The core spliceosome as target and effector of non-canonical ATM signalling

Maria Tresini1, Daniël O. Warmerdam2, Petros Kolovos3, Loes Snijder1, Mischa G. Vrouwe4, Jeroen A. A. Demmers5, Wilfred F. J. van IJcken6, Frank G. Grosveld3, René H. Medema2, Jan H. J. Hoeijmakers1, Leon H. F. Mullenders4, Wim Vermeulen1 & Jurgen A. Marteijn1

In response to DNA damage, tissue homoeostasis is ensured by protein networks promoting DNA repair, cell cycle arrest or apoptosis. DNA damage response signalling pathways coordinate these processes, partly by propagating gene-expression-modulating signals. DNA damage influences not only the abundance of messenger RNAs, but also their coding information through alternative splicing. Here we show that transcription-blocking DNA lesions promote chromatin displacement of late-stage spliceosomes and initiate a positive feedback loop centred on the signalling kinase ATM. We propose that initial spliceosome displacement and subsequent R-loop formation is triggered by pausing of RNA polymerase at DNA lesions. In turn, R-loops activate ATM, which signals to impede spliceosome organization further and augment ultraviolet-irradiation-triggered alternative splicing at the genome-wide level. Our findings define R-loop-dependent ATM activation by transcription-blocking lesions as an important event in the DNA damage response of non-replicating cells, and highlight a key role for spliceosome displacement in this process.

The DNA damage response (DDR), an intricate protein network that promotes DNA repair, translesion synthesis, cell cycle arrest or apoptosis, has evolved to counteract the detrimental effects of DNA lesions1–3. At the core of the DDR, the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) signalling pathways coordinate these processes in response to distinct types of DNA damage: ATR to single-stranded DNA damage, and ATM to double-strand DNA breaks (DSBs) and chromatin modifications4,5,6. These signalling networks utilize post-translational modifications and protein–protein interactions to elicit the initial stages of the cellular response. Later DDR stages involve changes in gene expression. Emerging evidence supports that DNA damage influences not only the expression levels of its target genes, by altering transcription rates and mRNA half-life, but also exon selection and ultimately their coding potential5,7.

Production of mature, protein-coding transcripts depends on the selective intron removal catalysed by the spliceosome, a dynamic ribonucleoprotein complex consisting of five small nuclear ribonucleoprotein (snRNP) complexes (U1, U2, U4, U5 and U6), and a large number of accessory proteins7,8. Exon/intron definition by U1 and U2 snRNPs stimulates the recruitment of the pre-assembled U4/U6.U5 tri-snRNP and numerous non-snRNP proteins. Following U1/U4 displacement and extensive conformational rearrangements, the two-step splicing reaction is catalysed by the mature, catalytically active spliceosome composed of U2, U5 and U6 snRNPs8.

The vast majority of mammalian genes are alternatively spliced to produce multiple mRNA variants from a single gene5, thus expanding protein diversity. Numerous mechanisms have evolved to provide the spliceosome with the plasticity required for selective exon inclusion, without compromising splicing fidelity9. These range from the presence of cis-acting elements on the transcript itself to post-translational modifications of spliceosomal proteins, which are subject to intracellular and environmental cues. Additionally, since most introns are spliced co-transcriptionally within the chromatin environment, splicing decisions are subject to spatiotemporal control imposed by transcribing polymerases and interaction with chromatin remodelers and histone marks10–12. Exon selection is also influenced by DNA damage6,13. There is evidence for a broad range of damage-induced alternative splicing events, including alternative exon inclusion and exon skipping, and production of proteins with altered (often pro-apoptotic) function13–16. DNA-damage-induced alternative splicing has been attributed to changes in the processivity rate of RNA polymerase16 (kinetic coupling), or changes in the interactions between the polymerase and splicing regulators4,15 (recruitment coupling), under the assumption that the core spliceosome is largely unaffected. Here we present evidence that DNA damage triggers specific profound changes in spliceosome organization, primarily that of late-stage spliceosomes. Additionally, we identify reciprocal regulation between ATM-controlled DDR signalling and the core spliceosome, and show that in response to transcription-blocking DNA lesions, non-canonical ATM activation contributes to the selection of genetic information ultimately included in mature transcripts.

DNA damage targets core spliceosomes

To gain mechanistic insight on the influence of DNA damage to chromatin-associated DDR processes, we used stable isotope labelling with amino acids in cell culture (SILAC)-based quantitative proteomic analysis17 to characterize ultraviolet (UV)-irradiation-triggered changes in chromatin composition (Extended Data Fig. 1a–c). Indirect effects of replication stress were avoided by use of quiescent, human dermal fibroblasts (HDFs). UV-induced photolesions inhibit transcription by impeding RNAPII progression, and as anticipated we observed a UV-dependent chromatin-depletion of core splicing factors. Surprisingly though, this depletion was selective; chromatin abundance of all detected U2 and U5 snRNP splicing factors was...
Figure 1 | DNA-damage-triggered chromatin displacement of activated spliceosomes. a, b, UV-induced changes in chromatin association of spliceosome components in quiescent HDFs. a, Immunoblots (right) and quantification (left) of splicing factor–chromatin association. b, Chromatin-associated snRNAs assayed by quantitative PCR (qPCR) and normalized to HotAir non-coding RNA (n = 4, mean ± s.d., t-test). c, d, Immunoblots (right) and quantification (left) of splicing factor–chromatin association in U2OS cells. c, Time post UV irradiation. d, UV dose-response and lack of influence of the proteasome inhibitor MG132. Graphs in c, d show signal intensities normalized to H2A (n = 3, mean ± s.d., t-test and one-way ANOVA). ***P < 0.01, ****P < 0.001.

The proteomic results were validated by chromatin fractionation and immunoblotting, for U1 (U1A, U1C), U2 (SF3a1, SF3b2), U4 (PRP3, NHP2L1) and U5 (SNRNP40, PRP8) snRNPs–specific proteins (Fig. 1a). We also assayed by qPCR the chromatin association of all spliceosomal snRNAs. UV irradiation resulted in preferential chromatin depletion of U2, U5 and U6 snRNAs, while U1 and U4 were essentially unaffected (Fig. 1b). Depletion of U2 and U5 snRNPs proteins was time- (Fig. 1c) and dose-dependent (Fig. 1d), but independent of proliferation status and cell type (Fig. 1a, c, d). Chromatin-depletion of U2 and U5 snRNPs splicing factors was independent of proteasome activity (Fig. 1d), suggesting that depletion was not caused by splicing factor degradation but rather by relocation. In agreement, total cellular levels of all tested splicing factors were unaffected by DNA damage (Extended Data Fig. 1e). Splicing factor relocation was verified by immunofluorescence microscopy in cells in which DNA damage was inflicted in a small subnuclear area. A representative example in Fig. 2a depicts depletion of the U5-associated protein SNRNP40 from DNA damage sites that were identified by cyclobutane pyrimidine dimer (CPD) immunodetection. Re-localization was monitored in real-time, using validated cell lines (Extended Data Fig. 2a–d) stably expressing GFP-tagged members of U2 (SF3a1) and U5 (SNRNP40, PRP8) snRNPs. Subnuclear damage infliction by UVC microbeam irradiation resulted in rapid depletion from irradiated sites of GFP-tagged U2 and U5 snRNPs splicing factors but not of U1 and U4 (Fig. 2b and Extended Data Fig. 3a–c). Inhibition of transcription-initiation prevented this depletion indicating that the displaced proteins were actively involved in splicing (Extended Data Fig. 3d). Irradiation of the entire cell resulted in prominent changes in splicing factor localization as evidenced by speckle reorganization and enlargement (Extended Data Fig. 4a, b). To further investigate the relocation kinetics of GFP-tagged SFs, we measured their mobility by fluorescence recovery after photobleaching (FRAP). We observed substantial and UV-dose-dependent increases in the mobility of U2 and U5 snRNPs factors...
but not of U1 and U4, at 1 hour post-irradiation (Fig. 2c, d). In agreement with the chromatin fractionation assays (Fig. 1d), mobilization was independent of proteasome activity, confirming that the UV-triggered mobilization is not caused by proteasome-dependent degradation (Extended Data Fig. 5d).

The UV-dependent chromatin depletion of snRNAs and proteins participating in late-stage spliceosomes, loss of association with elongating RNAPII (Extended Data Fig. 1f), rapid displacement from DNA damage sites and mobilization of U2 and U5 snRNP factors, indicate that UV irradiation influences late-stage RNAPII-associated spliceosomes.

**DNA-damage–specific spliceosome mobilization**

Next we used FRAP to address whether spliceosome mobilization is caused by specific DNA lesions or is a general response to macromolecular damage. Significant splicing factor mobilization was caused by genotoxins inflicting transcription-blocking DNA lesions (UV irradiation, Iulldin S), but not oxidative damage (tert-butyl-hydroxide, rotenone, ionizing radiation), DSBs (ionizing radiation) or DNA inter-strand crosslinks (mitomycin C). This specificity argues that the observed mobilization does not result from non-specific RNA/DNA damage but only from DNA lesions that interrupt transcription (Fig. 3a and Extended Data Fig. 5a, b) and are substrates of the transcription-coupled nucleotide excision repair (TC–NER) pathway20,21. Notably, HDFs deficient in either TC–NER, or global-genome (GG)-NER (lacking CSB and XPC activities respectively), or in both (lacking XPA), show no impairments either in damage-triggered spliceosome mobilization (Fig. 3b) or in chromatin-displacement of endogenous U2 and U5 snRNP splicing factors (Extended Data Fig. 5c). Thus, the influence of transcription-blocking lesions in splicing factor localization is independent from NER complex assembly indicating that pausing of elongating RNAPII is necessary and sufficient to trigger chromatin displacement of late-stage spliceosomes.

**Spliceosome mobilization by DDR signals**

Transcription inhibition by chemicals that target RNAPII mobilize splicing factors of all snRNPs, unlike UV irradiation that preferentially targets those participating in late-stage complexes (Fig. 4b and Extended Data Fig. 5e). This preferential mobilization implies distinct mechanisms of action between UV-irradiation-dependent and chemically-induced transcription inhibition. However, to formally exclude the possibility that transcription-blocking DNA lesions mobilize spliceosomes exclusively through RNAPII arrest, we used 5,6-dichloro-1-β-β-ribofuranosyl-benzimidazole (DRB) to inhibit transcription to the same extent as UV irradiation. Transcription arrest was evaluated by measuring reduced 5-ethyluridine (5EU) incorporation into newly synthesized RNA (Fig. 4a). Both treatments increased spliceosome mobility (Fig. 4b and Extended Data Fig. 6a) and their combination had an additive effect (Extended Data Fig. 6b). Notably, UV irradiation had a more profound splicing-factor-mobilizing effect than DRB (at equal transcription-inhibiting doses), indicating that transcription inhibition alone is not sufficient to attain the extensive mobilization triggered by UV irradiation (Fig. 4b and Extended Data Fig. 6a).

Pausing of RNAPII at DNA lesions not only halts transcription, but also activates DDR signalling pathways that modulate the cellular response via post-translational modifications22. Considering that many core splicing factors have been identified as DDR-kinase substrates22,23, we used the broad-range DDR-kinase inhibitor caffeine to evaluate if DDR signalling influences spliceosome organization. Caffeine partially suppressed the UV-dependent spliceosome mobilization but had no influence on the DRB-dependent mobilization, confirming that the two processes are, in part, mechanistically distinct (Fig. 4d).

To dissect which DDR signalling system augments the UV-triggered spliceosome mobilization, cells were treated with specific inhibitors of the major caffeine-sensitive DDR kinases: ATM, ATR and DNA-dependent protein kinase (DNA-PK). Neither ATR nor DNA-PK inhibition had a significant effect (Fig. 4c and Extended Data Fig. 6d). Surprisingly, ATM inhibition in non-replicating cells suppressed splicing factor mobilization to levels similar to caffeine (Fig. 4c and Extended Data Fig. 6d), while it had no influence on DRB-mediated mobilization (Extended Data Fig. 6h). The dependency of UV-triggered spliceosome mobilization on ATM signalling was confirmed by the impaired splicing factor mobilization in HDFs derived from an ataxia telangiectasia patient compared to those of a healthy donor (Fig. 4e and Extended Data Fig. 6c). Thus DNA-damage-triggered spliceosome mobilization results from the combined contribution of transcription inhibition and ATM signalling.

To evaluate the impact of ATM-dependent spliceosome mobilization on pre-mRNA processing, we assessed splicing efficiency in a select panel of DDR- and cell-cycle-related genes24. Quiescent RPE cells were UV irradiated in the absence or presence of the ATM inhibitor and intron retention was assayed by reverse-transcription PCR (RT–PCR)24. UV irradiation resulted in increased ATM-dependent intron retention (Fig. 4f and Extended Data Fig. 6f), while transcription inhibition by DRB had minimal, and ATM-independent, effects. Specificity of the ATM inhibitor was confirmed by small-interfering RNA (siRNA)-mediated ATM silencing which gave identical results (Extended Data Fig. 6e).

To investigate the genome-wide influence of UV irradiation on alternative splicing, as well as the ATM contribution in UV-irradiation-dependent gene expression and mRNA processing changes, we performed RNA sequencing (RNA-sequencing) on cells that were untreated or UV-irradiated in the presence or absence of the ATM inhibitor. We observed that a substantial number of UV-induced gene expression changes depend on ATM activity (Extended Data Fig. 6g), revealing a previously unknown contribution of ATM signalling in the UV-regulated transcriptome. Importantly, UV irradiation resulted in widespread splicing changes, a subset of which (up to 40%) was partly ATM-dependent, demonstrating the genome-wide influence of ATM not only in mRNA abundance but also in UV-induced alternative splicing (Fig. 4g and Supplementary Table 2).

Collectively, these findings demonstrate that UV irradiation influences gene expression in an ATM-dependent manner, and that ATM
Figure 4 | ATM modulates spliceosome mobilization and influences splicing decisions upon DNA damage. a, RNA synthesis measured by SEU pulse labelling (n = 150, mean ± s.e.m., t-test). Top, representative images obtained at 20× magnification. Bottom, graph of quantification (n = 150, mean ± s.e.m., t-test). b–e, FRAP of splicing factors in quiescent HDFs (n = 25, mean ± s.e.m., one-way ANOVA). b, Response to UV or DRB treatment. c–e, SNRNP40 response to: c, UV irradiation with or without ATM, ATR or DNA-PK inhibitors (ATMi, ATRi and DNA-PKi, respectively); d, UV or DRB participates in the selection of the genetic information contained in mature transcripts, thus revealing a novel non-canonical function of ATM in DDR.

Spliceosome–ATM reciprocal regulation

The ATM-dependency of splicing-factor-mobilization in quiescent cells indicates that UV irradiation activates ATM via a mechanism distinct from its canonical activation by replicative-stress- and ionizing-radiation-inflicted DSBs. UV irradiation of quiescent HDFs activated ATM, as evidenced by its auto-phosphorylation and phosphorylation of CHK2 (Fig. 5a and Extended Data Fig. 7a–e) to levels similar to the topoisomerase I inhibitor camptothecin (CPT) and the AE-9, AE-10, and AE-11 responses to: a, UV irradiation of HDFs from an ataxia telangiectasia (AT) patient or a healthy donor (Ctrl). b, DRB- or UV-triggered and ATM-dependent intron inclusion assayed by RT–PCR in quiescent cells. Signal intensity expressed as unspliced/spliced ratio (n = 4, mean ± s.d., one-way ANOVA). c, UV irradiation activates ATM via a mechanism distinct from its canonical activation by replicative-stress- and ionizing-radiation-inflicted DSBs. UV irradiation of quiescent HDFs (n = 25, mean ± s.e.m., one-way ANOVA) results in robust ATM activation (Extended Data Fig. 8d, e) and intron-retention levels comparable to UV irradiation (Extended Data Fig. 8f). To explain our observations we formulated the following hypothesis: RNAPII arrest at DNA lesions displaces a subset of splicing factors engaged in co-transcriptional splicing. Spliceosome displacement, in combination with negative supercoiling behind RNAPII, facilitates hybridization of naked pre-mRNA (still containing intronic sequences) to the DNA template strand. The resulting R-loop activates ATM, which then amplifies the mobilization signal and stimulates further spliceosome displacement either by promoting disassembly or preventing assembly of late-stage spliceosomes. Accordingly, we predicted that: (1) R-loops are formed at sites of UV-induced DNA damage; and (2) manipulation of R-loop levels will alter spliceosome mobility.

To visualize and resolve R-loops in UV-irradiated cells we exploited the ability of RNaseH1 to bind and hydrolyze RNA at RNA–DNA duplexes. For indirect, real-time visualization of R-loops, we used HDFs stably expressing GFP-tagged RNaseH1 (D145N), a binding-competent but catalytically inactive RNaseH1. RNaseH1 (D145N) was rapidly recruited to UV microbeam-irradiated sites in a transcription-dependent but ATM-independent manner (Fig. 5b and Extended Data Fig. 9d), suggesting R-loop formation at DNA-damage sites. The ability of RNaseH1 (D145N) to detect R-loops was confirmed.

U2 or U5 snRNP splicing factors, or combined RNase H1/H2A silencing, resulted in ATM activation in the absence of other treatments (Extended Data Fig. 8a, b, g). Similarly, treatment of quiescent cells with pladienolide B, which arrests late-stage spliceosomes and mobilizes U5, and to a lesser extent U2 snRNPs, resulted in robust ATM activation (Extended Data Fig. 8d, e).

In agreement, siRNA-mediated silencing of treatment with or without caffeine; e, UV irradiation of HDFs from an ataxia telangiectasia (AT) patient or a healthy donor (Ctrl). f, DRB- or UV-triggered and ATM-dependent intron inclusion assayed by RT–PCR in quiescent cells. Signal intensity expressed as unspliced/spliced ratio (n = 4, mean ± s.d., one-way ANOVA). g, Genome-wide identification by RNA-seq of UV-induced alternative splicing (AS) events. Right, types of alternative splicing events; left, number of total and ATM-dependent events. *P < 0.05, **P < 0.01, ***P < 0.001.
by overexpression of active RNaseH1 or by silencing of RNaseH2, which prevented or potentiated, respectively, recruitment of RNaseH1(D145N) at UVC microbeam-irradiation sites (Extended Data Fig. 9a). Formation of R-loops at these sites was verified using immunofluorescence signals at nuclear areas irradiated with UV or CPT treatment. (c–f, after RNaseH1/H2A silencing; g, h, n = 2, mean ± s.d., one-way ANOVA). i, Model of UV-triggered and R-loop/ATM-activated spliceosome mobilization. a, b, c, Images were obtained at 40× (a) and 63× (b, c) magnification. *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion

Here we present evidence that the core spliceosome is a target and an effector of the cellular response to transcription-blocking DNA damage, and we define a previously uncharacterized ATM-dependent branch of genome surveillance. Transcription-blocking DNA lesions cause selective chromatin displacement of late-stage spliceosomes by a two-step mechanism involving a stochastic (cis) and an ATM-signalling-mediated (trans) stage. Our hypothesis is that displacement of assembled co-transcriptional spliceosomes is required to remove steric inhibition that would otherwise prevent back-tracking (or removal) of RNAPII from DNA lesions, which is critical for subsequent DNA repair34. The initial spliceosome displacement probably results in naked (intron-retaining) pre-mRNA readily available for hybridization with template single-strand DNA at the transcription bubble. This culminates in R-loop formation at damaged DNA sites, which in turn activate ATM. Previously, R-loop mediated ATM activation has been linked to replication-induced DSBs because of collision of arrested transcription complexes with the replication machinery35,37. Here, we demonstrate that neither DSBs nor replication are required for R-loop-dependent ATM activation. While the exact mode of UV-triggered ATM activation remains to be determined, it does have significant biological consequences. It influences gene expression and plays a fundamental role in augmenting spliceosome displacement and alternative pre-mRNA splicing genome-wide.

ATM activation and spliceosome displacement are subject to reciprocal regulation, which has two unanticipated implications. First, in response to transcription-blocking lesions, changes in spliceosome organization activate ATM signalling irrespective of replication. Second, ATM modulates DDR, not only by controlling expression levels of its target genes, but also by influencing pre-mRNA processing. These observations provide new insights into the mechanisms and consequences of ATM activation in post-mitotic tissues, which is critical for proper cellular function, as evidenced by the severe neurodegeneration in ataxia telangiectasia patients38.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.T. designed the study, performed the majority of experiments, analysed the data and authored the manuscript with contributions from W.V. and J.A.M. J.A.A.D. performed the liquid-chromatography tandem mass spectrometry analysis, L.S. assisted in fractionation/immunoblotting experiments, J.A.M. performed S9.6 antibody immunofluorescence and assisted in UVC micro-irradiation experiments, D.W. and R.H.M performed RT–PCR splicing assays, P.K., F.G.G. and W.v.IJ. performed antibody immunofluorescence and assisted in UVC micro-irradiation experiments, L.S. assisted in fractionation/immunoblotting experiments, J.A.M. performed S9.6 antibody immunofluorescence and assisted in UVC micro-irradiation experiments, D.W. and R.H.M performed RT–PCR splicing assays, P.K., F.G.G. and W.v.IJ. performed RNA-seq experiments, L.H.M and M.G. V. generated RNaseH1 constructs and cell lines. L.H.M. and J.H.J.H. provided advice. All authors reviewed and commented on the manuscript.

Author Information RNA-seq data have been deposited in the Sequence Read Archive with accession number SRP053034. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.T. (mtsresi@erasmusmc.nl), W.V. (w.vermeulen@erasmusmc.nl) or J.A.M. (j.marteijn@erasmusmc.nl).
Nanoflow liquid chromatography–PCR cells were lysed 6 h after treatment.

In brief, samples containing MNase-digested chromatin were size-fractionated (1100 series capillary liquid chromatography system (Agilent Technologies)) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) operating in positive mode. Raw mass spectrometry data were analysed using the MaxQuant software. A false discovery rate of 0.01 for proteins and 0.05 for peptides was used. Proteins were identified with the Andromeda search engine which was used to search MS/MS spectra against the International Protein Index (IPI) human database. Statistical analysis was performed with Perseus (1.5.0.30)17.

**Cloning.** Human full-length cDNA clones used for subcloning were: PRPF8/PRPF8/DHX16 (CS116070), SF3A1 (SC321295), RNPRF40 (SC12670) and RNaseH1 (SC319446) from Origene and U1A/SNPRPA (HM6278-20826169), NH2PL1 (MH5208-208293930) and PRP3/PRPF3 (MH5208-208262260) from Dharmaco. To generate vectors expressing GFP- and mCherry-tagged proteins the open reading frames (minus the stop codon) of human U1A, SF3A1, PRP3, NH2PL1, PRP8 and SNPRPA40 were PCR amplified using oligonucleotides containing restriction enzyme sites. PCR products were subcloned into a PLHCX retroviral expression vector (Clontech Laboratories) modified to contain a GFP lacking the initiation codon. XPA and RNaseH1 lacking the mitochondrial localization signal (amino acids 1–28) were subcloned in modified PLHCX vectors containing either eGFP or mCherry lacking their stop codons. PCR amplifications were performed on a MJ Scientific, Inc., PCT-100 Thermostyler using high-fidelity Phusion polymerase (Biok). Amplified cDNAs were purified using the Promega Wizard kit. Following restriction digestion of insert and vectors, alkaline phosphatase treatment of the vectors, and agarose gel electrophoresis, the gel-excised DNAs were purified using the Promega Wizard kit. DNA inserts were ligated into vectors at a 3:1 molar ratio. Plasmid DNAs were validated by restriction digestion and sequencing.

**Infections/transfections.** CSRo-T, CSRo-AT-2, U2OS and VH-10T cell lines stably expressing GFP-tagged proteins were generated by retroviral infection followed by hygromycin selection. For retrovirus production Gruffyd A cells were transfected with the appropriate expression vector using FuGene6 (Roche) according to the manufacturer's instructions. Viral supernatants were harvested 48 h post-transfection, filtered through 0.45-μm filters (Millipore Corp.) and used immediately to infect subconfluent cell cultures in the presence of 5 μg/mL polybrene. U2OS cells were transiently transfected with RNaseH1–mCherry (pLHCX) using FuGene6. For gene silencing the following siRNAs were purchased from Dharmacon: HR-2 (anti-RNaseH1), and HR-4 (anti-RNaseH2A). Human RNaseH2A siRNA (L-003535-01-0005) targeting the catalytic subunit of RNaseH2, ON-TARGETplus RNaseH1 siRNA (L-012595-01-0005), ON-TARGETplus PRP8 siRNA (L-012225-01-0005), On-TARGETplus SF3A1 siRNA (L-016651-01-0005), On-TARGETplus ATM siRNA (L-003201-00-0005) and a control/scrambled siRNA duplex (D-001210-05-00). For gene silencing, U2OS cells were transfected with RNAiMax (Invitrogen), and CSRoT and RPE cells with HiPerfect (Qiagen), as recommended by the manufacturers. To inhibit endogenous RNaseH activity cells were transfected with a (1:1) mixture of siRNAs targeting RNaseH1 and RNaseH2.

**Preparation of whole cell lysates, chromatin fractionation, and immunoprecipitations.** Whole-cell lysates were prepared by lysis of equal cell numbers in 60 mM Tris-Ci (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.1% bромоморфолинол. DNA chromatin was isolated after Triton-X 100 extraction and MNase digestion. All fractionation steps were performed at 4 °C. Cells pellets were suspended in a non-denaturing isosmotic buffer (10 mM PIPES (pH 7.0), 3 mM MgCl2, 100 mM NaCl, 300 mM sucrose, 0.5 mM Na2VO4, 5 mM Naf, 5 mM NaPiP3, 10 mM β-glycerolphosphate, 0.1 mM PMSF, 1 mM EDTA, 1× EDTA-free protease inhibitor cocktail (Roche), 15 μM MG132, 10 mM N-ethylmaleimide and 20 μM PR-619 (LifeSensors) and extracted in tris-buffered saponin (TBS)-buffered (0.5% (w/v) saponin) for 5 min. Following centrifugation (16,000 g, 5 min), nuclei depleted from soluble nucleoplasms were washed with MNase digestion buffer (50 mM Tris-Ci (pH 7.5), 14 mM MgCl2, 50 mM KCl, 300 mM sucrose, 0.5 mM NaVO4, 5 mM NaF, 5 mM NaPiP3, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM EDTA, 1× EDTA-free protease inhibitor cocktail) and subsequently incubated with 0.3 U MNase (Sigma)/1 ml culture for 30 min. After incubation, chromatin was mechanically sheared by passing through a 27G syringe, 40 times. Particular matter was removed by centrifugation (20 min at 16,000 g) and supernatants containing equal amounts of proteins were used for immunoprecipitation.

GFP-tagged proteins were immunoprecipitated directly or after MNase digestion which was used to cleave DNA and RNA and disrupt ternary complexes. Samples were incubated (2 h, 4 °C) with pre-equilibrated GFP-Trap coupled to agarose beads (ChromoTek), and after extensive washing (10 mM Tris-Ci (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40), immunocomplexes were desorbed from the beads by heating for 10 min at 95 °C, in 120 mM Tris-Ci (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue. For immunoprecipitation of elongating RNAPII, cells were treated and extracted as described for isolation of crude chromatin with the exception that instead of MNase...
digestion, chromatin was mechanically sheared. Immunoprecipitations were performed by O/N incubation with either the anti-RNAIP1 CTD phospho-Ser2 antibody or rabbit IgG, followed by incubation with protein A/protein G agarose beads (Upstate Biotechnology).

**Immunoblotting.** Protein samples were size-fractionated on 5–20% gradient SDS-polyacrylamide gels (BioRad) and electro-transferred onto nitrocellulose membranes using a Bio-Rad Mini-Protein electrophoresis system. Abundance of proteins of interest was assayed using antibodies at concentrations recommended by their manufacturers. Membranes were incubated with primary antibodies overnight and then with secondary antibodies (1:1000 dilution in PBS) for 1 h. Membranes were washed, proteins of interest were visualized using the Odyssey CLX Infrared Imaging System (LI-COR Biosciences). Signal intensities were quantified using the ImageQuant TL software (GE Healthcare Life Sciences).

**RNA synthesis.** Transcription levels were determined following 2-hour incubation with ethynyluridine (EU) added directly in the culture (serum-free) media. EU incorporation was visualized using Click-IT conjugation of AlexaFluor647 (Invitrogen) according to the manufacturer’s protocol. Images were obtained using a Zeiss LSM 780 confocal microscope equipped with a 63× Plan-Apochromat 1.4 NA oil-immersion lens (Carl Zeiss Inc.). Fluorescence-signal intensities were quantified using the ImageJ software (NIH). In each experiment >150 cells per condition were analysed.

**Immunofluorescence and live-cell confocal laser-scanning microscopy.** For immunofluorescence experiments, cells were fixed with 3.7% paraformaldehyde (37°C for 10 min) followed by 0.1% Triton-X 100/PBS before immunofluorescence experiments, cells were fixed with 3.7% paraformaldehyde and 50 mM PFA. For R-loop immunodetection cells were fixed in 2% PFA (4°C for 15 min), washed, permeabilized with 0.5% Triton-X 100/PBS and blocked in 3% BSA/PBS. R-loop immunodetection was performed using primary antibodies against single-stranded DNA (ssDNA) and Alexa Fluorochrome-conjugated secondary antibody and extensive washing, proteins of interest were visualized using the Odyssey CLX Infrared Imaging System (LI-COR Biosciences). Signal intensities were quantified using the ImageQuant TL software (GE Healthcare Life Sciences).
one-way ANOVA for more than two groups. No statistical methods were used to
predetermine sample size. One-way ANOVA was followed by post hoc analysis
either by Dunnett’s test (for comparison of experimental conditions to control) or
Bonferroni’s test (comparison between groups). *P < 0.05, **P < 0.01 and
***P < 0.001. Proteomic data statistical analysis was performed with Perseus
(1.5.0.30)17. Significance B was calculated by estimating the variance of the dis-
tribution of all protein ratios, taking into account the dependency of the distri-
bution on the summed protein intensity17. Peptides with a significance B value
P ≤ 0.05 in either the forward or reverse experiment were considered significant
and indicated by (+) in Extended Data Fig. 1d and Supplementary Table 1.
Significant alternative splicing events were identified by MATS45; only UV-trig-
gered events with P < 0.05 were used for further analysis.

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Extended Data Figure 1 | Chromatin association of splicing factors.

**a** Schematic overview of the proteomic experiments for the identification of proteins that display UV-dependent chromatin association. **b** Schematic outline of cell fractionation. **c** Validation of the chromatin-isolation protocol for NER proteins that are recruited to chromatin in response to DNA damage. Mock-treated or UV-irradiated quiescent HDFs (20 J m\(^{-2}\), 1 h post-irradiation) were fractionated as outlined in **b**. Equal protein amounts from each fraction were analysed by immunoblotting using antibodies against the indicated NER proteins. Abundance of H2A is shown as a control for chromatin-isolation efficiency.

**d** UV-triggered changes in chromatin association of core splicing factors, identified by quantitative SILAC proteomics. Proteomic experiments were performed with HDFs as outlined in **a**. The table lists representative examples of splicing factors that participate in distinct snRNP complexes and their chromatin association in response to UV irradiation (20 J m\(^{-2}\), 1 h). U2 and U5 snRNP splicing factors show significantly reduced chromatin association (P ≤ 0.05, significance B\(^{17}\)) and are indicated with a cross. Significance B was calculated by estimating the variance of the distribution of all protein ratios, taking into account the dependency of the distribution on the summed protein intensity\(^{17}\). ND, not detected.

**e** Abundance of splicing factors in total cell lysates. Total lysates were prepared from U2OS cells that were mock-treated or UV-irradiated (20 J m\(^{-2}\), 1 h post-irradiation) and splicing factor abundance was assayed by immunoblotting. Abundance of H2A is shown as a loading control. Right, immunoblots; left, quantification of splicing factor signal intensities normalized to H2A. *n* = 3, mean ± s.d., one-way ANOVA/Bonferroni.

**f** UV-dependent interaction between elongating RNAPII and SFs. Quiescent HDFs were prepared as outlined in **b** except that, instead of MNase digestion, chromatin was mechanically sheared. Elongating RNAPII was immunoprecipitated with an antibody that recognizes specifically the Ser2-phosphorylated RNAPII C-terminal domain (CTD) and its interaction with the U2 snRNP splicing factors SF3a1 and SF3b2 was assayed by immunoblotting.
Extended Data Figure 2 | Validation of HDFs stably expressing GFP-tagged splicing factors. a, Whole-cell lysates from HDFs stably expressing eGFP tagged PRP8, SF3a1, SNRNP40 or free eGFP, were analysed by immunoblotting using antibodies against GFP (left) or against PRP8, SF3a1 and SNRNP40 (right). Ectopically produced proteins were expressed at near or below endogenous levels. b, Fluorescent microscopy images of GFP-tagged splicing factors showing the expected punctuated nuclear distribution. Images were obtained at 40× magnification. c, Localization of SNRNP40–GFP in nuclear speckles which were visualized by immunofluorescence detection of the speckle marker SRSF2/SC35. Images were obtained at 63× magnification. d, Interaction of SNRNP40–GFP with endogenous splicing factors and elongating RNAPII. Quiescent SNRNP40–GFP-expressing HDFs were mock-treated or irradiated with 20 J m⁻² UVC. After a 3-h recovery period, cells were lysed under native conditions and chromatin was sheared by mechanical force. SNRNP40–GFP was immunoprecipitated from whole-cell lysates using GFP-Trap agarose beads, and its association with endogenous splicing factors and the large subunit of RNAPII was assayed by immunoblotting. Non-transfected cells are shown as a negative control. SNRNP40–GFP interacts with U2 and U5 snRNP components, suggesting that the GFP tag does not interfere with complex formation. Interaction of SNRNP40 with its U5 snRNP partner PRP8 is partially maintained even after MNase digestion, consistent with its presence in U4/U6.U5 tri-snRNP complexes. Participation of SNRNP40–GFP in co-transcriptional splicing complexes is confirmed by co-immunoprecipitation of the active (hyperphosphorylated RNAPIIo) large subunit of RNAPII.
Extended Data Figure 3 | Displacement of mature spliceosomes from subnuclear sites of UV-inflicted DNA damage. a, U2OS cells stably expressing GFP-tagged splicing factors were UV-irradiated (60 J m\(^{-2}\)) through isopore membranes resulting in DNA lesion formation in small subnuclear areas. DNA damage sites (circled) were visualized by immunofluorescence using an antibody against the NER recognition factor XPC. Scale bar, 5 μm. b, SF3a1–GFP and PRP8–GFP depletion from UVC laser microbeam irradiation sites. Quantification of 20 cells from two independent experiments. eGFP localization at sites of DNA damage is used to demonstrate that depletion of eGFP-tagged splicing factors is not caused by photobleaching. c, UVC laser microbeam irradiation results in preferential displacement of U2- and U5-associated splicing factors from DNA damage sites. Quiescent HDFs were irradiated in a ~1-μm-diameter nuclear area via a UVC laser. GFP signal intensity, reflecting the abundance of GFP-tagged U1, U2, U4 and U5 snRNP components at UVC DNA damage sites, was quantified in the irradiated and in a non-irradiated nuclear area (undamaged control). Plotted is the fluorescence signal intensity expressed as a percentage of that before irradiation, at the 1-min time point. Cells expressing free eGFP were used as negative control. Representative from three independent experiments (n = 12, mean ± s.e.m., paired t-test). d, Depletion of splicing factors from UVC laser irradiation sites depends on active transcription. Transcription initiation was inhibited in quiescent HDFs by prolonged α-amanitin treatment (10 μM, ≥24 h) before subnuclear UVC laser irradiation. Plotted is the SNRNP40–GFP abundance in irradiated and non-irradiated nuclear areas at 1-min post-irradiation. Representative from three independent experiments (n = 12, mean ± s.e.m., one-way ANOVA/Bonferroni).
Extended Data Figure 4 | SNRNP40 reorganization and speckle enlargement in response to UV irradiation. Representative microscopic images showing SNRNP40–GFP distribution in quiescent HDFs before, and 1 h post UVC irradiation with 20 J m$^{-2}$. a, Live cells. b, Fixed cells. Images were obtained at 63× magnification.
Extended Data Figure 5 | Transcription stalling mobilizes spliceosomes independent from NER complex assembly and proteasome activity. a, RNA synthesis is inhibited preferentially by genotoxins that inflict bulky DNA lesions. Influence of genotoxins on RNA synthesis of quiescent HDFs was measured by 5EU pulse labelling combined with click chemistry. Top, representative images; bottom, quantification of fluorescence intensity (n = 150, mean ± s.e.m., one-way ANOVA/Bonferroni). Images were obtained at 40× magnification. b, Mobilization of U2 and U5 snRNPs by genotoxins inflicting transcription-blocking DNA lesions. Mobilization of GFP-tagged SF3a1 (left) and PRP8 (right) assayed by FRAP in quiescent HDFs exposed to different types of genotoxins (n = 30, mean ± s.e.m., one-way ANOVA/Bonferroni). IR, ionizing radiation. c, Chromatin displacement of mature spliceosomes is not TC–NER-dependent. Left, chromatin abundance of U2 and U5 snRNP splicing factors was assayed by immunoblotting in XPA-deficient (left), XPA–GFP-corrected (middle) and CSB-deficient (right) HDFs. Cells were UV-irradiated (20 J m⁻²) and chromatin was isolated at the indicated times. Top, immunoblots; bottom, quantification of splicing factor signal intensities normalized to H2A (n = 3, mean ± s.d., one-way ANOVA/Bonferroni). d, Proteasome activity is not required for UV-damage-induced spliceosome mobilization. Mobilization of SNRNP40–GFP assayed by FRAP in quiescent HDFs exposed to UV radiation in the presence or absence of the proteasome inhibitor MG132 (50 µM) (n = 30, mean ± s.e.m., t-test). e, SNRNP40–GFP mobilization by transcription inhibitors. FRAP of SNRNP40–GFP in quiescent HDFs after inhibition of transcription initiation (10 µg ml⁻¹ α-amanitin, 24 h) or elongation (1 µg ml⁻¹ actinomycin D or 50 µM DRB, 1 h) (n = 30, mean ± s.e.m., one-way ANOVA/Dunnett’s).
Extended Data Figure 6 | ATM-dependency of UV induced spliceosome mobilization, alternative splicing and gene expression changes. 

a, UV irradiation and DRB-dependent mobilization of SNRNP40. Quiescent HDFs expressing SNRNP40–GFP were UV-irradiated or DRB treated with doses that inhibit transcription to similar levels. Splicing factor mobility was assayed by FRAP. b, Additive effect of combined UV and DRB treatments. FRAP of SNRNP40–GFP in quiescent HDFs treated with DRB, UV, or a combination of both, each at a dose that inhibits RNA synthesis by ~50%. c, Impaired UV-dependent SF3a1 mobilization in cells lacking ATM activity. SF3a1–GFP mobilization was measured by FRAP in quiescent HDFs derived from an ataxia telangiectasia (AT) patient or a healthy donor. d, ATM-dependent spliceosome mobilization. Quiescent HDFs with or without ATM (KU55933), ATR (VE821) or DNA-PK (NU7441) inhibitors before irradiation. GFP-tagged SF3a1 or PRP8 mobility was assayed by FRAP. ATM, but not ATR or DNA-PK inhibition partially prevented the UV-induced splicing factor mobilization. 

a–d, n = 25, mean ± s.e.m., one-way ANOVA/Bonferroni.

e, Reduced UV-induced intron retention in response to ATM silencing. Intron inclusion in retina pigment epithelium (RPE) cells transfected either with control or ATM-silencing siRNAs and subsequently mock-treated or UV-irradiated (20 J m⁻², 6 h) was assayed by RT–PCR.

f, ATM-dependent changes in intron retention. Intron inclusion was assayed by RT–PCR in untreated, UV-irradiated and DRB-treated quiescent cells in the presence or absence of 10 µM ATM inhibitor. 

g, Heat map of UV-triggered and ATM-dependent transcriptome changes. Quiescent cells were mock-treated or UV-irradiated in the presence or absence of the ATM inhibitor. Transcriptome profiles were generated by RNA-seq. Differentially expressed genes between untreated and UV-irradiated cells (P < 0.05) and UV-irradiated cells in the presence or absence of the ATM inhibitor (P < 0.05), were clustered in a heat map using Pearson correlation. 

n = 1,676 differentially expressed transcripts. The observed correlation indicates that UV-inducible transcriptome changes can be, in part, prevented by ATM inhibition.

h, Lack of influence of ATM inhibition on DRB-mediated splicing factor mobilization. Splicing factor mobility was measured by FRAP in untreated or DRB-treated HDFs in the presence or absence of 10 µM ATM inhibitor (n = 30, mean ± s.e.m., one-way ANOVA/Bonferroni).
Extended Data Figure 7 | Canonical and non-canonical ATM activation.

**a** ATM autophosphorylation (Ser1981) was assayed in quiescent HDFs 1 h after the indicated treatments. In non-replicating cells UV and trichostatin A (TSA) activate ATM via non-canonical pathways. Transcription inhibition by DRB has no influence on ATM activity.

**b** The quiescent status of serum-deprived HDFs was verified by immunodetection of the cell cycle marker Ki67, which is not expressed by quiescent (G0) cells.

**c** Immunofluorescence detection of active ATM in quiescent HDFs treated with DDR kinase inhibitors.

**d** Immunoblotting analysis of nuclear extracts derived from quiescent HDFs treated as in **c** using a phospho-specific ATM (Ser1981) antibody (top) and an antibody recognizing ATM (bottom).

**e** Differences in autophosphorylated-ATM distribution in quiescent HDFs treated with various ATM activators. Left, multiple cells; right, single-cell magnification illustrating pan-nuclear localization of phosphorylated ATM after UV irradiation and focal accumulation after CPT or ionizing radiation treatments. Magnified cells are indicated by arrows (left panel).

**f** Differences in amounts of DNA damage-foci formation indicative of DSBs, in response to CPT, UV and ionizing radiation. Quiescent HDFs were pre-treated with the ATR inhibitor (10 μM, 1 h) and subsequently exposed to the indicated genotoxins. DSB foci were visualized by immunofluorescence using antibodies against γH2AX and p53BP1. Left, multiple cells; right, single-cell magnification; images were obtained at 40× and 63× magnification, respectively. Magnified cells are indicated by arrows in the left panel.
Extended Data Figure 8 | ATM activation by interference with spliceosome assembly or RNaseH1/H2A silencing. a, ATM autophosphorylation was assayed by immunofluorescence in HDFs after silencing of SF3a1, PRP8 or combined silencing of RNaseH1 and RNaseH2A. b, Immunoblotting analysis of silenced proteins in total cell lysates. Tubulin is shown as a loading control. c, Splicing factor mobilization by the spliceosome inhibitor pladienolide B (PL) was assayed by FRAP in quiescent HDFs. Consistent with its function in interfering with spliceosome maturation following pre-spliceosome assembly, cell treatment with pladienolide B resulted in extensive mobilization of U5 snRNP factors (PRP8 and SNRNP40), partial mobilization of the U2 snRNP SF3a1, and had no influence on the U1 snRNP factor U1A (n = 30, mean ± s.e.m., one-way ANOVA/Bonferroni). d, e, ATM activation by Pladienolide B. Quiescent HDFs were either treated with 5 μM pladienolide B or exposed to 1 Gy ionizing radiation (IR) and autophosphorylated ATM was detected by immunofluorescence (d) or immunoblotting (e). f, Effect of pladienolide treatment on intron retention. RNA isolated from mock-treated, UV-irradiated or pladienolide B-treated RPE cells. Intron retention, as assayed by RT–PCR on transcripts of the indicated genes, shows that pladienolide B influences splicing to the same extent as UV irradiation. U/S, ratio of relative abundance of unspliced (U) to spliced (S) introns. g, Efficiency of RNaseH1 and H2A silencing at the single-cell level, assayed by immunofluorescence. Images were obtained at 40× magnification.
Extended Data Figure 9 | UV-induced R-loop formation enhances spliceosome mobilization. 

**a**, Recruitment of RNaseH1(D145N)–GFP at local DNA damage sites depends on endogenous levels of RNaseH activity. DNA damage was inflicted via a UVC laser in ~1-μm-diameter subnuclear areas of cells after silencing of RNaseH2A or overexpression of RNaseH1–mCherry. Recruitment of RNaseH1(D145N)–GFP at the irradiated sites was monitored by live-cell imaging. Plotted is the fluorescence intensity of RNaseH1(D145N)–GFP at 1 min post-irradiation, at the irradiated and in a non-irradiated nuclear area. Representative from three independent experiments (n = 10, mean ± s.e.m., one-way ANOVA/Bonferroni).

**b, c**, R-loop formation at sites of local UVC laser irradiation. Immunofluorescence detection of R-loops using the DNA–RNA hybrid-specific S9.6 antibody. Sites of irradiation are visualized by XPC immunodetection. Dashed boxes indicate the magnified areas shown in the right panels. The dashed lines indicate the line-scan track used to quantify fluorescence intensity of S9.6 and anti-XPC (shown in the graph). Specificity of the antibody was confirmed by its increased sensitivity after RNaseH2A silencing and its ability to detect R-loops when suboptimal doses of UVC irradiation were applied. 

**d**, RNaseH1 accumulation at local DNA damage sites depends on active transcription but not ATM activity. Transcription initiation was inhibited in quiescent HDFs by α-amanitin (10 μg ml⁻¹, 24 h) before local UVC laser irradiation. Plotted is the fluorescence intensity at 1 min post-irradiation of RNaseH1(D145N)–GFP at the irradiated area and in a non-irradiated nuclear area for untreated, ATM-inhibitor- and α-amanitin-treated cells. Representative from three experiments (n = 10, mean ± s.e.m., one-way ANOVA/Bonferroni).

**e**, RNaseH1 overexpression inhibits the UV-dependent spliceosome mobilization. FRAP of U2OS cells stably expressing GFP-tagged SF3a1 and PRP8 and transiently transfected with RNaseH1–mCherry. 

**f**, RNaseH1 and H2A silencing potentiates the UV-dependent spliceosome mobilization. RNaseH1 and H2 were silenced in U2OS cells expressing SF3a1–GFP or PRP8–GFP and splicing factor mobility was assayed by FRAP. 

**g**, FRAP of SNRNP40–GFP in quiescent HDFs after RNaseH1/H2 silencing. 

**e–g**, n = 30, mean ± s.e.m., one-way ANOVA/Bonferroni.
Extended Data Figure 10 | Combined transcription inhibition and ATM activation results in extensive mobilization of mature spliceosomes.

a, Combinatorial effect of DRB and ionizing radiation on spliceosome mobilization. Quiescent HDFs were exposed to ionizing radiation in the presence or absence of DRB, and SF3a1–GFP and PRP8–GFP mobility was assayed by FRAP. b, The ionizing-radiation-mediated increase of DRB-dependent spliceosome mobilization depends on ATM activity. FRAP of GFP-tagged SNRNP40 in quiescent HDFs treated with DRB and/or ionizing radiation in the presence or absence of an ATM inhibitor. c, Spliceosome mobilization by CPT. Quiescent HDFs were treated with 25 μM CPT, 25 μM DRB and 20 J m⁻² UV at doses that inhibit transcription to approximately 30% and their influence on SF3a1, PRP8 and SNRNP40 mobilization was measured by FRAP. Mobilization of GFP-tagged SF3a1, PRP8 and SNRNP40 in quiescent HDFs was measured by FRAP. a–c, n = 30, mean ± s.e.m., one-way ANOVA/Bonferroni. d, Inhibition of RNA synthesis by the treatments shown in c was assayed in quiescent HDFs by 5EUs incorporation and click chemistry (n = 150, mean ± s.e.m., one-way ANOVA/Dunnett’s).