the requirement for Mn²⁺ for ATM kinase activity (5, 15). To further document the role of ATM in DDX1 phosphorylation, we carried out in vitro phosphorylation experiments with extracts from BT (ATM-proficient) and L3 (ATM-deficient) cells. As shown in Fig. 4D, recombinant DDX1 was phosphorylated by ATM immunoprecipitates derived from BT cells but not those from L3 cells.

Next, we examined the in vivo phosphorylation status of DDX1 as a consequence of exposure to IR. HeLa cells were metabolically labeled with 32P, subjected to 5 Gy of IR, and lysed 1 h later. Endogenous DDX1 was immunoprecipitated from the cell lysates, electrophoresed through an SDS-PAGE gel, and transferred onto nitrocellulose membranes. Although there was a basal level of phosphorylated DDX1 prior to IR, phosphorylation was significantly increased (>3-fold) in response to IR (Fig. 4E). The treatment of cells with wortmannin prior to irradiation greatly reduced levels of phosphorylated DDX1 (Fig. 4E). These results suggest a link between DDX1 phosphorylation and DDX1 relocalization in response to IR.

**DDX1 IRIF and RNA transcription.** DEAD box proteins are putative RNA helicases that alter RNA secondary structure. These proteins bind RNA and have RNA-dependent ATPase and ATP-dependent RNA-unwinding activities. The accumulation of a putative RNA-unwinding protein at sites of DNA DSBs suggests a connection between RNA and DNA DSB repair. To address the possibility that DDX1 may be associated with sites of active transcription at DNA DSBs, we carried out coimmunostaining with anti-RNA polymerase II (active-form) antibodies and anti-DDX1 antibodies. There was no accumulation of RNA polymerase II at DDX1 IRIF, with RNA polymerase II showing a speckled staining pattern throughout the nucleus (see Fig. S2A in the supplemental material). Furthermore, neither the phosphorylated (active) nor the unphosphorylated (inactive) form of RNA polymerase II coimmunoprecipitated with DDX1 before or after IR (see Fig. S2B in the supplemental material). Attempts to correlate DDX1 IRIF with newly synthesized RNA by labeling with 5-fluorouridine (5-FUrd) for 15 min prior to irradiation were equally unsuc-
cessful, with anti-BrdU antibody staining showing a speckled pattern throughout the nucleus, with intense staining in the nucleolus (see Fig. S3 in the supplemental material).

As an alternative strategy to address a possible link among DDX1, transcription, and DNA DSBs, we tested the effect of actinomycin D on the formation of DDX1 IRIF. Actinomycin D targets transcription templates by preferentially intercalating into d(GpC) at “transcription bubbles,” thus blocking transcription. At 6 μg/ml, actinomycin D inhibits transcription by RNA polymerases I, II, and III. HeLa cells were treated with 6 μg/ml of actinomycin D for 30 min prior to exposure to 5 Gy of IR. Cells were then immunostained with anti-DDX1 and anti-γ-H2AX antibodies. Whereas γ-H2AX foci were abundant in actinomycin D-treated irradiated cells, anti-DDX1 immunostaining revealed a speckled pattern without discrete foci (Fig. 5A). The absence of DDX1 IRIF suggests a dependence on RNA transcription.

We also tested whether DDX1 IRIF could form during mitosis, a stage of the cell cycle when there is no RNA synthesis (29, 53). Ten hours after being released from a thymidine block (with ~60% of cells in mitosis), cells were irradiated and immunostained. As shown in Fig. 5B, DDX1 IRIF were not detected in mitotic cells, although IR-induced γ-H2AX IRIF were readily apparent in these cells. Note that the numbers and appearance of DDX1 IRIF are completely normal in the two interphase cells flanking the mitotic cell in Fig. 5B.

RNase H treatment dissociates DDX1 from the IRIF. To further investigate a possible role for RNA in the formation of DDX1 foci in response to IR, we tested the effects of DNase I and RNases A and H on the formation of DDX1 IRIF. HeLa cells exposed to 5 Gy of IR were permeabilized with 2% Tween 20 and incubated with DNase I, RNase A, or RNase H for 15 min (Fig. 6). The cells were then fixed and immunostained with anti-γ-H2AX and anti-DDX1 antibodies. Consistent with the data in a previous report (61), DNase I digestion abolished IR-induced γ-H2AX foci. DNase I-treated cells were also completely devoid of DDX1 IRIF (Fig. 6B), indicating that both γ-H2AX and DDX1 IRIF depend on the presence of chromosomal DNA for their proper localization. RNase A, which digests single-stranded RNAs, had no effect on IR-induced γ-H2AX and DDX1 foci (Fig. 6C). However, RNase H treatment resulted in the dissociation of DDX1 IRIF, but not γ-H2AX foci, in ~80% cells (Fig. 6D). As RNase H specifically degrades RNA molecules in RNA-DNA duplexes, the dissociation of DDX1 from the IRIF upon RNase H digestion suggests that RNA-DNA duplex structure is required for the maintenance of DDX1 at the DNA DSB sites.

**DDX1 unwinds RNA-DNA and RNA-RNA duplexes in vitro.** The results from the RNase H treatment suggest that DDX1 at IRIF is involved in binding, unwinding, or otherwise altering RNA-DNA duplex structures. To investigate a possible role for DDX1 in RNA-DNA unwinding, we carried out in vitro unwinding assays using an RNA-DNA hybrid consisting of a 41-nucleotide (nt) RNA strand and a 29-nt DNA strand, with 29 bp of double-stranded structure and a 12-nt RNA overhang at the 5′ end (Table 1). R41(+)D29(−) (where * denotes the 32P-labeled strand) was purified from an acrylamide gel and...
FIG. 7. Characterization of DDX1 RNA-DNA-unwinding activity. (A) The RNA-DNA duplex R41(+)D29(−) was generated by the annealing of R41(+) with 5′-end 32P-labeled D29(−) as described in Materials and Methods. Fifty femtomoles of duplexes was incubated with
incubated with recombinant DDX1. While addition of DDX1 resulted in a band that migrated faster than the RNA-DNA hybrid alone (Fig. 7A, compare lanes 1 and 3), this band migrated at a considerably slower rate than the band corresponding to single-stranded D29 (Fig. 7A, compare lanes 2 and 3), suggesting RNase activity rather than unwinding activity. There was no difference in band migration whether ATP was included in the reaction mixture (Fig. 7A, lane 7), indicating that the nuclease activity is ATP independent. DDX1 was inactivated by boiling, resulting in mostly intact R41/D29 products (Fig. 7A, lane 8). Next, we tested the effects of different nucleotides (ATP, GTP, CTP, UTP, ADP, GDP, and AMP, as well as nonhydrolyzable ATP-γ-S) on DDX1 activity. Data obtained with CTP, GDP, AMP, and ATP-γ-S were similar to those obtained in the presence of ATP or the complete absence of nucleotides (Fig. 7A, lane 4, and data not shown). Both GTP and UTP had an inhibitory effect on DDX1 nuclease activity, with GTP being the stronger inhibitor (Fig. 7A, lane 6, and data not shown). However, the most striking effect was that of ADP, with virtually complete unwinding of the R41/D29 duplex observed in the presence of this nucleotide (Fig. 7A, lane 5). To ensure that these enzymatic activities were not caused by RNase contamination of our DDX1 preparations, we tested the effect of RNase A on the R41/D29 substrate. As shown in Fig. 7A (lanes 9 to 16), a single product migrating approximately halfway between the R41/D29 and D29 bands was observed under all conditions tested. As RNase A cleaves single-stranded RNAs at C and U residues, this product likely represents the R29/D29 heteroduplex.

The banding patterns observed upon the incubation of DDX1 with R41/D29 suggest two activities: an ATP-independent, GTP-inhibited nuclease activity responsible for removing the 5′ single-stranded RNA overhang and an ADP-dependent RNA-DNA-unwinding activity. To further address DDX1 nuclease activity, we incubated DDX1 with single-stranded R29(−)* under different buffer conditions. As shown in Fig. 7B, DDX1 effectively digested this substrate in an ATP-independent manner (lanes 2 and 5). R29 degradation was inhibited by GTP (Fig. 7B, lane 4), strictly dependent on Mg** (Fig. 7B, lane 6), and abolished when DDX1 was boiled for 2 min (Fig. 7B, lane 7). Incubation with ADP resulted in the formation of smaller digestion products than those observed with ATP (Fig. 7B, lane 3). In comparison, the incubation of R29(−)* with RNase A resulted in the complete disappearance of R29, with the same rapidly migrating band observed under all conditions tested (Fig. 7B, lanes 8 to 14). The incubation of DDX1 with the DNA counterpart of R29, D29(−)* (Table 1), revealed no DNase activity under any of the conditions tested (Fig. 7B, far-right panel).

To further characterize the RNA-DNA-unwinding activity of DDX1, we generated two heteroduplexes: (i) D41(+)/R29(−), with the same nucleotide sequences as the R41(+)/D29(−) duplex but with a 12-nt DNA overhang at the 5′ end (Fig. 7C and E), and (ii) blunt-ended D29(+)/*R29(−) (Fig. 7D and F). When the DNA strands were labeled, we observed effective unwinding of both the D41/R29 (Fig. 7C) and D29/R29 (Fig. 7D) duplexes in the presence of DDX1 and ADP but not under any of the other conditions tested. When the RNA strands were labeled, significant reductions in the levels of both the D41(+)/*R29(−)* and D29(+)/*R29(−)* duplexes were observed upon incubation with DDX1 in the presence of ADP. (Fig. 7E and F, lanes 4). The absence of single-stranded R29(−)* products in lanes 4 in Fig. 7E and F reflects the single-stranded-RNA degradation activity of DDX1 once the double-stranded molecules had been unwind. In contrast to the RNA-DNA duplex with an RNA overhang (Fig. 7A), where the RNA overhang was digested in a nucleotide-independent manner by DDX1, the D41(+)/*R29(−)* duplex remained intact under all conditions tested except when incubated in the presence of ADP. These observations are in keeping with the inability of DDX1 to degrade single-stranded DNA. Together, the results obtained with all three RNA-DNA duplexes tested indicate that overhangs are dispensable for DDX1-mediated unwinding.

As DEAD box proteins are classically defined as RNA helicases or RNA-unwinding/destabilizing proteins, we tested the effect of DDX1 on an RNA-RNA duplex. R41(+)/*R29(−)* was generated by annealing R41(+) with 32P-labeled R29− (Table 1). The incubation of this substrate with DDX1 produced results similar to those observed with the R41(+)/*D29(−)* duplex: i.e., largely ATP-independent degradation of the 5′ RNA overhang (Fig. 8A, lanes 3 and 6) and complete unwinding of the 29-bp double-stranded RNA in the presence of ADP, followed by the degradation of the single-stranded products (Fig. 8A, lane 4). In comparison, RNase A digestion of the RNA-RNA duplex generated similarly migrating bands [representing double-stranded R29(+)/*R29(−)*] under all conditions tested (Fig. 8A, lanes 9 to 12). In Fig. 8B, we show that DDX1 cannot degrade or unwind a DNA-DNA duplex prepared by hybridizing D41(+) to 32P-labeled D29(−)* (Table 1). DNA-DNA duplexes with as few as 16 bp of double-stranded structure could not be unwound by DDX1 (data not shown).

**DISCUSSION**

**Recruitment of DDX1 to sites of DNA DSBs.** To this day, DEAD box proteins remain a puzzling family of proteins. In spite of having reasonably well characterized biochemical functions (as ATPases and RNA helicases), the great majority of
these proteins have no clear biological roles. Few natural RNA substrates for DEAD box proteins have been identified, perhaps reflecting transient interactions with a wide spectrum of RNAs or simply our lack of understanding of the complex environment under which these proteins operate. Here, we demonstrate that DDX1 is part of the intricate machinery that is rapidly engaged when a cell is exposed to agents that cause DNA DSBs. Similar to pATM and γ-H2AX, DDX1 is recruited to sites of DNA DSBs within minutes of the cell’s being exposed to IR, suggesting a requirement for DDX1 early in the DNA damage response. DDX1 IRIF are also observed when cells are exposed to the radiomimetic drug bleomycin. In contrast, exposure to UV and cisplatin does not initially lead to DDX1 IRIF formation, although DDX1 IRIF form at later time points, presumably at sites of unrepaired DNA damage resulting in DSBs. These combined data suggest a role for DDX1 that is specific to DNA DSBs.

**Role of ATM in DDX1 recruitment to sites of DNA DSBs.** The cellular response to DNA DSBs involves a well-orchestrated series of signaling events, from checkpoint activation and cell cycle arrest to the repair of the damaged DNA and the resumption of cell cycle progression. ATM is a key player in damage sensing, signal transduction, and the coordination of the diverse pathways that are part of the DSB response (33, 54). The main mechanism by which ATM relays damage signals is the direct phosphorylation of its substrate proteins. The complete absence of DDX1 IRIF in ATM-defective cells, combined with the fact that at least some DDX1 exists in a complex with ATM, suggests that DDX1 may be a substrate of ATM. In support of this possibility, we have shown that DDX1 is phosphorylated by endogenous ATM in vitro and that DDX1 phosphorylation is dependent on Mn$^{2+}$ and inhibited by wortmannin, both characteristics of ATM kinase phosphorylation (5). Furthermore, DDX1 phosphorylation is induced in vivo upon

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**FIG. 8.** DDX1 unwinds RNA-RNA but not DNA-DNA duplexes. (A) The RNA-RNA duplex R41(+)/R29(−)* was generated by annealing R41(+) with 5′-end $^{32}$P-labeled R29(−). Fifty femtomoles of the substrate was incubated with either 0.3 μg of recombinant DDX1 (lanes 3 to 6) or 0.1 U of RNase A (lanes 9 to 12) in the presence of 1 mM ATP, ADP, or GTP as indicated. The R29(−)* oligonucleotide in lanes 1 and 7 and the R41(+)/R29(−)* duplex in lanes 2 and 8 were incubated in unwinding buffer without protein. ATP was omitted from reaction mixtures loaded into lanes 6 and 12. All reactions were carried out for 20 min at 37°C prior to electrophoresis in a 12% polyacrylamide gel, followed by autoradiography. R, RNA; D, DNA; +, with; −, without. (B) Fifty femtomoles of D41(+)/D29(−)* was incubated with 0.3 μg of recombinant DDX1 (lanes 2 to 6) at 37°C for 20 min, and the mixture was then subjected to electrophoresis and autoradiography.
the exposure of cells to IR, and this response is inhibited by wortmannin. These data are compatible with the direct mediation of the recruitment of DDX1 to DSBs through ATM phosphorylation. However, we cannot exclude the possibility that the recruitment of DDX1 to these sites is an indirect consequence of the ATM-mediated phosphorylation of other proteins. Two recent studies involving systematic searches for ATM substrates in DNA DSB-induced cellular responses have yielded hundreds of putative novel ATM interactors (39, 40). It is noteworthy that a number of DEAD box proteins (DDX6, DDX17, DDX18, and DDX47) were identified in these screens, suggesting a broad-scope relationship between DEAD box proteins and ATM.

**DNA/RNA helicases in the DNA damage response.** DNA helicases implicated in the DNA damage response include RecQ DNA helicases BLM, WRN, and RECQL4, as well as a DEAD box helicase called senataxin (57). BLM, defective in individuals with Bloom’s syndrome, interacts directly with ATM and exhibits ATM-dependent phosphorylation upon IR (9). At sites of DNA breaks, BLM disrupts the Rad51-single-stranded-DNA filament, a species that promotes HR (13). As BLM can also stimulate DNA repair, it has been suggested previously that BLM plays a dual function in promoting and inhibiting HR (13).

A few reports indicate a possible role for DEAD box proteins in the DNA DSB response. For example, Ghabrial and Schupbach (23) suggested that Vasa (DDX4) in *Drosophila melanogaster* may be a target of a meiotic checkpoint that is activated by the accumulation of DNA DSBs. In addition, the results of studies using human p68 (DDX5) indicate that this DEAD box protein may be required for p53-regulated gene expression upon exposure to etoposide, a DNA DSB-inducing agent (8). The data presented here provide evidence that DDX1 plays an integral role in the cellular response to DNA DSBs. However, only a subset of DNA DSBs are targeted by DDX1, as evidenced by the fact that only 29% of γ-H2AX IRIF colocalized with DDX1 IRIF. Strikingly, RNase H and DNase I treatment, but not RNase A treatment, eliminated DDX1 IRIF. As RNase H removes RNA from RNA-DNA double-stranded molecules, these results suggest a requirement for both DNA and RNA at sites of DDX1 IRIF. The involvement of RNA at DNA DSBs has been reported previously by Pryde et al. (44), who demonstrated that treatment with RNase A, but not RNase H, dissociates 53BP1 from IRIF. These authors proposed that RNA is required for 53BP1 retention at DSBs. The results obtained with 53BP1 and DDX1 suggest different types of nucleic acid interactions among the proteins recruited at DNA DSBs.

**Recruitment of DDX1 to sites of DNA DSBs containing RNA.** There are an estimated 5,000 to 8,000 actively transcribed genes in the nucleus of a cycling HeLa cell (17). These genes are believed to be transcribed in foci enriched with RNA polymerase II, called transcription factories (28). Although the effect of DSBs on transcription remains poorly understood, one would expect either stalling or dismantling of the transcriptional machinery in the general vicinity of DNA DSBs. A communostaining analysis did not reveal any colocalization of RNA polymerase II and DDX1, either before or after irradiation. Furthermore, our 5-FUrd incorporation analysis indicates that there is no enrichment of newly synthesized RNA at DDX1 IRIF. As the limit of detection of the confocal microscopy is ~0.2 μm, thus requiring thousands of fluorescently labeled molecules for detection, the only conclusion one can reach from these studies is that there is no accumulation of large quantities of newly synthesized RNA at DDX1 IRIF.

In keeping with a need for RNA at DDX1 IRIF, actinomycin D, a drug that intercalates into double-stranded DNA at the transcription bubble and interferes with RNA elongation by all three RNA polymerases, effectively inhibited DDX1 (but not γ-H2AX) IRIF formation. Others have shown that RNA is released from its site of transcription within minutes of being exposed to actinomycin D (19). Interestingly, actinomycin D itself can induce the formation of γ-H2AX foci and has been proposed to distort DNA structures at transcriptionally active sites (42). Of note, cells in mitosis, the only stage of the cell cycle when transcription is silent, did not undergo DDX1 IRIF formation. Together with the RNase H results discussed earlier, our data support a role for RNA in the recruitment or retention of DDX1 at DNA DSBs.

**DDX1 can unwind RNA-DNA substrates.** In a previous study, recombinant DDX1 was unable to unwind RNA-RNA duplexes containing either 10 or 14 bp of complementary sequence (16). Here we have shown that DDX1 can unwind both RNA-RNA and RNA-DNA duplexes containing 29 bp of double-stranded structure in an ADP-dependent manner. Furthermore, in addition to unwinding activity, DDX1 has an ATP-ADP-independent RNase activity toward single-stranded RNA that is inhibited by GTP and requires Mg2⁺. Although no other DEAD box proteins with unwinding and RNase activities have been described in the literature to date, the WRN DNA helicase has been reported to have a 5’→3’ exonuclease activity that removes one DNA strand in a DNA-DNA duplex and the RNA strand in an RNA-DNA duplex (58). In contrast to DDX1, WRN does not digest single-stranded RNA (or DNA), and its exonuclease activity is dependent on unwinding.

Other DEAD box proteins have been shown to have both RNA-RNA- and RNA-DNA-unwinding activities. For example, elf4A can unwind both RNA-RNA and RNA-DNA duplexes containing 12 to 17 bp of double-stranded structure in the presence of ATP (49). Yeast Has1p, a DEAD box protein involved in 18S rRNA maturation, unwinds RNA-DNA duplexes containing 16 to 17 bp of double-stranded structure, also in an ATP-dependent manner (46). While most DEAD box proteins unwind RNA-RNA or RNA-DNA duplexes in an ATP-dependent manner, a requirement for ADP is not without precedent. For example, the RNA-unwinding and -annealing activities of yeast DEAD box protein DED1 have recently been shown to be controlled by relative ATP and ADP concentrations (62). Although ADP itself does not allow DED1 to unwind RNA, a higher ADP concentration promotes annealing over unwinding. A second yeast DEAD box protein, Dbp5, appears to function in a manner analogous to that of Ran, shifting between ATP-bound and ADP-bound states (59). The ADP-bound form of Dbp5 is required for RNP remodeling, with ADP-bound Dbp5 having a different conformation from that of ATP-bound Dbp5.

**Role of DDX1 at DNA DSBs.** The two main pathways involved in DNA DSB repair are error-prone nonhomologous end joining and HR, which involves template-guided repair using undamaged homologous sequences. Although few stud-
ies have addressed heterogeneity in the repair of DNA DSBs, one could surmise that it would be of benefit to the cell to use a template-guided mechanism for the repair of transcriptionally active genes. In fact, it has been suggested previously that only DSBs associated with transcription can induce the chromosomal exchange events related to HR (45). Template-guided repair requires the unwinding of the DNA double strands to allow the copying of the undamaged DNA into the damaged DNA strand. Unwound DNA may be particularly susceptible to binding with complementary transcripts. Conversely, RNA released from transcription factories as the result of DNA DSBs may be particularly prone to pairing with its complementary genomic DNA. DDX1 may therefore play a role in clearing DNA of opportunistic DNA-RNA interactions resulting from DSBs. In this context, DDX1 may play an active role in template-guided repair or may simply facilitate the replacement of the transcription machinery with the DNA repair machinery.

DDX1 accumulated at a subset of DNA DSBs which invariably (that is, in the case of >90% of DDX1 IRIF) contained pATM. As DDX1 IRIF did not form in the absence of ATM, we propose the following model. DDX1 is phosphorylated and recruited to DNA DSBs by ATM. The presence of DNA-RNA duplex structures at DSB sites is critical for the retention of DDX1. Relative enrichment with and/or the accessibility of ADP at these sites activates DDX1 unwinding activity, likely through a conformational alteration of DDX1 as observed previously for other ADP binding proteins (4, 59). The RNA-DNA-unwinding activity of DDX1, combined with its RNase activity, ensures the complete removal and degradation of RNA from these sites. Thus, we propose a role for DDX1 in RNA clearance from sites of DSBs to facilitate repair. In support of this model, a role in RNA clearance has recently been proposed for Drosophila DEAD protein DDx5 (p68), specifically to remove RNA from transcribed genes in order to reset the chromatin to a transcriptionally inactive state (14).

In summary, we have shown that DDX1 rapidly accumulates at sites of DNA DSBs in an ATM-dependent manner upon exposure to IR. DDX1 has ADP-dependent RNA-DNA- and RNA-RNA-unwinding activities, as well as ADP/ATP-independent RNase activity toward single-stranded RNA. Our data suggest that the presence of RNA at DNA DSBs is a prerequisite for DDX1 recruitment at these sites. We propose that the combined RNase and RNA-DNA-unwinding activities of DDX1 provide a powerful tool for the rapid clearance of RNA from sites of DNA DSBs, thus facilitating DSB repair. Determining whether DDX1 marks RNA-containing DNA DSB sites for special repair, e.g., by a template-guided mechanism, will be the subject of future investigations.

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REFERENCES


