Spliceosome Mutations Induce R Loop-Associated Sensitivity to ATR Inhibition in Myelodysplastic Syndromes

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Abstract

Heterozygous somatic mutations in spliceosome genes (U2AF1, SF3B1, ZRSR2, or SRSF2) occur in >50% of patients with myelodysplastic syndrome (MDS). These mutations occur early in disease development, suggesting that they contribute to MDS pathogenesis and may represent a unique genetic vulnerability for targeted therapy. Here, we show that RNA splicing perturbation by expression of the U2AF1(S34F) mutant causes accumulation of R loops, a transcription intermediate containing RNA:DNA hybrids and displaced single-stranded DNA, and elicits an ATR response. ATR inhibitors (ATRi) induced DNA damage and cell death in U2AF1(S34F)-expressing cells, and these effects of ATRi were enhanced by splicing modulating compounds. Moreover, ATR-induced DNA damage was suppressed by overexpression of RNaseH1, an enzyme that specifically removes the RNA in RNA:DNA hybrids, suggesting that the ATRi sensitivity of U2AF1(S34F)-expressing cells arises from R loops. Taken together, our results demonstrate that ATRi may represent a novel therapeutic target in patients with MDS carrying the U2AF1(S34F) mutation and potentially other malignancies harboring spliceosome mutations.

Significance: This study provides preclinical evidence that patients with MDS or other myeloid malignancies driven by spliceosome mutations may benefit from ATR inhibition to exploit the R loop–associated vulnerability induced by perturbations in splicing. Cancer Res. 78(18): 5363–74. ©2018 AACR.

Introduction

Myelodysplastic syndrome (MDS), a heterogeneous group of clonal hematopoietic stem cell disorders, is the most common adult myeloid malignancy (1). MDS is characterized by peripheral blood cytopenias and progenitor expansion, and can transform to chemoresistant secondary acute myelogenous leukemia (sAML; ref. 2). Somatic heterozygous mutations in the spliceosome genes such as U2AF1, SF3B1, SRSF2, and ZRSR2 occur in over 50% of patients with MDS and are mutually exclusive (3–11). We and others have identified U2AF1 (U2 small nuclear RNA auxiliary factor) mutations in 11% of patients with MDS, making it one of the most commonly mutated genes in this disease (3, 4). U2AF1 mutations are typically present in the founding clones of MDS tumors, suggesting that U2AF1 mutations are important in disease initiation. Although the mechanism by which U2AF1 mutations contribute to MDS pathogenesis remains unclear, the occurrence of U2AF1 mutations early in disease development suggests that these are likely driver mutations (9, 10), raising the possibility that they generate specific oncogenic stresses.

Bone marrow cells from patients and genetically engineered mouse models carrying spliceosome mutations displayed RNA splicing abnormalities (12–24). Expression of MDS-associated U2AF1, SF3B1, ZRSR2, or SRSF2 mutants in cell lines also altered RNA splicing. These studies suggest that cells harboring spliceosome mutations have altered splicing programs, which may contribute to MDS pathogenesis. Intriguingly, hotspot mutations in different spliceosome proteins (U2AF1, S34F/Y or Q157P/Q; SF3B1, K700E; SRSF2, P95H) altered splicing of distinct groups of transcripts, raising an important question as to how these spliceosome mutations converge on similar disease mechanisms. We recently demonstrated that RNA splicing perturbation by either pharmacologic modulation or expression of the U2AF1 S34F (U2AF1S34F) mutant increased levels of R loops, a transcription intermediate containing an RNA:DNA hybrid and displaced single-stranded DNA (ssDNA; ref. 25). Although R loops have physiological functions, aberrant levels and distributions of R loops are associated with genomic instability (26–28). Because RNA splicing normally occurs in a transcription-coupled manner, splicing perturbations may interfere with transcription elongation and increase R loop formation (29). The associations of RNA splicing perturbation, R loop accumulation, and genomic instability...
prompted us to investigate whether the spliceosome mutations in MDS generate a common vulnerability that can be exploited therapeutically.

Replication protein A (RPA), a ssDNA-binding heterotrimeric complex, has diverse functions in DNA replication, DNA repair and other cellular processes (30). During responses to DNA damage and replication problems, RPA functions as a key sensor of ssDNA at sites of DNA damage and stalled DNA replication forks. RPA-coated ssDNA (RPA-ssDNA) acts as a platform to recruit the ATR checkpoint kinase and its regulators and substrates (31). We recently found that RPA is present at R loops and is important for R loop suppression through its interaction with RNaseH1, an enzyme that specifically removes the RNA moiety within RNA:DNA hybrids (25). Given the role of RPA as a master sensor of genomic stress arising from diverse sources, our results raised the possibility that the RPA at R loops may enable ATR to respond to aberrant R loops or the genomic instability that they induce.

Here, we report that cells expressing mutant splicing factors accumulated R loops and elicited an R loop-associated ATR response. ATR inhibition using specific ATR inhibitors (ATRi) induced more DNA damage in cells expressing the U2AF1S34F mutant than in cells expressing wild-type U2AF1 (U2AF1WT), killing U2AF1S34F-expressing cells preferentially. The spliceosome modulator E7107, which specifically targets the SF3B1 complex, induced further R loop accumulation and an ATR response in U2AF1S34F-expressing cells, rendering cells more sensitive to ATRi. Consequently, combination of E7107 and ATRi induced significantly higher levels of DNA damage in U2AF1S34F-expressing cells compared with U2AF1WT-expressing cells, resulting in an increase in apoptosis. Finally, expression of RNaseH1 attenuated the E7107+ATRi-induced DNA damage in U2AF1S34F-expressing cells, suggesting that the DNA damage induced by E7107 and ATRi arises from R loops. These results suggest that ATR plays an important role in suppressing the R loop-associated genomic instability in U2AF1S34F-expressing cells and maintaining cell viability.

Altogether, our results provide a preclinical rationale to test ATR inhibitors in MDS and other myeloid malignancies driven by the U2AF1S34F mutation. Furthermore, they provide a basis to characterize other spliceosome mutations and possibly exploit the R loop-associated vulnerability induced by splicing perturbations.

Materials and Methods

Cell culture

The HeLa cells used in this study were obtained from Dr. Stephen Elledge’s laboratory, and have been analyzed by RNA-seq. The K562 cells were obtained from the ATCC and have been analyzed by RNA-seq. The OCI-AML3 cells were obtained from DSMZ without any further authentication. All cell lines used in this study were tested for Mycoplasma and passaged for less than 2 months after thawing. HeLa cells were cultured in DMEM supplemented with 10% FBS, 2 mmol/L glutamine, and 1% penicillin/streptomycin. The HeLa-derived cell lines that inducibly express GFP-tagged nuclear RNaseH1 were generated by lentiviral infection and neomycin selection. All HeLa-derived cell lines were cultured in medium supplemented with Gluta-Max (600 µg/mL). RNaseH1-GFP expression was induced by doxycycline (200 ng/mL) for 48 hours. Viruses expressing indicated Flag-tagged wild-type or mutant U2AF1 and SRSF2 containing an IRES-GFP were used to infect HeLa cells (22). The plasmids also contain an IRES-GFP, which was used to sort for transfected cells. K562 cells stably expressing Flag-tagged U2AF1WT and U2AF1S34F containing a P2A-mCherry were grown in RPMI-1640 medium supplemented with 10% FBS, 1× Gluta-Max and 1× penicillin/streptomycin. OCI-AML3 cells carrying doxy-cycline inducible Flag-tagged wild-type or mutant U2AF1 were cultured in α-MEM supplemented with 20% FBS, 1× Gluta-Max and 1× penicillin/streptomycin.

Inhibitors and antibodies

Cells were treated with inhibitors: Pladienolide B (EMD Millipore), E7107 (H3 Biomedicine), ATR inhibitors (VE-821, VE-822, AZ 20; SelleckChem), ATMi (KI55933, SelleckChem). Antibodies used in this study include AQR (A302-547A-T, Bethyl), pChk2 (2661, Cell Signaling Technology), Flag (F7425, Sigma), GADPH (AB516, Millipore), GFP (A11122, Thermofisher Scientific), γH2AX (9718S, Cell Signaling Technology), Ku70 (GTX70271, Genetex), PCNA (sc-56, Santa Cruz Biotechnology), pRPA (A300-246A, Bethyl), U2AF1 (ab197591, Abcam), and S9.6 (Protein A purified from hybridoma S9.6 by Antibodies Incorporated).

RNA extraction, reverse transcription, and alternative RNA splicing assay

RNA material was extracted from cells with RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instructions. Total DNA (2 µg) was used for reverse transcription to cDNA using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by PCR using Platinum Blue PCR Supermix (Invitrogen). Primer sequences for indicated genes are listed in Supplementary Table S1. PCR products were visualized using gel imager G:Box (Syngene) after separation in agarose gel electrophoresis or SDS-PAGE. The intensity of each segregated band was quantified using ImageJ.

Cell viability assay

Cell viability measurement for HeLa-derived, OCI-AML3, and K562-derived cells was performed using Cell TiterGlo and CellTiter 96AQueous One Solution Cell Proliferation Assay Kit (Promega), respectively.

Cell apoptosis assay

Cell apoptosis assay was performed according to the manufacturer’s protocol (BD Biosciences). Briefly, cells were washed once with PBS, followed by addition of fluorophore-conjugated Annexin V and DAPI or 7-AAD. After a 15-minute incubation at room temperature, cells were analyzed using flow cytometric analysis on a Fortessa X-20 (BD Biosciences).

Isolation and ex vivo culture of primary human CD34+ hematopoietic cells

Deidentified umbilical cord blood units (UCB) was obtained from New York Blood Center and was deemed by the center as research-grade. Informed consent was given previously from the donor to the New York Blood Center. The authors in this study have no contacts with donors. The use of UCB was conducted under Institutional Review Board approval from Massachusetts General Hospital (2014P000922/MGH) and were processed within 48 hours after collection. The UCB was diluted at 1:1 ratio at room temperature in 1× PBS (Gibco).
supplemented with 2% FBS and 1 x penicillin/streptomycin. Total mononuclear cells (MNC) were fractionated by gradient centrifugation in Ficoll-Paque Plus (GE Healthcare). After removal of platelet cells and erythrocytes, CD34+ cells were enriched from the total MNCs using autoMACS-positive selection system and CD34 MicroBead Kit UltraPure Human (Miltenyi Biotec) according to the manufacturer’s instructions. The isolated CD34+ cells were recovered overnight in X-Vivo-15 media (Lonza) supplemented by 50 ng/mL of each cytokine (human interleukin-3, stem cell factor, thrombopoietin and FLT-3 ligand) at 37°C in the 5% CO2 incubator. All cytokines were purchased from Peprotech. Isolated CD34+ cells were recovered and assessed by flow cytometry analysis using an APC-conjugated CD34 antibody (BD Biosciences). CD34+ cells were cultured in 24-well plates (2 x 10^5 cells/well) in X-Vivo-15 media supplemented with cytokines and 4 µg/mL of Polybrene (Millipore). Cells were infected with lentiviral vectors carrying U2AF1WT or U2AF1S34F (1,180 ± 104 cells/well) and treated with 4 µg/mL of each cytokine and 50 ng/mL of each cytokine (human interleukin-3, stem cell factor, thrombopoietin and FLT-3 ligand) at 37°C in the 5% CO2 incubator. The relationship between R loops and aberrant splicing in MDS patient bone marrow and shown to be caused by U2AF1S34F-induced alternative RNA splicing occurs independently of R loops.

**Immunofluorescence**

For immunofluorescence in HeLa cells, samples were prepared as previously described (25). For immunofluorescence using phospho-H2AX antibody in K562 and primary CD34+ cells, cells were pelleted at 500 rpm for 5 to 8 minutes, then washed once with PBS. Cells were then fixed with 3% paraformaldehyde/2% sucrose for 15 minutes and cytospun onto coated glass slides. Subsequently, cells were permeabilized with 1x PBS containing 0.5% Triton X-100, and treated in blocking buffer (1x PBS, 3% BSA, 0.05% Tween-20, and 10% milk) before primary antibody incubation for 2 hours at room temperature. After the incubation in primary antibody, cells were washed three times with 1x PBS containing 0.05% Tween-20 and incubated with anti-rabbit secondary antibody for 1 hours. To visualize nuclei, cells were stained with DAPI after the final wash in PBS. The images were captured using a Nikon 90i microscope and analyzed using ImageJ software.

**Results**

**Expression of U2AF1S34F induces an R loop–associated ATR response**

We recently showed that RPA localizes to R loops and interacts with RNaseH1, an enzyme that specifically hydrolyzes RNA:DNA hybrids to promote R loop resolution (25). In the absence of R loop suppressor AQR, R loop levels were elevated, and the RPA32 colocalized with R loops was phosphorylated at Ser33 (RPA32-pS33; p-RPA; ref. 25), suggesting that a kinase is active at R loops. Similar to AQR depletion, expression of U2AF1S34F increased R loops (25), prompting us to test whether U2AF1S34F also activates the kinase that phosphorylates RPA. We generated HeLa cells stably expressing either U2AF1WT or U2AF1S34F at levels below that of endogenous U2AF1 (Supplementary Fig. S1A). Even at levels lower than U2AF1WT and endogenous U2AF1, U2AF1S34F exerted dominant effects and induced R loop accumulation (25). Compared with cells expressing U2AF1WT, U2AF1S34F-expressing cells exhibited higher levels of p-RPA in both immunofluorescence and Western blot (Fig. 1A–C). Similar to that in HeLa cells, expression of U2AF1S34F in the leukemia cell line K562 also induced p-RPA (Fig. 1D). To test whether p-RPA arises from U2AF1S34F-induced R loops, we generated a U2AF1S34F-expressing HeLa cell line that inducibly produces GFP-tagged nuclear RNaseH1 (Supplementary Fig. S1B–C). Induction of RNaseH1 in U2AF1S34F-expressing cells reduced p-RPA staining to a level similar to that in U2AF1WT-expressing cells (Fig. 1A and B), showing that U2AF1S34F induces p-RPA in a R loop-dependent manner. Next, we tested whether U2AF1S34F-induced RPA phosphorylation is mediated by the ATR kinase, which interacts with RPA (31, 32). The p-RPA in U2AF1S34F-expressing HeLa and K562 cells was reduced by an inhibitor of ATR (ATRI; VE-821) in a dose-dependent manner, but not by an inhibitor of the related kinase ATM (ATMi; KU55933; Fig. 1C and D; Supplementary Fig. S1D; refs. 33, 34). In contrast to ATR, ATM was not activated in U2AF1S34F-expressing cells as indicated by the lack of Chk2 phosphorylation (Supplementary Fig. S1E). However, Chk2 was phosphorylated in U2AF1S34F-expressing cells after Camptothecin (CPT) treatment and the phosphorylation was abolished by ATMi (Supplementary Fig. S1E), showing that the ATM pathway is intact in these cells and the ATM is effective. Together, these results suggest that U2AF1S34F expression induces R loop accumulation and activates ATR but not ATM in an R loop-dependent manner.

**U2AF1S34F induces alternative splicing independently of R loops**

The U2AF1-S34F mutation in MDS and AML leads to alternative splicing of a subset of genes, principally by promoting exon skipping and utilization of cryptic 3’ splice sites (4, 12, 35). The relationship between R loops and aberrant splicing in U2AF1S34F-expressing cells is not known. To test whether U2AF1S34F-induced R loops contribute to aberrant splicing, we examined the splice isoform changes in five transcripts (MED24, RMT2D/MLL, BCO1, PICALM, and H2AFY) that were previously identified in MDS patient bone marrow and shown to be caused by U2AF1S34F (Fig. 2A–E; ref. 35). As previously reported, U2AF1S34F expression altered the splicing of these transcripts. However, induction of RNaseH1 in U2AF1S34F-expressing cells did not affect levels of the alternative splice isoforms. These results suggest that U2AF1S34F-induced alternative RNA splicing occurs independently of R loops and associated ATR response. The protein levels of several known suppressors of R loops were similar in U2AF1WT and U2AF1S34F-expressing cells (Supplementary Fig. S2), suggesting that alternative splicing of R loop suppressors is unlikely the cause of U2AF1S34F-induced R loops. Thus, the dominant effects of U2AF1S34F on the spliceosome may induce alternative splicing and R loop accumulation independently.

**U2AF1S34F and other MDS-associated spliceosome mutants sensitize cells to ATRi**

The ATR response triggered by U2AF1S34F expression prompted us to investigate whether ATR is functionally important in U2AF1S34F-expressing cells. We treated U2AF1WT- and

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U2AF1S34F-expressing HeLa cells with ATRi or ATMi for 48 hours, and then measured DNA damage levels using γH2AX as a marker (Fig. 3A). Without altering the cell cycle significantly, ATRi, but not ATMi, induced γH2AX in U2AF1S34F-expressing cells (Fig. 3A; Supplementary Fig. S3A). Furthermore, ATRi induced higher levels of γH2AX in U2AF1S34F-expressing cells than in U2AF1WT-expressing cells (Fig. 3A). ATRi also reduced the viability of U2AF1 S34F-expressing cells more than that of U2AF1WT-expressing cells (Fig. 3B). In contrast, U2AF1WT- and U2AF1S34F-expressing cells exhibited similar sensitivity to ATMi (Fig. 3B). To confirm the results from HeLa cells, we inducibly expressed U2AF1 WT and U2AF1 S34F in the AML cell line OCI-AML3 (Supplementary Fig. S3B; ref. 36). Neither U2AF1WT nor U2AF1S34F rendered OCI-AML3 cells sensitive to ATMi (Fig. 3C). However, compared with U2AF1WT, OCI-AML3 cells, U2AF1S34F-OCI-AML3 cells were more susceptible to growth inhibition by ATRi (Fig. 3C). These results show that ATRi but not ATMi promotes the survival of U2AF1S34F-expressing cells by suppressing DNA damage.

RNA-splicing modulators induce R loops and associated ATR response

Having established that U2AF1S34F induces R loops and associated ATR response, we investigated whether perturbation of RNA splicing by splicing modulators could also activate ATR through induction of R loops. E7107, a splicing modulator that targets the SF3B1 complex, induces splicing alterations such as intron retention (37–39). As previously described, we generated HeLa cell lines that stably express the S34Y, Q157P, or Q157R mutant alleles at similar levels (Fig. 3D). In all the cell lines expressing these U2AF1 mutants, ATRi induced higher levels of γH2AX than in U2AF1WT-expressing cells (Fig. 3D). To test whether mutations in splicing factors other than U2AF1 also sensitize cells to ATRi, we generated stable cells expressing wild-type SRSF2 (SRSF2 WT) and its MDS-associated mutant derivative, SRSF2P95H. SRSF2P95H-expressing cells exhibited higher levels of γH2AX than SRSF2WT-expressing cells in the absence of ATRi (Fig. 3E). ATRi treatment further increased γH2AX levels in SRSF2P95H-expressing cells (Fig. 3E). Collectively, these results suggest that the MDS-associated U2AF1 and SRSF2 mutations create a dependency on ATR to suppress DNA damage.

Figure 1. U2AF1S34F-expressing cells induce R loop-associated ATR-mediated RPA32 phosphorylation. A and B, HeLa cells stably expressing either U2AF1WT or U2AF1S34F were cultured for 24 hours. RNaseH1 expression was induced by the addition of doxycycline as indicated. Individual cells were analyzed by immunofluorescence using an RPA32 phospho-specific S33 (RPA32 pS33) antibody. Representative images are shown in A, and intensities of RPA32 pS33 staining in individual cells were analyzed (n > 350). Red bars, the mean RPA32 pS33 intensities of the indicated cell populations. A.U., arbitrary unit. ***, P ≤ 0.001. C and D, HeLa and K562 cells stably expressing either U2AF1WT or U2AF1S34F were cultured for 48 hours, followed by treatment with either ATRi or ATMi (10 μmol/L) for 1 hour. Levels of indicated proteins were analyzed by Western blot and the ratios of transgenic (tg)/endogenous (endo) U2AF1 are shown.
used the monoclonal antibody S9.6 that specifically recognizes DNA:RNA hybrids to detect nuclear R loops by immunofluorescence (25, 40). E7107-treated HeLa cells showed an increase in S9.6 staining compared with control cells treated with DMSO (Fig. 4A). We also observed colocalization of p-RPA and S9.6 staining in E7107-treated cells but not control cells (Fig. 4B). Notably, p-RPA and S9.6 staining increased together in individual E7107-treated cells (Fig. 4C), suggesting that they reflect two associated events induced by E7107.

To test whether p-RPA arises from E7107-induced R loops, we generated a stable cell line that inducibly expresses GFP-tagged nuclear RNaseH1 (Supplementary Fig. S4A). Expression of RNaseH1 in E7107-treated cells significantly suppressed p-RPA (Fig. 4D). Furthermore, when HeLa cells were first treated with E7107 and then exposed to ATRi, the p-RPA in E7107-treated cells was reduced by ATRi in a concentration-dependent manner (Fig. 4E; Supplementary Fig. S4B). Consistent with these results, Pladienolide B (Plad-B), another splicing modulator that induces R loops (25), also increased p-RPA levels (Fig. 4F). The p-RPA in Plad-B treated cells was eliminated by ATRi but not ATMi (Fig. 4F). Collectively, these results suggest that RNA splicing perturbation by splicing modulating compounds leads to accumulation of R loops and elicits an ATR response.

**RNA splicing modulators sensitize cells to ATRi**

Given the induction of R loops and ATR response by splicing modulators, we asked whether splicing modulators sensitize cells to ATRi. We treated HeLa cells with E7107 for 24 hours in the presence or absence of ATRi or ATMi (Fig. 5A). Only

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**Figure 2.** RNaseH1 overexpression does not affect alternative splicing in U2AF1S34F-expressing cells. Quantification of exon skipping (MED24, KMT2D) in A and B, alternative splice site utilization (BCOR, PICALM) in C and D, or mutually exclusive exons (H2AFY) in E were detected and measured by gel electrophoresis (n = 3 replicates). Representative gel images (middle) and corresponding diagrams of the events measured (bottom) are shown. n.s., nonsignificant; *, P ≤ 0.05; **, P ≤ 0.01.
ATRi but not ATMi induced γH2AX in E7107-treated cells in a dose-dependent manner (Fig. 5A; Supplementary Fig. S5A). Similarly, in Plad-B–treated cells, γH2AX was only induced by ATRi but not ATMi (Fig. 5B). In addition to VE-821, two other ATR inhibitors, AZ20 (ATRi #2) and VE-822 (ATRi #3), also induced γH2AX in Plad-B–treated cells (Fig. 5C). These results suggest that splicing modulators create a dependence on ATR to suppress DNA damage.

To understand how ATR suppresses DNA damage in cells treated with splicing modulators, we analyzed individual cells for γH2AX by immunofluorescence. A significant fraction of the cells treated with Plad-B and ATRi, but not those treated with Plad-B alone, ATRi alone, or Plad-B and ATMi, displayed γH2AX staining (Fig. 5D and E). The majority of the γH2AX-positive cells induced by Plad-B and ATRi showed discrete γH2AX foci, suggesting formation of DNA double-strand breaks (DSB; Fig. 5D). A subset of the cells treated with Plad-B and ATRi displayed strong pan-nuclear γH2AX staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) signals, indicating severe chromosome fragmentation and apoptosis (Fig. 5D and E; Supplementary Fig. S5E). Consistent with the Plad-B results, a significant fraction of E7107–treated cells displayed γH2AX staining in the presence of ATRi (Supplementary Fig. 5D).

The collision between R loops and replication forks is a potential source of DNA damage in cells treated with splicing modulators and ATRi. Consistent with this possibility, E7107 reduced S-phase cells (Supplementary Fig. S5E), indicating a checkpoint response to replication problems. To test whether DNA damage arises during DNA replication in cells treated with both E7107 and ATRi, we compared the effects of these compounds on S-phase and non–S-phase cells (Supplementary Fig. S5F). We used PCNA to distinguish S-phase and non–S-phase cells, and γH2AX to measure DNA damage. E7107 and ATRi induced much more DNA damage in S-phase cells than in non–S-phase cells, supporting the idea that the DNA damage induced by E7107 and ATRi primarily arises from the collision between R loops and replication forks (Supplementary Fig. S5F).

Next, to test whether ATRi affects the survival of cells treated with splicing modulators, we treated HeLa cells with ATRi in the presence or absence of 0.5 nmol/L Plad-B (Fig. 5F). ATMi was tested in parallel with ATRi in this experiment. Plad-B only increased the sensitivity of cells to ATRi but not ATMi. Thus, splicing modulators induce R loops and generate replication problems in S-phase cells, possibly through the collision between R loops and replication forks. Upon the inhibition of ATR, the collision between R loops and replication forks likely give rise to high levels of DSBs, which ultimately drive cells into apoptosis.

E7107 potentiates the ATRi sensitivity of U2AF1S34F-expressing cells

We next tested whether splicing modulators can exacerbate R loop accumulation in cells expressing U2AF1S34F and increase their ATRi sensitivity. We treated U2AF1WT- and U2AF1S34F-expressing cells with E7107 for 24 hours and analyzed R loop levels. The baseline levels of R loops are higher in...
U2AF1S34F-expressing cells than in U2AF1WT-expressing cells (Fig. 6A, lane 1 vs. 3). After E7107 treatment, R loop levels were increased in both U2AF1WT- and U2AF1S34F-expressing cells (Fig. 6A, lane 1 vs. 2, and 3 vs. 4). Importantly, the levels of R loops were much higher in U2AF1S34F-expressing cells than in U2AF1WT-expressing cells (Fig. 6A, lane 2 vs. 4), suggesting that the U2AF1S34F mutant potentiates the induction of R loops by E7107. The increased R loop accumulation in E7107-treated U2AF1S34F-expressing cells was accompanied by an increase in p-RPA (Fig. 6B). Suppression of R loops by RNaseH1 in E7107-treated U2AF1S34F-expressing cells reduced p-RPA levels (Fig. 6B), suggesting that the increased of p-RPA reflects an R loop-associated ATR response. The combination of E7107 and ATRi (E7107 + ATRi) induced higher levels of γH2AX in U2AF1S34F-expressing cells than in U2AF1WT-expressing cells (Fig. 6C). The γH2AX levels in U2AF1S34F-expressing cells treated with E7107 + ATRi were also higher than those in cells treated with either E7107 or ATRi alone. Importantly, induction of RNaseH1 in U2AF1S34F-expressing cells treated with E7107 and ATRi significantly reduced γH2AX levels (Fig. 6D), showing that the DNA damage induced by E7107 and ATRi arises from R loops. Consistent with the increase in γH2AX, ATRi reduced the viability of U2AF1S34F-expressing cells more than that of U2AF1WT-expressing cells in the presence of E7107 (Fig. 6E). The combination of E7107 and ATRi increased apoptosis more efficiently in U2AF1S34F-expressing cells than in U2AF1WT-expressing cells (Fig. 6F and G; Supplementary Fig. S6). Therefore, splicing modulators increase R loop levels in cells expressing U2AF1S34F, rendering them more sensitive to ATRi.

ATRi induces DNA damage in primary human CD34+ hematopoietic cells expressing U2AF1S34F

Because spliceosome mutations often occur in the founding clones of MDS, we next asked whether U2AF1S34F
expression in primary human hematopoietic progenitor cells sensitizes them to ATR inhibition. CD34\(^+\) hematopoietic progenitor cells were isolated from human UCB (Fig. 7A). Using \(\gamma\)-irradiated CD34\(^+\) progenitor cells, we first confirmed that the formation of DSBs in these cells can be reliably monitored with \(\gamma\)H2AX immunofluorescence (Supplementary Fig. S7). Next, we expressed GFP-tagged U2AF1 WT and U2AF1S34F in CD34\(^+\) progenitor cells by lentiviral infection. The CD34\(^+\) cells expressing U2AF1WT or U2AF1S34F were isolated by cell sorting and R-loop staining was performed. Consistent with our observation in HeLa and K562 cells, U2AF1S34F-expressing CD34\(^+\) cells displayed higher levels of R loops compared with U2AF1WT-expressing CD34\(^+\) cells (Fig. 7B). Next, we treated the two CD34\(^+\) cell populations with ATRi and used \(\gamma\)H2AX to measure the induction of DNA damage. ATR inhibition in U2AF1WT-expressing CD34\(^+\) cells did not significantly alter \(\gamma\)H2AX levels (Fig. 7C, lanes 1 vs. 2). However, ATRi caused a significant increase of \(\gamma\)H2AX in U2AF1S34F-expressing CD34\(^+\) cells compared with DMSO-treated U2AF1S34F-expressing cells (Fig. 7C, lanes 3 vs. 4) and ATRi-treated U2AF1WT-expressing cells (Fig. 7C, lanes 2 vs. 4). Finally, we used Annexin V staining to compare the ATRi induced cell death in the two CD34\(^+\) cell populations. ATRi inhibition, in a dose-dependent manner, induced higher levels of cell death in U2AF1S34F-expressing CD34\(^+\) cells than in U2AF1WT-expressing CD34\(^+\) cells (Fig. 7D). Collectively, these results demonstrate that expression of U2AF1S34F in human primary CD34\(^+\) hematopoietic progenitor cells indeed induces R loops, rendering them susceptible to ATR inhibition.

**Discussion**

Growing evidence has shown that defects of RNA processing factors, including spliceosome components, trigger R loop accumulation and R loop-associated genomic instability (26–28). We previously reported that expression of the MDS-associated U2AF1S34F mutant caused accumulation of R loops (25). Here, we present evidence that the R loop accumulation in U2AF1S34F-expressing cells elicits an ATR response, which is consistent with a recent report (41). We further show that R loop accumulation is not required for the splicing alterations induced by U2AF1S34F expression. Whether splicing alterations contribute to R loop accumulation is still unclear. Because splicing factor mutations often arise in the founding clones of MDS, both R loops and splicing alterations may contribute to initiation of the disease (Fig. 7E). R loops can not only induce genomic instability but also alter gene expression. Although R loop accumulation alone may not be sufficient to promote wide-spread genomic instability in MDS, which is characterized by a low mutation burden (42, 43), aberrant R loops may potentiate specific oncogenic events. Importantly, aberrant R loops become a significant source of DNA damage when ATR is inhibited. The ATRi-induced and R loop-associated DNA damage in U2AF1S34F-expressing cells is...
sufficient to reduce cell viability. Our results collectively suggest a strategy to therapeutically target the MDS cells harboring U2AF1 and SRSF2 mutations. Future studies will be necessary to test the ability of other MDS-associated splicing factor mutations to induce R loops and R loop-associated ATRi sensitivity.

RPA is a crucial sensor of DNA replication stress and DNA damage in eukaryotic cells (30). At stalled replication forks and resected DSBs, RPA-ssDNA acts as a key platform to recruit the ATR kinase and its regulators and substrates. Like replication forks and repair intermediates, R loops contain ssDNA. We recently showed that the RPA associated with R loops is phosphorylated at S33 (25). Here, we show that RPA is phosphorylated by ATR at R loops. How ATR is activated at R loops remains to be elucidated. The most straightforward possibility is that the RPA–ssDNA in R loops recruits the ATR–ATRIP complex, which in turn phosphorylates RPA. It is also possible that R loops need to be processed by certain repair and recombination factors to trigger the ATR response. Additional proteins are likely required to activate ATR at R loops. In S phase, R loops may activate ATR by stalling replication forks (44). Regardless of how ATR is activated at R loops, its function is needed to suppress R loop-associated DNA damage. Consistent with this notion, the combination of ATRi and E7107 induced DNA damage primarily in S-phase cells. In the absence of ATR, R loops may lead to collapse of DNA replication forks or be aberrantly processed by nucleases. These events could give rise to DSBs, driving cells into apoptosis. Therefore, ATR inhibition abrogates an important protective mechanism at R loops, leaving R loops to generate DNA damage and...
promote cell death (Fig. 7E). The synthetic lethal relationship between R loop accumulation and ATR inhibition may have broad implications in cancer therapy.

Heterozygous somatic mutation in the spliceosome genes such as U2AF1, SF3B1, SRSF2, or ZRSR2 occur in over 50% of patients with MDS (3–10). These spliceosome mutations occur in the founding clones of MDS, implicating RNA splicing perturbation in disease initiation. Intriguingly, the splicing alterations observed in U2AF1 mutant cells are distinct from those in SF3B1 and SRSF2 mutant cells (13–24). In addition, we and others showed that U2AF1S34F/Y and U2AF1Q157P/R mutants induced alternative splicing in different transcripts (17, 42). These findings raise an important question as to how mutually exclusive spliceosome mutations converge on similar MDS phenotypes. Here, we show that ATRi treatment of cells expressing the MDS-associated U2AF1 and SRSF2 mutants induced DNA damage. These results raise the possibility that aberrant R loop accumulation may be a common vulnerability in MDS cells harboring spliceosome mutations. In addition, it is tempting to speculate that various spliceosome mutations in MDS may promote disease initiation through R loop induction. In future studies, it will be important to test whether other

Figure 7.
U2AF1S34F expression in primary CD34+ hematopoietic progenitor cells confer sensitivity to ATR inhibition. A, Representative flow cytometry plots of GFP-sorted cell populations primary CD34+ hematopoietic progenitor cells from human UCB that were isolated and infected with lentiviruses expressing either U2AF1WT or U2AF1S34F. The x-axis depicts GFP expression. The y-axis is autofluorescence (used for gating). B, Cells were isolated, cultured for an additional 72 hours, and subjected to immunofluorescence analysis using an S9.6 antibody. Black bars, the median S9.6 intensities of the indicated cell populations. **, P < 0.01. C, Cells were either treated with DMSO or ATRi (1 μmol/L) for 2 days, followed by cell sorting and cytospin. Intensities of γH2AX staining in individual cells were analyzed by immunofluorescence from three biological replicates. Black bars represent the mean γH2AX intensities of the indicated cell populations (n > 180). **, P < 0.01; ***, P < 0.001; n.s., nonsignificant. D, Primary CD34+ hematopoietic progenitor cells from human UCB were isolated similar to A. After 24 hours recovery, cells were seeded and treated with indicated concentrations of ATRi for 48 hours. The percentage of cell death was analyzed by Annexin V staining. **, P < 0.01; n.s., nonsignificant. E, RNA splicing perturbation in U2AF1S34F cells potentially induces two independent pathways. First, cells expressing U2AF1S34F mutant induce RNA splicing changes that may promote tumorigenesis. Second, U2AF1S34F cells also accumulate R loops, which elicit an ATR-mediated response. Inhibition of ATR increases DNA damage and ultimately cell death. The addition of spliceosome modulators, Plad-B or E7107, further enhances R loop accumulation and an ATR-mediated response.

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MDS-associated spliceosome mutants also induce R loops, and whether these R loops have common functional effects in myeloid progenitor cells. Furthermore, it will be important to go beyond cell models and directly test these hypotheses in patients with MDS and genetic models. Regardless of whether and how R loops contribute to MDS pathogenesis, they may create a common dependency of MDS cells on ATR for survival, rendering ATR a potential therapeutic target in patients with MDS carrying spliceosome mutations. Although our current results are limited to U2AF1 and SRSF2 mutations, our study provides a rationale to characterize other spliceosome mutations and a possibly way to target them in therapy.

Both splicing modulators and ATR inhibitors have already advanced into clinical trials for different cancers (45–47). Splicing modulator therapy is being pursued in patients with MDS with spliceosome mutations who have failed first-line therapies (NCT02841540). ATR inhibitors (VX-970 and AZD6738) are currently being tested in several clinical trials in patients with advanced solid malignancies (46). Our results suggest that ATR inhibitors may also benefit patients with MDS with U2AF1 and SRSF2 mutations. Furthermore, a recent pan-cancer analysis suggested that as many as 119 splicing factor genes are recurrently mutated and may contribute to oncogenesis not only in MDS but also in other cancers, including AML, CLL, lung adenocarcinoma, head and neck squamous cell carcinoma, endometrial carcinoma, bladder urothelial carcinoma, breast adenocarcinoma, and colorectal carcinoma (48, 49). In future studies, it will be important to address whether all these cancer-associated splicing factor mutations induce R loops. If aberrant R loop accumulation is common in cancer cells harboring spliceosome mutations, ATR inhibitors may have therapeutic potential in a broad spectrum of cancers. Notably, combinations of splicing modulators and ATRi killed U2AF1S34F-expressing cells more effectively than splicing modulators or ATRi alone. Our results suggest that the mechanisms of action for ATRi and splicing modulators are distinct. Although splicing modulators promote R loop accumulation, ATRi allows R loops to induce DNA damage (Fig. 7E). These findings suggest that splicing modulators can be used to potentiate the effects of ATRi in the presence of spliceosome mutations, making these drugs more effective in cancer cells at tolerable concentrations. Together, our studies establish a scientific rationale to test ATR inhibitors and splicing modulators in patients with MDS carrying U2AF1 and SRSF2 mutations, providing a therapeutic strategy that could potentially be applied to other cancers with spliceosome mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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