ATR and H2AX Cooperate in Maintaining Genome Stability under Replication Stress

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Chromosomal abnormalities are frequently caused by problems encountered during DNA replication. Although the ATR-Chk1 pathway has previously been implicated in preventing the collapse of stalled replication forks into double-strand breaks (DSB), the importance of the response to fork collapse in ATR-deficient cells has not been well characterized. Herein, we demonstrate that, upon stalled replication, ATR deficiency leads to the phosphorylation of H2AX by ATM and DNA-PKcs and to the focal accumulation of Rad51, a marker of homologous recombination and fork restart. Because H2AX has been shown to play a facilitative role in homologous recombination, we hypothesized that H2AX participates in Rad51-mediated suppression of DSBs generated in the absence of ATR. Consistent with this model, increased Rad51 focal accumulation in ATR-deficient cells is largely dependent on H2AX, and dual deficiencies in ATR and H2AX lead to synergistic increases in chromatid breaks and translocations. Importantly, the ATM and DNA-PKc phosphorylation site on H2AX (Ser139) is required for genome stabilization in the absence of ATR; therefore, phosphorylation of H2AX by ATM and DNA-PKcs plays a pivotal role in suppressing DSBs during DNA synthesis in instances of ATR pathway failure. These results imply that ATR-dependent fork stabilization and H2AX/ATM/DNA-PKcs-dependent restart pathways cooperatively suppress double-strand breaks as a layered response network when replication stalls.

Genome maintenance prevents mutations that lead to cancer and age-related diseases. A major challenge in preserving genome integrity occurs in the simple act of DNA replication, in which failures at numerous levels can occur. Besides the misincorporation of nucleotides, it is during this phase of the cell cycle that the relatively stable double-stranded nature of DNA is temporarily suspended at the replication fork, a structure that is susceptible to collapse into DSBs. Replication fork stability is maintained by a variety of mechanisms, including activation of the ATR-dependent checkpoint pathway.

The ATR pathway is activated upon the generation and recognition of extended stretches of single-stranded DNA at stalled replication forks (1–4). Genome maintenance functions for ATR and orthologs in yeast were first indicated by increased chromatid breaks in ATR−/− cultured cells (5) and by the “cut” phenotype observed in Mec1 (Saccharomyces cerevisiae) and Rad3 (Schizosaccharomyces pombe) mutants (6–9). Importantly, subsequent studies in S. cerevisiae demonstrated that mutation of Mec1 or the downstream checkpoint kinase Rad53 led to increased chromosome breaks at regions of the genome that are inherently difficult to replicate (10), and a decreased ability to reinitiate replication fork progression following DNA damage or deoxyribonucleotide depletion (11–14).

In vertebrates, similar replication fork stabilizing functions have been demonstrated for ATR and the downstream protein kinase Chk1 (15–20). Several possible mechanisms have been put forward to explain how ATR-Chk1 and orthologous pathways in yeast maintain replication fork stability, including maintenance of replicative polymerases (α, δ, and ε) at forks (17, 21), regulation of branch migrating helicases, such as Blm (22–25), and regulation of homologous recombination, either positively or negatively (26–29).

Consistent with the role of the ATR-dependent checkpoint in replication fork stability, common fragile sites, located in late-replicating regions of the genome, are significantly more unstable (5–10-fold) in the absence of ATR or Chk1 (19, 20). Because these sites are favored regions of instability in oncogene-transformed cells and preneoplastic lesions (30, 31), it is possible that the increased tumor incidence observed in ATR haploinsufficient mice (5, 32) may be related to subtle increases in genomic instability. Together, these studies indicate that maintenance of replication fork stability may contribute to tumor suppression.

It is important to note that prevention of fork collapse represents an early response to problems occurring during DNA replication. In the event of fork collapse into DSBs, homologous

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4 The abbreviations used are: DSB, double-strand break; MEF, murine embryonic fibroblast; 4-OHT, 4-hydroxytamoxifen; HR, homologous recombination; DMEM, Dulbecco’s modified Eagle’s medium; shRNA, short hairpin RNA; FB5, fetal bovine serum; BrdUrd, bromodeoxyuridine; shATR, shRNA targeting ATR; shCtrl, shRNA targeting control; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase.
recombination (HR) has also been demonstrated to play a key role in genome stability during S phase by catalyzing recombination between sister chromatids as a means to re-establish replication forks (33). Importantly, a facilitator of homologous recombination, H2AX, has been shown to be phosphorylated under conditions that cause replication fork collapse (18, 34).

Phosphorylation of H2AX occurs predominantly upon DSB formation (34–38) and has been reported to require ATM, DNA-PKcs, or ATR, depending on the context (37–42). Although H2AX is not essential for HR, studies have demonstrated that H2AX mutation leads to deficiencies in HR (43, 44), and suppresses events associated with homologous recombination, such as the focal accumulation of Rad51, BRCA1, BRCA2, ubiquitinated-FANCD2, and Ubc13-mediated chromatin ubiquitination (43, 45–51). Therefore, through its contribution to HR, it is possible that H2AX plays an important role in replication fork stability as part of a salvage pathway to reinitiate replication following collapse.

If ATR prevents the collapse of stalled replication forks into DSBs, and H2AX facilitates HR-mediated restart, the combined deficiency in ATR and H2AX would be expected to dramatically enhance the accumulation of DSBs upon replication fork stalling. Herein, we utilize both partial and complete elimination of ATR and H2AX to demonstrate that these genes work cooperatively in non-redundant pathways to suppress DSBs during S phase. As discussed, these studies imply that the various components of replication fork protection and regeneration cooperate to maintain replication fork stability. Given the large number of genes involved in each of these processes, it is possible that combined deficiencies in these pathways may be relatively frequent in humans and may synergistically influence the onset of age-related diseases and cancer.

**EXPERIMENTAL PROCEDURES**

**MEF Isolation, Lentivirus Infection, and ATR Deletion**—Murine embryonic fibroblasts (MEFs) were harvested from day 14.5 postcoitus embryos and grown in Dulbecco’s modified Eagle’s medium (DMEM, Cellgro) supplemented with 10% FBS (Hyclone) in a 3% oxygen incubator and frozen within 3 doublings after isolation. For shRNA lentivirus infections, cells were plated at 3 × 10^6 cells/10-cm plate in 0.5% FBS/DMEM in the presence of virus (as described below) and distributed into additional plates by split/plating 24 h later (1 × 10^6 cells per 10-cm plate in 0.5% FBS/DMEM). The next day, media was changed to 0.1% FBS/DMEM; 24 h later, cells were stimulated with 20% FBS/DMEM for 16–19 h to enrich and normalize for S phase (supplemental Fig. S1). Plates were then harvested or treated as described (“Results”). Lentivirus constructs expressing short hairpin RNAs (shRNAs) from the H1 promoter that target ATR (5’-gaattgtattgtggtaaatcaagagattggccacagtaacaattc) or a control sequence (5’-gtacctaggttacctaggttatcttcagagattggccacagtaacaattc) were generated using the H1UG1 vector, which co-expresses enhanced green fluorescent protein through the human ubiquitin C promoter. Lentiviruses were produced as described (52), titered by enhanced green fluorescent protein expression and delivered to MEFs at a multiplicity of infection of 5–10, which consistently yielded 95–98% infection rates. To delete ATR in ATR<sup>lox</sup>-Cre-ERT2<sup>+</sup> MEFs (18, 53), cells were enriched in G<sub>0</sub> as described above and treated with 0.2 μM 4-hydroxytamoxifen (4-OHT, Calbiochem) for 48 h prior to serum stimulation.

**Aphidicolin Sensitivity Assay**—MEFs were infected with lentiviral constructs on the first day of synchronization (above) and replated 1 day later at 2 × 10<sup>5</sup> cells per 10-cm plate. Upon serum stimulation, cells were left untreated or treated with 0.2 or 0.4 μM aphidicolin. Cells were grown for a total of 4 days, with an intervening replating at day 2. Media and aphidicolin were replenished every 24 h. At replating and final harvest, cells were counted and the total population doublings were determined.

**Chemical Inhibitors**—Aphidicolin (Calbiochem), a DNA polynucleoside phosphorylase inhibitor, was used at a final concentration of 5 μM unless otherwise indicated. ATM inhibitor KU-55933 (Sigma) and DNA-PK inhibitor NU7026 (Calbiochem) were added to media at a concentration of 10 μM 1 h prior to cell collection or aphidicolin treatment.

**Quantitative Reverse Transcriptase-PCR**—RNA was extracted from 1 × 10<sup>6</sup> cells with TRizol reagent (Invitrogen) according to the manufacturer’s instructions and cDNA was produced with the cDNA Archive Kit (Applied Biosystems). Quantitative PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). Primers against β-actin and ATR (Applied Biosystems, Mm00607939_s1, Mm01223656_m1) were used for ΔΔ<sub>C<sub>T</sub></sub> analysis. Analysis was performed using an Applied Biosystems 7900HT Sequencing Detection System, with amplification quantified by MGB probes.

**Immunoblotting**—Resuspended cells were lysed in 1× SDS lysis buffer (2× = 20% glycerol, 0.15 M SDS, 125 mM Tris, pH 6.8). Whole cell lysates were separated by SDS-PAGE (15% for H2AX; 4% for ATR) and blotted onto 0.45-μm polyvinylidene difluoride membranes. Blots were detected for non-phospho-H2AX (Bethyl), phospho-H2AX (Upstate, JBW301 clone), glyceraldehyde-3-phosphate dehydrogenase (U. S. Biological), ATR (Santa Cruz), and mTOR (Cell Signaling) according to the manufacturer’s instructions.

**Flow Cytometric Quantification of BrdUrd Incorporation, Phospho-H2AX, and Phospho-histone H3**—G<sub>0</sub>-enriched cells were harvested 16–19 h after serum stimulation and fixed in 70% EtOH prior to staining. Cells were analyzed for phospho-H2AX and DNA content as previously described (54). For quantification of S phase, cells were incubated with 10 μM BrdUrd (Roche) for 30 min, then harvested and fixed in 70% EtOH, acid denatured (3 N HCl containing 0.5% Tween 20), and neutralized with 0.1 M sodium borate, pH 8.5 (Sigma). Following staining with anti-BrdUrd (BD Pharmingen) and fluorescein isothiocyanate-conjugated secondary antibodies (Jackson), cells were stained with propidium iodide (50 μg/ml propidium iodide, 0.1% Triton X-100, 50 μg/ml RNase, 50 mM EDTA) for DNA content. To determine the percentage of phospho-H2AX-positive cells in mitosis, cells were first permeabilized and stained for phospho-H2AX (54) with aflavococinic-conjugated secondary antibody, followed by staining for phospho-histone H3 (Upstate) at a 1:200 dilution and fluorescein isothiocyanate-conjugated secondary antibody at a 1:500 dilution. For each procedure, cells were analyzed by FACS using a FACScalibur (BD Biosciences) and CellQuest software.
immunocytochemical Detection of BrdUrd, Rad51, and Phospho-H2AX—MEFs were plated on round coverslips and synchronized as described above. After stimulation, cells were treated with 10 μM BrdUrd for 30 min, fixed with 3% paraformaldehyde, 2% sucrose in phosphate-buffered saline for 10 min at room temperature, then permeabilized in 0.5% Triton X-100 in phosphate-buffered saline for 10 min on ice. Cells were stained using Anti-Rad51 (Santa Cruz) or anti-phospho-H2AX antibody (Upstate), followed by Alexa Fluor 594 secondary antibody (Invitrogen) detection. Cells/antibodies were then fixed (3% paraformaldehyde, 2% sucrose, phosphate-buffered saline) for 10 min at room temperature, denatured with 2 N HCl for 5 min at room temperature, washed, and stained with anti-BrdUrd (BD Pharmingen), Alexa Fluor 488 (Invitrogen) secondary antibody and 4',6-diamidino-2-phenylindole. Cells were visualized with a Nikon Eclipse 80i fluorescent microscope with a ×100 objective lens. Rad51 foci and phospho-H2AX staining were quantified from images by double-blind methods.

Metaphase Spreads and Spectral Karyotyping—To arrest cells in M phase, 0.5 μM nocodazole (Calbiochem) was added for 4 h, and mitotic spreads were prepared as described (18). SYTOX Green (Invitrogen) nucleic acid stain was used (1:50,000 in phosphate-buffered saline, pH 7.9). Metaphases were visualized using a Nikon Eclipse 80i fluorescent microscope with a ×100 objective lens. Spectral karyotyping was performed following the DNA spectral karyotyping hybridization and detection protocol from Applied Spectral Imaging. Images were captured on an ASI CCD camera and interferometer, using an Olympus BX61 microscope with a ×60 objective lens. Data were managed and analyzed using Case Data Manager software (version 5.0) from ASI.

H2AX Wild-type and H2AXS139A Add-back Constructs—Full-length murine H2AX and H2AXS139A were PCR amplified (PFU Turbo polymerase, Stratagene) from cDNA (Open Biosystems, MMM1013-64604) using the following primers: a common forward primer (5'-gacctagcttagccgaccacattgctggagcggcagcgcaag) and one of two differing reverse primers (5'-cagggatatcatcggagctgcatggcgcggcttt for H2AX wild-type and 5'-cagggatatcgattcgcgctgacctgcttctt for H2AXS139A). Amplified products were subsequently cloned into the HFUW lentiviral vector for expression from the human ubiquitin C promoter. Protein expression and virus titration was assayed by Western blot and FACS analysis for H2AX using a total H2AX antibody (Bethyl) using the manufacturer’s recommended concentrations (supplemental Fig. S3).

RESULTS

Intermediate Suppression of ATR Leads to Increased H2AX Phosphorylation in S Phase upon Replication Stress—We have previously shown that ATR deletion leads to increased H2AX phosphorylation upon replication stalling (18). However, through overexpressing a kinase-inactive mutant, ATR has also been reported to be responsible for H2AX phosphorylation in response to replication stress (40). We reasoned that these contrasting results may be due to differences in the levels of ATR pathway inhibition, as dominant-negative overexpression leads only to hypomorphic suppression (55).

To examine the possibility that levels of ATR suppression strongly influence its effects on H2AX phosphorylation, wild-type and ATR+/−/− MEFs were transduced with lentivirus expressing an shRNA targeting either ATR (shATR) or a non-targeting control shRNA (shCtrl). Quantitative reverse transcriptase-PCR analysis demonstrated that ATR mRNA was reduced 61% upon shATR expression in wild-type cells (Fig. 1A). As previously reported (5), ATR+/−/− cells maintained only 55% of the wild-type ATR mRNA level; however, this level decreased to 16% of wild-type when ATR+/−/− cells expressed shATR (ATRshATR, Fig. 1A). ATR protein levels decreased in a manner consistent with the decrease in mRNA (Fig. 1B).
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To determine whether partial suppression of ATR in combination with replication stress leads to H2AX phosphorylation, wild-type, ATR-suppressed (e.g. ATR\textsuperscript{hypo}), and ATR-deleted cells (ATR\textsuperscript{Δ/−}) were serum-starved at the time of ATR depletion and subsequently stimulated to enter the cell cycle. At peak S phase, cells were treated with aphidicolin (5 μM). For each condition, S phase levels were similar at the time of aphidicolin treatment (supplemental Fig. S1). Although only a small increase in H2AX phosphorylation was observed in wild-type cells following aphidicolin treatment (Fig. 1C, lanes 1–3), increasing levels of H2AX phosphorylation were observed upon aphidicolin treatment as ATR abundance was reduced, culminating with the highest levels observed in ATR\textsuperscript{Δ/−} cells.

The stimulatory effect of ATR suppression on H2AX phosphorylation implied that H2AX may perform a salvage pathway role in response to replication stress. If so, then dual suppression of ATR and H2AX in the presence of replication stress would be expected to diminish viability further than suppression of either gene alone. To test this hypothesis, cells were treated with aphidicolin (0.2 and 0.4 μM). These concentrations of aphidicolin only partially inhibit DNA polymerase processivity, and have been shown to cause increased fragile site expression in ATR-deficient cells (19). The expansion of wild-type and H2AX\textsuperscript{−/−} MEFs expressing shATR and ATR\textsuperscript{hypo} MEFs were only marginally inhibited by low doses of aphidicolin (20–30%). However, proliferation of ATR\textsuperscript{+/−}/H2AX\textsuperscript{−/−} MEFs expressing the shATR hairpin (ATR\textsuperscript{hypo}/H2AX\textsuperscript{−/−}) was significantly suppressed by low doses of aphidicolin, culminating in a greater than 60% reduction in the presence of 0.4 μM aphidicolin (Fig. 1D). These results indicate that H2AX plays an important function in the biological response to replicative stress specifically under conditions of ATR dysfunction.

H2AX Phosphorylation Occurs in S Phase and Is Not the Result of Premature M Phase Entry—It has been suggested previously that double-strand breaks resulting from aphidicolin treatment of ATR-deficient MEFs resist premature mitotic entry (19). By flow cytometry, aphidicolin treatment led to an increased frequency of ATR\textsuperscript{Δ/−} cells that exhibited detectable H2AX phosphorylation (Fig. 2A). However, co-detection of phosphorylated H2AX with phospho-histone H3, a mitotic marker, indicated that nearly all of aphidicolin-treated ATR\textsuperscript{Δ/−} cells with detectable levels of H2AX phosphorylation were phosphohistone H3 negative (Fig. 2A). These results indicate that H2AX phosphorylation is not the result of premature mitotic entry, consistent with previous findings that ATR\textsuperscript{Δ/−} MEFs resist premature mitotic entry under replication stress (18).

Co-staining for phospho-H2AX and DNA content (propidium iodide) indicated that the increased H2AX phosphorylation in ATR\textsuperscript{Δ/−} cells occurred in S phase, as it was observed predominantly in cells with intermediate DNA content, between 2N and 4N (Fig. 2B). Furthermore, 30 min incorporation of BrdUrd prior to aphidicolin treatment revealed that 99 ± 0.01% (S.E.) of phospho-H2AX-positive ATR\textsuperscript{Δ/−} cells treated with aphidicolin were also positive for BrdUrd as deter-

FIGURE 2. H2AX phosphorylation in response to ATR deficiency occurs in S phase and is not caused by premature mitotic entry. A and B, cell cycle flow cytometric detection of phospho-H2AX. Wild-type and ATR\textsuperscript{Δ/−} cells untreated or treated for 3 h with aphidicolin were detected for phospho-H2AX and phospho-histone H3, to quantify M phase cells (A), or propidium iodide for DNA content (B). As positive controls, wild-type cells were treated for 6 h with 0.5 μM nocodazole (noco) or exposed to 10 gray ionizing radiation (IR) and harvested 45 min later. Percentage of phospho-H2AX-positive cells is indicated for each condition, and frequency of both single-positive and double-positive cells for phospho-H2AX and phospho-histone H3 staining are indicated in A.
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Two compounds. To further confirm the roles of ATM and DNA-PKcs, \( \text{ATR}^{\text{hypo}} \text{ATM}^{-/-} \) and \( \text{ATR}^{\text{hypo}} \text{DNA-PKcs}^{-/-} \) MEFs were generated, synchronized, and treated with aphidicolin as described above (Fig. 1). Again, five phase levels were similar between all cell lines at the time of aphidicolin treatment (supplemental Fig. S1). As shown in Fig. 3B, ATM absence potently suppressed aphidicolin-induced H2AX phosphorylation (\( \text{ATR}^{\text{hypo}} \text{ATM}^{-/-} \) cells) in comparison to \( \text{ATR}^{\text{hypo}} \) cells. \( \text{ATR}^{\text{hypo}} \text{DNA-PKcs}^{-/-} \) cells also exhibited suppressed levels of H2AX phosphorylation (Fig. 3C). In either cell line, \( \text{ATR}^{\text{hypo}} \text{ATM}^{-/-} \) or \( \text{ATR}^{\text{hypo}} \text{DNA-PKcs}^{-/-} \), residual H2AX phosphorylation was largely ablated by chemical inhibition of the other kinase (Fig. 3, B, lanes 10–12, and C, lanes 10–12). These data indicate that the H2AX phosphorylation stimulated by replication stress in ATR-deficient cells is co-dependent on ATM and DNA-PKcs.

Increased Rad51 Foci Formation in ATR-deficient Cells Requires H2AX—Collapsed replication forks utilize homologous recombination to catalyze invasion of the DSB ends into the unbroken complimentary DNA on the sister chromatid, ultimately restoring active replication forks. Because H2AX has been implicated in modulating the efficiency of homologous recombination (43, 44) and Rad51 accumulation (37, 45–51), we asked whether ATR deletion led to an increased frequency of homologous recombination intermediates and if H2AX played a facilitative role in this process.

To do so, we quantified Rad51 focal accumulation in wild-type and ATR-deleted cells in the presence and absence of H2AX. \( \text{ATR}^{\Delta/-} \) and \( \text{ATR}^{\Delta/-} \text{H2AX}^{-/-} \) cells were generated by treating \( \text{G} \text{G}^{\text{enriched}} \text{ATR}^{\text{lox/cre}} \text{Cre-ERT2}^{+} \) and \( \text{ATR}^{\text{lox/cre}} \text{H2AX}^{-/-} \text{Cre-ERT2}^{+} \) MEFs with 4-OHT to acutely activate Cre recombinase (18, 53). Subsequently, 4-OHT was removed, and cells were stimulated to re-enter the cell cycle. Consistent with ATR absence leading to elevated DSB formation and an increased reliance on HR, \( \text{ATR}^{\Delta/-} \) cells exhibited a 2-fold or greater increase in Rad51 foci compared with wild-type or \( \text{H2AX}^{-/-} \) cells, both in the presence and absence of aphidicolin treatment (Fig. 4). Importantly, the elevated levels of Rad51 foci in \( \text{ATR}^{\Delta/-} \) cells were significantly suppressed in the absence of H2AX (\( \text{ATR}^{\Delta/-} \text{H2AX}^{-/-} \) cells), both in aphidicolin-treated and untreated cells (Fig. 4B). These results indicate that the focal accumulation of Rad51 that occurs upon ATR deletion is largely dependent on H2AX.

ATR and H2AX Cooperate in Maintaining Genome Stability under Replication Stress—Increased accumulation of Rad51 foci in ATR-deficient cells and suppression of these foci in the absence of H2AX are consistent with a co-dependence on ATR and H2AX for maintaining genome stability in S phase. That is, failure of the ATR pathway to maintain stalled replication forks leads to an increased dependence on H2AX to facilitate replication fork restart through homologous recombination. If this model is correct, then DSBs resulting from replication fork stalling in ATR-deficient cells should be more persistent in the absence of H2AX, leading to a synergistic increase in genomic instability. However, the decreased abundance of Rad51 foci in the absence of H2AX could also be an indication of accelerated repair and attenuated DSB persistence.

Figure 3. ATM and DNA-PKcs are responsible for H2AX phosphorylation in ATR-deficient cells. A, the effect of ATM and DNA-PKcs chemical inhibition on H2AX phosphorylation in wild-type and \( \text{ATR}^{\text{hypo}} \) cells. \( \text{G} \text{G}^{\text{enriched}} \) and restimulated MEFs were untreated or treated with aphidicolin for 1 or 2 hours at peak S phase (16–19 hour of G2) prior to SDS-PAGE sample collection. Phospho-H2AX was detected by immunoblot; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected as a loading control. B, phosphorylation of H2AX in \( \text{ATR}^{\text{hypo}}, \text{ATR}^{\text{hypo}} \text{ATM}^{-/-} \), and DNA-PKcs-inhibited \( \text{ATR}^{\text{hypo}} \text{ATM}^{-/-} \) cells. C, phosphorylation of H2AX in \( \text{ATR}^{\text{hypo}}, \text{ATR}^{\text{hypo}} \text{DNA-PKcs}^{-/-} \), and ATM-inhibited \( \text{ATR}^{\text{hypo}} \text{DNA-PKcs}^{-/-} \) cells. Samples for A and C were generated and detected for phospho-H2AX and glyceraldehyde-3-phosphate dehydrogenase as described in A.
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To discriminate between these possibilities, we quantified the effect of combined ATR and H2AX suppression on chromatid breaks in the presence and absence of aphidicolin (Fig. 5). As described previously (18), an increase in chromatid breaks was observed upon ATR deletion alone (ATR<sup>Δ</sup>-/ Δ- versus wild-type MEFs) (Fig. 5B). However, this level of breakage was further elevated when combined with H2AX deficiency, confirming that ATR deletion led to an increased dependence on H2AX to suppress chromatid breaks (ATR<sup>Δ</sup>-/ Δ- versus ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup>, Fig. 5B). The frequency of chromatid breaks in ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells was significantly greater than the combined frequency observed in cells deficient for ATR or H2AX alone (p = 0.045), indicating that the dual loss of these genes led to a synergistic increase in genomic instability.

To confirm these results and investigate the interdependence of ATR and H2AX during replication stress, metaphase spreads were analyzed from controls (wild-type and H2AX<sup>−/−</sup>, ATR<sup>Δ</sup>-/ Δ- and ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> MEFs expressing the shCtrl hairpin), ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> MEFs that were left untreated or pulse treated with aphidicolin for 2 h and then released into M phase. A significant increase in chromatid breaks was observed in ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells compared with either wild-type or H2AX<sup>−/−</sup> control cells, and this instability was further elevated following aphidicolin treatment (p ≤ 0.05). Moreover, similar to ATR<sup>Δ</sup>-/ Δ- cells, the genomic instability observed in untreated ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells was synergistically increased by H2AX deletion in comparison to suppression of ATR or H2AX alone (p value = 0.001). Interestingly, because ATR<sup>Δ</sup>-/ Δ- cells had significantly fewer chromatid breaks than ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells (p value = 0.05), these data indicate that partial loss of ATR in an H2AX-null background is more destabilizing than the complete deletion of ATR alone.

As noted above, the level of genomic instability generated by ATR deficiency was enhanced by aphidicolin treatment (Fig. 5B); however, this instability was significantly exacerbated by the absence of H2AX (p value = 0.045). The level of instability quantified from ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells treated with aphidicolin is likely underestimated, as spreads with greater than 20 chromatid breaks were frequently observed (Fig. 5A, lower right image) and were classified by the lowest reliable estimate (20 breaks). The increased genomic instability generated by dual ATR and H2AX deficiency demonstrates co-dependent roles for these genes in maintaining genome integrity under replication stress.

To investigate whether phosphorylation of H2AX was critical for genome stabilization in response to ATR suppression, lentiviruses encoding wild-type H2AX and a serine 139 to alanine mutant (H2AX<sup>S139A</sup>) were used to complement ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells 2 days prior to shRNA-mediated ATR suppression. Cells were then left untreated or pulse treated with aphidicolin and chromosome spreads were analyzed as described above. Consistent with an important genome stabilizing function for H2AX-Ser<sup>139</sup> phosphorylation, complementation of H2AX null cells with the H2AX<sup>S139A</sup> mutant failed to suppress the genomic instability observed in ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells in the presence of aphidicolin (Fig. 5B). In contrast, ectopic expression of wild-type H2AX decreased the genomic instability of ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells to a level similar to that observed in ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> cells. Together, these data indicate that ATR and H2AX cooperate in maintaining genome integrity during DNA synthesis and that phosphorylation of H2AX by ATM and DNA-PKcs (Fig. 3) plays a pivotal role in genome maintenance in instances of ATR pathway failure (Fig. 5B).

Loss of ATR and H2AX Leads to an Increase in Translocation Events—Our results are consistent with a model in which dual deficiency of ATR and H2AX leads to the accumulation of DSBs. According to this model, the absence of H2AX leads to an increased persistence of DSBs due to defective homologous recombination-mediated restart. One expectation of this model is that alternative, potentially error-prone DSB repair mechanisms may have a greater opportunity to generate mutagenic recombination events, such as chromatid translocations, in ATR/H2AX dual-deficient cells.

To accurately quantify the effect of ATR and H2AX deficiency on chromatid translocations, we performed spectral karyotyping (spectral karyotyping) analysis on wild-type, ATR<sup>Δ</sup>-/ Δ-, H2AX<sup>−/−</sup>, and ATR<sup>Δ</sup>-/ Δ-H2AX<sup>−/−</sup> metaphase spreads (Fig. 6). Although increases in chromatid translocation events were observed in H2AX<sup>−/−</sup> and ATR<sup>Δ</sup>-/ Δ- cells in comparison to wild-type cells (Fig. 6B), the combined elimination of ATR and
H2AX in ATR\textsuperscript{Δ−}/H2AX\textsuperscript{−/−} cells led to a dramatic increase in frequency of chromatid translocations, corresponding to 0.1 translocations per metaphase. This frequency was 4- and 7.5-fold greater than that observed in ATR\textsuperscript{Δ−} (p = 0.030) and H2AX\textsuperscript{−/−} cells (p = 0.004), respectively. The observed increase in translocation events in ATR\textsuperscript{Δ−}/H2AX\textsuperscript{−/−} cells is consistent with a decrease in Rad51-mediated HR repair in the absence of H2AX (Fig. 4), and an increased reliance on error-prone DSB repair mechanisms (Fig. 6).

**DISCUSSION**

Our studies indicate that ATR and H2AX provide cooperative safeguards to ensure genome stability during DNA replication. According to this model, activation of the ATR pathway serves as a primary mechanism to stabilize stalled forks and prevent their collapse into DSBs, a function that is well supported by studies in yeast, Xenopus extracts, and mammalian cells in which ATR or ATR orthologs have been mutated or depleted (10–13, 17–19, 21, 58). However, in instances of ATR pathway failure, DSBs are generated, stimulating the ATM- and DNA-PKcs-dependent phosphorylation of H2AX (Fig. 3). The co-dependence of H2AX phosphorylation on ATM and DNA-PKcs (Fig. 3) indicates that H2AX represents an important nexus at which these two DSB-responsive kinases converge.

Consistent with H2AX playing a facilitative role in replication fork stability by promoting homologous recombination-mediated fork restart, the increased focal accumulation of Rad51 in ATR-deleted cells was largely dependent on H2AX (Fig. 4). Furthermore, synergistic increases in chromatid breaks and translocations were observed in ATR-deleted cells that lacked H2AX, suggesting that the absence of H2AX enhanced persistence of DSBs and their accessibility to error-prone DNA repair mechanisms (Figs. 5 and 6). Thus, whereas ATR is an important player...
**ATR and H2AX Cooperatively Maintain Genome Stability**

In preventing replication fork instability, our data are consistent with ATM, DNA-PKcs, and H2AX providing supportive roles by acting in concert as a salvage pathway to assist in repairing collapsed replication forks and restoring DNA replication (Fig. 7).

The mechanism by which the ATR pathway preserves replication fork stability is unclear (59); however, several studies have indicated functions both in preserving the replication fork structure and in stimulating restart. Studies on ATR orthologs in yeast and ATR-depleted *Xenopus* extracts indicate that ATR regulates the continued association of replicative DNA polymerases at the fork, both upon stalling and during restart following camptothecin-mediated collapse (17, 21, 58). In addition, it has been shown that Chk1, a conventional protein kinase regulated by ATR, is required for the efficient formation of Rad51 foci following ionizing radiation-induced damage (27). In light of these findings, it is conceivable that the increased level of Rad51 accumulation in ATR-deleted cells (Fig. 4) is underrepresented, due to deficiencies in Chk1 activation. Our results in no way contradict the possibility that ATR plays important roles both in the prevention of fork collapse and reinitiation of replication. However, because Rad51 foci in ATR<sup>−/−</sup> cells increase significantly over that observed in wild-type cells (Fig. 4B), our data further supports a role for ATR in preventing the emergence of DSBs following replication fork stalling. In this context, H2AX assumes an ATR-independent support function that assists in Rad51 accumulation and the suppression of persistent DSBs.

Although several lines of evidence demonstrate that H2AX plays a facilitative, but non-essential, role in homologous recombination (43, 44), the exact mechanism of its involvement remains unclear. Rad51 accumulation at DSBs is not fully dependent on H2AX (43, 48); however, several studies have indicated a partial requirement (43, 45–51). For example, focal accumulation of BRCA1, which is required for efficient Rad51 foci formation, is reduced in H2AX-deficient murine cells (43, 51). Similarly, general ubiquitination of chromatin and the focal accumulation of ubiquitinated-FANCD2 following DNA damage is strongly impaired by the absence of H2AX, and, these events appear to be required for accumulation of Rad51 at damage sites (45–47, 49, 50). On the other hand, in DT40 cells, the involvement of H2AX phosphorylation in Rad51 recruitment is predominantly early in the DSB response and is strongly compensated for by an H2AX-independent mechanism catalyzed by XRCC3 (48). An H2AX-dependent mechanism for Rad51 accumulation that is distinct from the relatively well-defined core pathway (BRCA1, BRCA2, XRCC3, etc.) is additionally supported by the further suppression of homologous recombination achieved by combining H2AX deficiency with hypomorphic levels of BRCA1 and Rad51 (44).

Regardless of the specific mechanism of involvement, the accumulation of Rad51 resulting from ATR deletion appears to largely rely on H2AX (Fig. 4). Consistent with decreased Rad51-mediated HR and elevated usage of error-prone repair, combined deletion of ATR and H2AX led to a significant increase in translocation events. It is not clear at the present time whether increased translocations represent 1) default repair mechanisms acting under conditions of decreased HR, or 2) the products of misdirected ATM and DNA-PKcs activity in the absence of H2AX.

Together, our data are consistent with a layered checkpoint response governing genome stability upon replication stress. According to the model described herein, this process is regulated by two main functional groups of proteins, the ATR fork-stabilizing pathway and the ATM-, DNA-PKcs-, and H2AX-
mediated DSB salvage pathway. Given the large number of genes associated with each of these networks (the ATR- and ATM-dependent checkpoint pathways, the Rad52 epistasis group, ubiquitin transfer complexes, etc.), combinations of synthetic interactions between these two groups may occur frequently in human populations.

Both ATR and H2AX have been implicated in suppressing cancer (18, 60–62). Because combined reductions in fork stabilizing and restart pathways can lead to dramatic increases in DSBs during normal DNA synthesis, such combined deficiencies would be expected to strongly influence the onset of age-related diseases (via tissue degeneration) and cancer (via tumor suppressor gene loss of heterozygosity, translocation events, etc.). Thus, our studies are consistent with the genetic etiology of cancer and other age-related diseases residing both in specific mutations and their context within deficiencies in networks that govern upstream stabilization or salvage pathways.

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