

Alpha Thalassemia/Mental Retardation Syndrome X-linked Gene Product ATRX Is Required for Proper Replication Restart and Cellular Resistance to Replication Stress*

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Justin Wai-Chung Leung, Gargi Ghosal, Wenqi Wang, Xi Shen, Jiadong Wang, Lei Li, and Junjie Chen¹

From the Department of Experimental Radiation Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

Background: ATRX is involved in genome maintenance.

Results: Somatic ATRX knock-out cells displayed hypersensitivity to hydroxyurea (HU) and defects in checkpoint activation and replication restart.

Conclusion: ATRX is required for replication stress tolerance, proper checkpoint activation, and replication restart at stalled replication forks.

Significance: These results reveal an unanticipated role of ATRX in maintaining genomic stability upon replication stress.

Alpha thalassemia/mental retardation syndrome X-linked (ATRX) is a member of the SWI/SNF protein family of DNA-dependent ATPases. It functions as a chromatin remodeler and is classified as an SNF2-like helicase. Here, we showed somatic knock-out of ATRX displayed perturbed S-phase progression as well as hypersensitivity to replication stress. ATRX is recruited to sites of DNA damage, required for efficient checkpoint activation and faithful replication restart. In addition, we identified ATRX as a binding partner of MRE11-RAD50-NBS1 (MRN) complex. Together, these results suggest a non-canonical function of ATRX in guarding genomic stability.

DNA replication is tightly regulated to ensure accurate duplication of genetic information. In response to DNA damage or replication stress, replication forks stall in front of DNA lesions. A stalled replication fork is arrested, but is still capable of resuming replication once the lesion is repaired or bypassed. It is believed that DNA damage or stalled replication forks activates ataxia-telangiectasia-mutated (ATM)² and Rad3-related (ATR) kinases (1, 2). Activated ATM and ATR phosphorylate downstream targets including checkpoint kinases, which are able to protect the stalled replication forks and prevent the fork from collapsing. In the absence of proper DNA damage repair or replication checkpoints, stalled replication forks will dissociate, leading to the generation of DNA double strand

breaks (DSBs), genomic instability, and eventually tumor development.

SNF2 family proteins are implicated in a wide range of cellular functions including transcription regulation, DNA repair, and mitotic recombination (3). Recently, several groups including our laboratory identified that some SNF2 family members, including INO80, SMARCL1, and ZRANB3, play important roles in DNA damage response and replication stress tolerance (4–9). As a matter of fact, SWI/SNF family proteins are implicated in cancers and other human syndromes with mental retardation (MR) and genomic instability, including alpha thalassemia/mental retardation syndrome X-linked (ATRX) (10, 11).

ATRX was originally identified in alpha thalassemia patients with urogenital abnormalities and facial dysmorphism (12). The ATRX protein contains several highly conserved domains, including the N-terminal ATRX-DNMT3-DNMT3L (ADD) domain and the C-terminal SNF2 ATPase and HELIC domain that confer helicase activity (Fig. 1A). Many of the SNF2 proteins use the energy generated from ATP hydrolysis to translocate along DNA and thereby remodel DNA structures or DNA-protein interactions. Most of the ATRX mutations were found in the ADD domain and the helicase domain, which is highly associated with the ATRX syndrome (13, 14).

ATRX was initially suggested to be involved in the regulation of globin gene expression (12, 15). Mutations of ATRX not only induce alpha thalassemia, but also mental retardation with facial abnormalities and gonadal dysgenesis. However, later studies suggest that ATRX has many important functions in the control of telomere stability and chromosome cohesion (16–18). ATRX can bind directly to double-stranded and structured DNA *in vitro* (19, 20). Recent studies reported that ATRX is localized at G-rich tandem repeats (TRs), which potentially form G4-quadruplex at telomeres and euchromatin (20). ATRX interacts strongly with DAXX to deposit histone H3.3 specifically at telomeres to maintain telomere integrity (17, 21, 22). In addition, ATRX is implicated in mitotic and meiotic regulation. For example, in ATRX knockdown HeLa cells, a subset of meta-

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¹ Recipient of an Era of Hope Scholar award from the Dept. of Defense (W81XWH-05-1-0470) and a member of the M.D. Anderson Cancer Center (CA016672). To whom correspondence should be addressed: Department of Experimental Radiation Oncology, Unit 66, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Room Y3.6006, Houston, TX 77030. Tel.: 713-792-4863; Fax: 713-794-5369; E-mail: jchen8@mdanderson.org.

² The abbreviations used are: ATM, ataxia-telangiectasia-mutated; ATRX, alpha thalassemia/mental retardation syndrome X-linked; HU, hydroxyurea; IR, irradiation; APH, aphidicolin; MMC, mitomycin C; PML-NB, promyelocytic leukemia nuclear body.

phase chromosomes failed to condense and the sister chromatids lacked centromeric cohesion, which coincided with spindle checkpoint activation in these cells (16). Presence of micronuclei following G1 entry was also observed in ATRX knockdown cells (16). These and other observations all indicate that ATRX deficiency may lead to genomic instability (11, 16, 23–25). However, it is unknown whether or not ATRX is directly involved in DNA damage response.

Here, we sought to uncover the functions of ATRX in DNA damage response. By knocking out ATRX gene somatically, we were able to show that ATRX is involved in replication stress tolerance. We also demonstrated that ATRX is recruited to the DNA damage site. We showed that ATRX is required for proper S phase progression and replication restart. Moreover, we uncovered MRE11-RAD50-NBS1 (MRN) complex as ATRX-associated proteins *in vivo*. Taken together, our findings reveal a novel role for ATRX in the maintenance of genomic stability.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—HCT116 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. HEK293T cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and kept at 37 °C in a humidified incubator containing 5% CO₂.

Plasmids—Full-length ATRX cDNA was a generous gift from Dr. Yang Shi in Harvard Medical School. The cDNAs were cloned into gateway compatible pDONR 201 and subcloned into an expression vector harboring N-terminal SFB tags for expression in mammalian cells.

Antibodies, Mutagens, and Chemical Inhibitors—The primary antibodies used in this study were purchased as follows: polyclonal anti-ATR_X antibody, monoclonal anti-PML antibody, monoclonal anti-Chk1 antibody were from Santa Cruz Biotechnology, Inc.; monoclonal anti-Flag antibody, monoclonal anti-BrdU antibody were from Sigma Aldrich; polyclonal anti-pHistone3 (S10) antibody, polyclonal anti-Histone3 antibody were from Millipore; polyclonal anti-bromodeoxyuridine antibody was from Abcam, Inc; rhodamine-conjugated anti-mouse IgG, and FITC-conjugated anti-rabbit IgG were from Jackson ImmunoResearch, Inc.; Alexa Fluor 488-conjugated anti-rat antibody was from Molecular Probes, Inc.; polyclonal anti-γH2AX were produced in house used as previously described (26). Thymidine, hydroxyurea, and aphidicolin were purchased from Sigma Aldrich and used at the indicated concentrations.

Somatic Knock-out of ATR_X—Generation of somatic knock-out cells was performed as previously described using adeno-associated virus-based strategy (27). Briefly, homology arms targeting exon 5 were cloned into the targeting vector. The targeting adeno-associated viruses were packaged in 293T cells by transfecting 3 μg of the targeting vector together with pHelper and pRC plasmids. Viruses were harvested at 72 h after transfection. Human colon cancer cell line HCT116 was infected for 48 h and selected with geneticin for 20 days. The

targeted allele was screened by genomic PCR. The protein expression was examined by Western blot analysis.

Cell Survival Assay—ATR_X-deficient HCT116 cells and wild-type HCT116 cells were exposed to differing doses of irradiation (IR) or aphidicolin (APH), hydroxyurea (HU), mitomycin C (MMC) for 24 h. Cells were then washed free of drugs and incubated in fresh medium for another 14 days. The cells were then fixed and stained with 0.5% crystal violet in 20% ethanol. Colonies containing more than 50 cells were counted and normalized for plating efficiencies.

Immunoblotting Analysis—Cells were lysed in NETN (150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl, pH 8.0, 0.5% Nonidet P-40 (v/v)) containing protease inhibitors. For immunoblotting analysis, proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, incubated overnight in primary antibodies as indicated, followed by 1 h of incubation in horseradish peroxidase-conjugated secondary antibodies.

Immunofluorescence Staining—Cells were cultured on coverslips and fixed in 3% paraformaldehyde and permeabilized in 0.5% Triton solution. Cells were then incubated with indicated primary antibodies for 30 min, washed, and incubated with secondary antibodies for 30 min. Cells were then counterstained with DAPI and mounted with anti-fade solution.

Double Thymidine Block and Fluorescence-activated Cell Sorting (FACS) Analysis—For BrdU incorporation analysis, cells were synchronized with 1 mM thymidine and released according to standard protocol (28). The cells were pulsed with 10 μM BrdU for 30 min prior to harvesting. Cells were trypsinized and fixed in 70% ethanol overnight. Cells were then washed and treated with 2 M HCl for 30 min. Cells were washed again with PBS three times and stained with primary BrdU antibody followed by goat anti-mouse FITC-conjugated secondary antibody. For pH3 (Ser-10) staining, cells were incubated with 2 mM HU for 24 h. After extensive washing, cells were incubated in fresh medium containing nocodazole; the proportion of mitotic cells at indicated time points was measured. For FACS analysis, fixed cells were stained with propidium iodide (4 μg/ml), treated with RNase (2 μg/ml) at room temperature for 30 min. The samples were then analyzed in flow cytometer using FACS Flow Jo software.

Chromatin Fractionation—Preparation of chromatin fractions was described previously (26). Briefly, cells were harvested at indicated times after treatment. The soluble fraction was extracted with NETN buffer at 4 °C for 10 min followed by centrifugation with 15,000 rpm for 30 min. The pellet was resuspended in 0.2 M HCl. The soluble fraction was neutralized with 1 M Tris-HCl, pH 8.0 before further analysis.

Laser-induced Micro-irradiation—Cells were seeded on 35-mm glass bottom dishes (MatTek Corp.) and incubated overnight. Cells were micro-irradiated with a Micropoint Ablation System (Photonics Instruments, St. Charles, IL) with the laser output set to 35%. Average of 20 cells were micro-irradiated and further cultured for 6 h prior to immunostaining and then visualized with a Nikon Elipse TE2000-U inverted microscope (29).

DNA Fiber Analysis—Cells were labeled with 20 μM IdU for 15 min followed by 20 μM thymidine for 15 min to chase out the IdU. After pre-labeling, the cells were treated with 2 mM HU for

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2, 6, or 24 h. Cells were then washed with PBS and incubated with 100 μ M CldU for 15 min.

For DNA fiber length measurement, cells were synchronized with the addition of 100 nM nocodazole in culture media. After 8 h of incubation, rounded mitotic cells were shaken off and washed three times with PBS. The cells were then replated in fresh medium. 9 h after replating, when the majority of the cells were in early S-phase, cells were labeled with IdU for 15 min followed by 6 h of incubation of 4 mM HU. Cells were then washed with PBS and pulsed with 100 μ M CldU for indicated time points. DNA spreads were prepared as previously described (30). DNA fiber staining was performed as previously described (25). The significance of the difference between the means was determined by Student's *t* test.

Tandem Affinity Purification—HEK293T cells stably expressing SFB-tagged ATR_X were established. Expression of exogenous ATR_X was confirmed by Western blotting and immunofluorescence staining. Tandem affinity purification was conducted at 4 °C. Cells were lysed in NETN buffer for 30 min. The lysates were cleared by centrifugation at 14,000 rpm for 30 min and rocked with streptavidin-conjugated beads (Amersham Biosciences) for 2 h. The immunocomplexes were washed with NETN for three times and eluted with 2 mg/ml biotin. The eluent was incubated with S-protein agarose beads (Novagen) for 2 h. The beads were then washed, eluted, and analyzed by Taplin Mass Spectrometry Facility at Harvard Medical School (Boston, MA.).

Co-precipitation Assay—HEK293T cells were harvested in NETN buffer containing protease inhibitors 24 h after transiently transfected with plasmids encoding SFB-tagged proteins. Crude lysates were cleared by centrifugation at 14,000 rpm for 30 min at 4 °C. Cell lysates were then incubated with streptavidin beads for 2 h at 4 °C. The complexes were washed with NETN for three times, eluted by boiling in 1 \times Laemmli buffer, and then subjected to Western blotting analysis.

RESULTS

Generation of ATR_X Somatic Knock-out Cells—To study ATR_X gene function in DNA damage response, we employed a loss-of-function approach by generating somatic ATR_X knock-out cell lines. We designed a targeting construct and specifically deleted exon 5 of the ATR_X gene to generate a transcription frameshift (Fig. 1, *B* and *C*). Absence of ATR_X expression in knock-out cells was confirmed by PCR, Western blotting as well as immunofluorescence staining (Fig. 1, *D–F*).

ATR_X is predominately localized in nuclei with nuclear bodies that co-localize with promyelocytic leukemia nuclear bodies (PML-NBs). PML-NBs contain PML protein and are associated with various nuclear functions including DNA repair (31). Notably, ATR_X-deficient cells consistently showed bigger and brighter PML bodies than those observed in parental wild-type HCT116 cells (Fig. 1*G*). This observation is consistent with a previous report (32). These larger PML-NBs are speculated to be the ALT-associated PML-NBs, which are present in ALT-positive cells and correspond to telomere location in these cells (33).

ATR_X Is Involved in Replication Stress—Because of the genomic instability reported in ATR_X knockdown cells as well

as in ATR_X-deficient ALT cells (11, 16), we speculated that ATR_X may play a role in DNA damage response. To test this hypothesis, we treated wild-type and two independent clones of ATR_X-deficient cells with different genotoxic agents. As shown in Fig. 2*A*, ATR_X-deficient cells are more sensitive to HU and APH treatment. However, ATR_X-deficient cells did not show any increased sensitivity to MMC or irradiation (IR) (Fig. 2*A*), suggesting that ATR_X may be specifically involved in replication stress response.

Cells undergoing replicative stress often encounter problems while progressing through S phase and therefore show an increase of S phase cells. Indeed, we observed an increased S phase population in ATR_X deficient cells (Fig. 2*B*). In addition, ATR_X-deficient cells showed a significant increase in BrdU-positive S phase population 4 or 8 h after double thymidine block/release when compared with wild-type cells (Fig. 2, *C* and *D*). Strikingly, ATR_X-deficient cells displayed an increase in γ H2AX positive cells 4 h after release from thymidine block (Fig. 2*E*) as well as 2 h following HU treatment (Fig. 3, *A* and *B*), suggesting that more DSBs were generated during normal and stressed replication in the absence of ATR_X. This may explain the increased sensitivity to replication stress observed in ATR_X-deficient cells (Fig. 2*A*).

ATR_X Localizes to DNA Damage Sites—To determine whether ATR_X is directly involved in DNA damage response, we examined ATR_X localization upon DNA damage. Endogenous ATR_X co-localized with single-strand DNA-binding protein RPA2 following laser-induced micro-irradiation (Fig. 3*C*), indicating that ATR_X localizes to DNA damage sites. However, we were not able to clearly demonstrate ATR_X foci formation upon HU treatment. This is likely due to the fact that ATR_X is concentrated at heterochromatin as well as PML bodies, which make it difficult to spot damage-induced ATR_X foci. Therefore we used fractionation assay and indeed observed HU-induced chromatin enrichment of ATR_X (Fig. 3*D*). Together, these data suggest that ATR_X may participate in replication stress pathway.

ATR_X Is Required for Replication Checkpoint Control and Replication Restart—To further explore the functional role of ATR_X upon replication stress, we treated the cells with increasing doses of HU and examined checkpoint activation using CHK1 phosphorylation as readout. Consistently, pCHK1 (S317) is greatly reduced in ATR_X-deficient cells treated with low dose of HU (Fig. 3*E*), indicating that ATR_X is required for efficient checkpoint activation in response to replication stress.

Cells with defective replication checkpoint often do not recover efficiently from HU arrest. Notably, ATR_X-deficient cells exhibited slower mitotic entry after release from HU (Fig. 4*A*). Moreover, we observed a dramatic increase of stalled replication forks and a reduction in new origin firing in ATR_X-deficient cells after treatment of HU for 2 and 6 h (Fig. 4, *B* and *D*). However, both wild-type and ATR_X-deficient cells showed similar increase of stalled forks after 24 h of incubation of HU, which is similar to a previous report (34). These data suggest that ATR_X only delay but not block fork collapse following replication stress. There is no significant difference in the lengths of DNA fibers after HU release over time in wild-type or

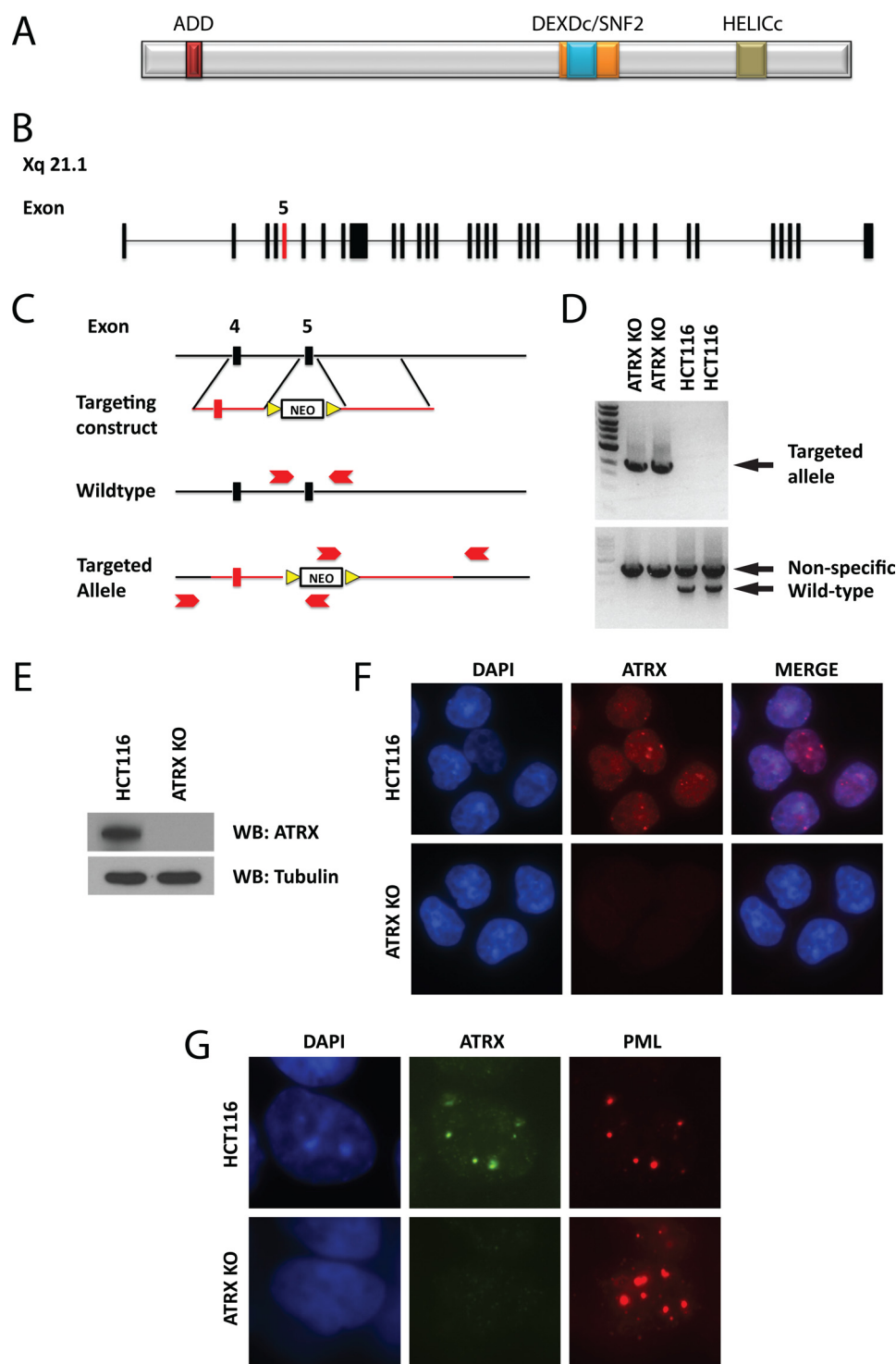


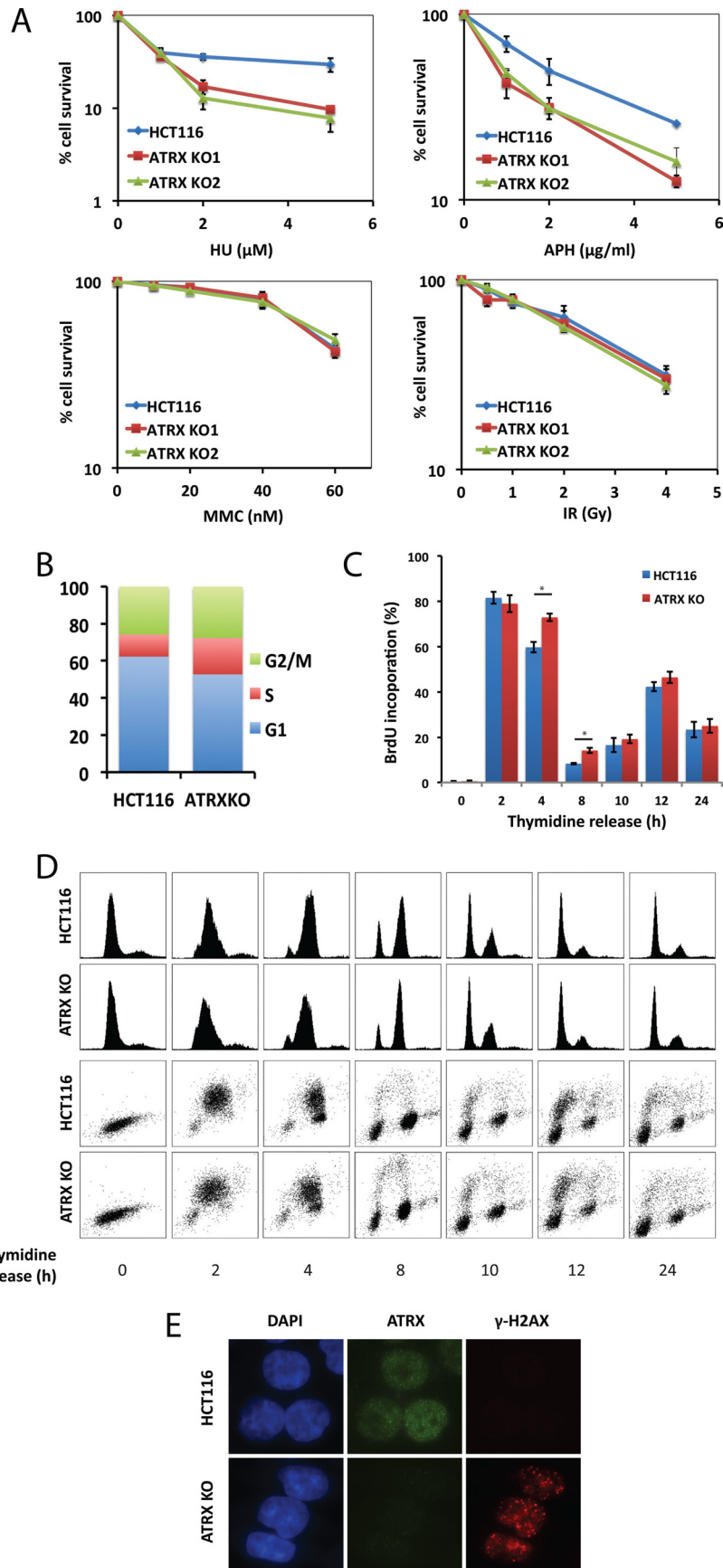
FIGURE 1. Generation of ATR_X somatic knock-out cells. *A*, schematic diagram of the conserved functional domain of ATR_X. *B*, schematic representation of the ATR_X genomic DNA and exon distribution. *C*, targeting and screening strategies for generation of somatic knock-out cells. Homology arms were generated by PCR and cloned into an AAV vector flanked by neo and two loxP sites. Primers for screening were designed outside the homology arms as indicated. *D*, knock-out cells were verified by PCR as indicated. *E* and *F*, ATR_X KO cells were confirmed by immunostaining and Western blotting analysis. *G*, representative micrographs showing immunofluorescence staining of ATR_X and PML nuclear bodies in control and ATR_X KO cells.

ATR_X knock-out cells (Fig. 4C), indicating that ATR_X does not play any role in regulating the rate of DNA replication.

ATR_X Interacts with MRN Complex *in Vivo*—In an attempt to understand the underlying mechanism by which ATR_X participates in replication stress response, we established HEK293T cells stably expressing hATR_X harboring N-terminal

SFB (S-protein, Flag, Streptavidin)-tag and performed tandem affinity purification to identify potential ATR_X-interacting proteins by mass spectrometry analysis. DAXX, a well-known ATR_X binding partner, co-purified with ATR_X (Fig. 5A). Interestingly, the MRN complex (MRE11A-RAD50-NBS1) also co-purified from ATR_X. To further confirm this interaction, we

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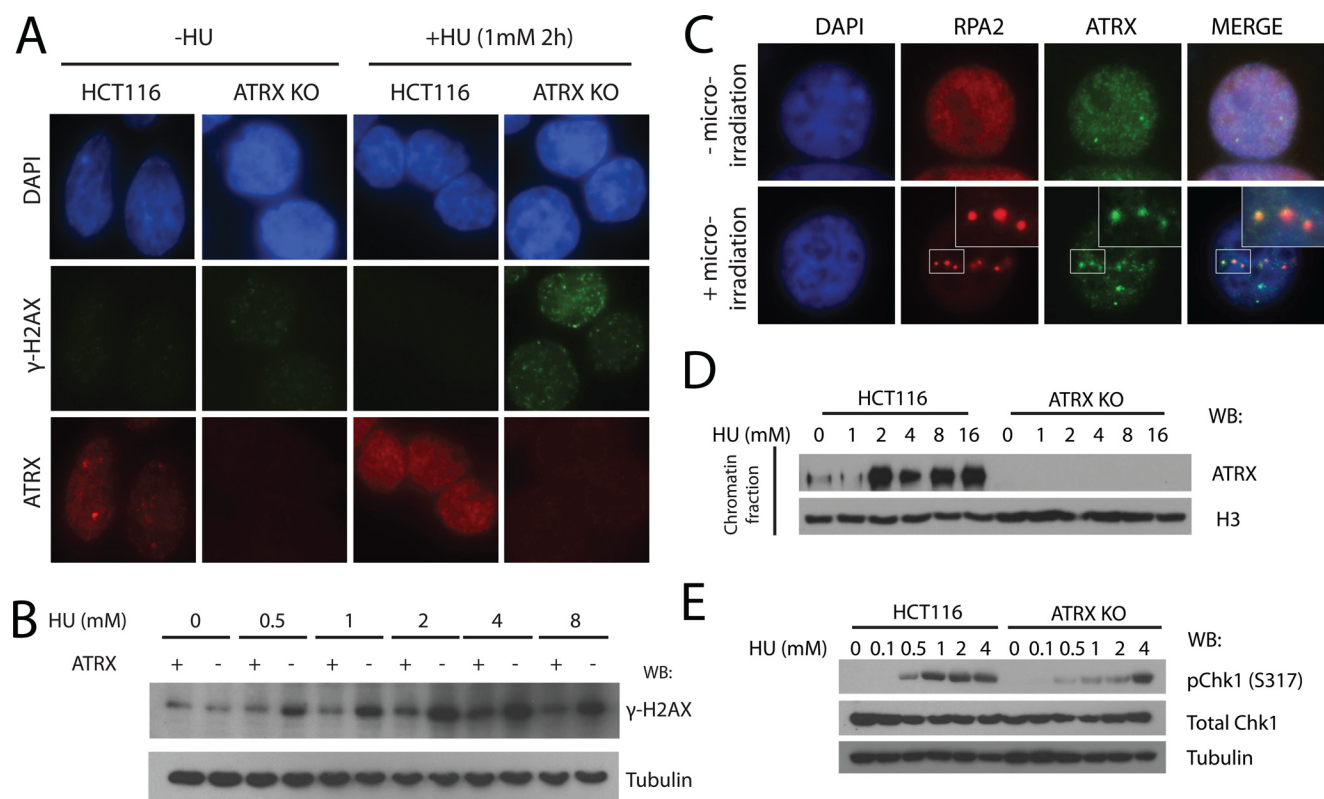


FIGURE 3. ATR_X is recruited to the DNA damage site and promotes checkpoint activation. *A*, wild-type and ATR_X-deficient HCT116 cells were mock-treated or treated with 4 mM HU for 2 h. Cells were fixed and stained with indicated antibodies and counterstained with DAPI. *B*, cells were treated with indicated doses of HU for 2 h and whole cell extracts were subjected to Western blotting using indicated antibodies. *C*, HeLa cells were treated with laser induced micro-irradiation and stained with indicated antibodies. *D*, wild-type and ATR_X knock-out HCT116 cells were treated with the indicated concentrations of HU. Cells were harvested, fractionated, and Western blotting was conducted using indicated antibodies. *E*, cells were treated with indicated doses of HU. Whole cell extracts were prepared and subjected to Western blotting as indicated.

transiently co-expressed SFB-ATR_X with myc-MRE11A, myc-RAD50, or myc-NBS1 in HEK293T cells and performed co-precipitation experiments. Our data showed that ATR_X pulled down NBS1, but not the other two components in the MRN complex (Fig. 5*B*). These data suggest that ATR_X is a putative binding partner of MRN complex, possibly through its interaction with NBS1, and may function with MRN in DNA damage response.

DISCUSSION

Impaired replication fork progression and increased replication-dependent DNA damage were found in early stages of tumor development (35, 36). Tight regulation of DNA replication, including efficient reactivation of stalled replication forks, is essential to maintain faithful replication and genome stability.

ATR_X-deficient cells fail to resume replication after HU treatment, indicating that the stalled replication forks may be collapsed. The slower progression through S phase and delayed mitotic entry observed in ATR_X-deficient cells could be due to stalled replication forks in the absence of ATR_X. Indeed, a

recent study showed a similar observation on the delayed S-phase progression upon ATR_X inactivation (37). These replication defects in ATR_X deficient cells may lead to reduced number of replication forks in these cells, and therefore indirectly affect Chk1 activation in response to HU, as we observed in this study. A previous report demonstrated that Chk1 inhibits origin initiation (38). However, in the current study, ATR_X knock-out cells displayed reduced pChk1 and also a reduction in origin firing. It is possible that inefficient CldU incorporation in ATR_X-deficient cells after releasing from HU may contribute to this phenomenon.

ATR_X, as a chromatin remodeler that binds to specific DNA structures including unusual DNA structures, short repetitive sequences and G-4 quadruplex, is recruited to telomere ends and participates in the resolution of G4-quadruplex structure (20). This may subsequently facilitate the resumption of replication and mitotic entry. However, ATR_X may also play a more direct role in replication checkpoint control. ATR_X could function with other proteins, such as MRN complex, to protect stalled replication forks upon replication stress. Recently stud-

FIGURE 2. ATR_X is involved in replication stress. *A*, clonogenic survival assay of wild-type and two independent clones of ATR_X-deficient HCT116 cells following HU, APH, MMC, and IR treatment as indicated. *B*, cell-cycle distributions of wild-type and ATR_X-deficient HCT116 cells was analyzed by FACS and presented as percentages of cells in G1, S, and G2/M phases. *C*, cells were released from double thymidine block (1 mM) and pulsed with BrdU 30 min prior harvest. BrdU incorporation was analyzed by staining with anti-BrdU antibody and quantified by flow cytometry. Results were the average of three independent experiments and presented as mean ± S.E. *, *p* < 0.01. *D*, FACS analysis of cell cycle and BrdU incorporation after double thymidine block and release. *E*, cells were fixed 4 h after being released from double thymidine block and stained with antibodies as indicated.

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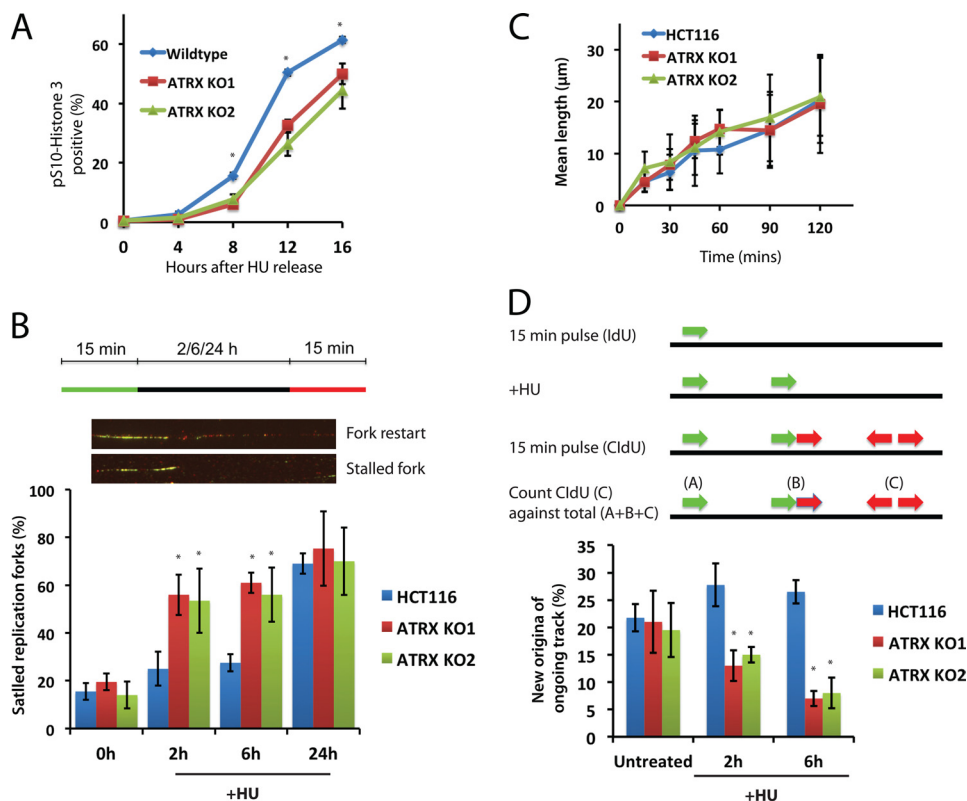


FIGURE 4. ATRX is required for replication restart. *A*, mitotic entry after HU release in wild-type and ATRX-deficient HCT116 cells. Cells were pre-treated with 2 mM HU for 24 h before releasing. Data represented averages of three independent experiments and were shown as mean \pm S.E. *B*, replication restart after HU treatment. At least 100 DNA fibers were counted per condition. Error bar represented S.E. *C*, synchronized cells in early S phase were labeled with IdU for 15 min, treated with HU for 6 h, released and labeled with CldU for 15–120 min before preparing DNA fibers. The average length of at least 80 replication tracks was plotted for each time point. *D*, outline of the protocol used to quantify the sites of newly initiated replication fork at indicated time points after the removal of HU. The percentage of newly initiated replication forks was determined by dividing the number of CldU-containing track (C) by the number of total tracks (A+B+C). *, $p < 0.001$. CldU, 5-chloro-2-deoxyuridine; IdU, 5-iodo-2-deoxyuridine; HU, hydroxyurea.

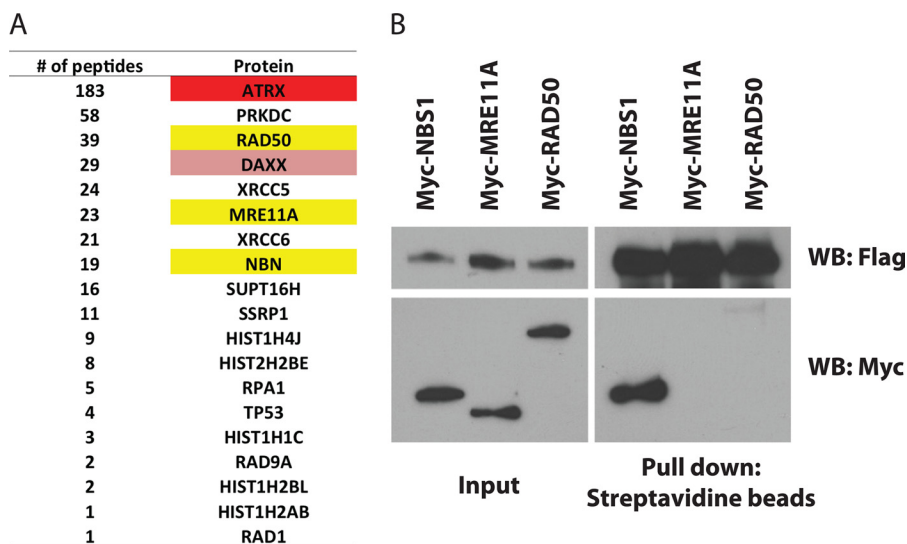


FIGURE 5. ATRX interacts with MRN complex. *A*, HEK293T cells stably expressing SFB-ATRX were subjected to tandem affinity purification and mass spectrometry analysis. Red indicates the bait protein, and yellow indicates the MRN complex identified by TAP. The number of peptides recovered from mass spectrometry study was also presented. *B*, HEK293T cells were transfected with constructs encoding SFB-ATRX together with constructs encoding Myc-NBS1, Myc-MRE11A, or Myc-RAD50. Co-precipitation and immunoblotting were carried out as indicated.

ies showed that MRE11A and NBS1 are important for promoting replication restart (39, 40). A study in yeast demonstrated that MRN complex is recruited to stalled forks to stabilize replisome (41). In addition, MRN complex is required for promoting the resumption of DNA replication following stress (41). The

association between MRN complex and ATRX provides a possible explanation as to how ATRX may be involved in replication stress pathway. Unfortunately, due to the large size of ATRX protein, we were not able to reconstitute the ATRX knock-out cells with wild-type ATRX expression. Thus, we

were unable to further pursue the functional significance of ATRX/MRN interaction in replication checkpoint control.

In conclusion, we showed that ATRX promotes checkpoint activation upon modest replication stress. ATRX participates in restart of stalled replication forks and recovery from replication stress. Moreover, ATRX localized to DNA damage sites and associated with the MRN complex. These results suggest that ATRX may be directly involved in replication stress response, adding yet another imperative function to the expanding roles of ATRX in the maintenance of genomic stability.

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