ADAR1 RNA editing enzyme regulates R-loop formation and genome stability at telomeres in cancer cells

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ADAR1 is involved in adenosine-to-ino sine RNA editing. The cytoplasmic ADAR1p150 edits 3′UTR double-stranded RNAs and thereby suppresses induction of interferons. Loss of this ADAR1p150 function underlies the embryonic lethality of Adar1 null mice, pathogenesis of the severe autoimmune disease Aicardi-Goutières syndrome, and the resistance developed in cancers to immune checkpoint blockade. In contrast, the biological functions of the nuclear-localized ADAR1p110 remain largely unknown. Here, we report that ADAR1p110 regulates R-loop formation and genome stability at telomeres in cancer cells carrying non-canonical variants of telomeric repeats. ADAR1p110 edits the A-C mismatches within RNA:DNA hybrids formed between canonical and non-canonical variant repeats. Editing of A-C mismatches to I:C matched pairs facilitates resolution of telomeric R-loops by RNase H2. This ADAR1p110-dependent control of telomeric R-loops is required for continued proliferation of telomerase-reactivated cancer cells, revealing the pro-oncogenic nature of ADAR1p110 and identifying ADAR1 as a promising therapeutic target of telomerase positive cancers.
adenosine deaminase acting on RNA (ADAR) is the enzyme involved in adenosine-to-inosine RNA editing (A-to-I RNA editing), and three ADAR gene family members (ADAR1, ADAR2, and ADAR3) have been identified in vertebrates1-3. ADARs share common domain structures, such as multiple dsRNA-binding domains (dsRBBDs) and a separate catalytic domain6,7. Both ADAR1 (ADAR, DRADA) and ADAR2 (ADARB1) are catalytically active enzymes, whereas no catalytic activity of ADAR3 (ADARB2) has been shown so far1-4. A-to-I editing occurs most frequently in noncoding regions that contain repetitive elements Alu and LINE8,9, and many millions of editing sites have been identified in the human transcriptome of these repetitive sequences8-11.

Two ADAR1 isoforms, p150 and p110, are generated by the use of separate promoters and alternate splicing12. ADAR1p150 is mostly in the cytoplasm, whereas ADAR1p110 mainly localizes in the nucleus13. The cytoplasmic ADAR1p150 regulates the dsRNA-sensing mechanism mediated by melanoma-differentiation-associated protein 5 (MDA5), mitochondrial antiviral signaling protein (MAVS), and interferon signaling (MDA5-MAVS-IFN pathway) underlies the embryonic lethality of Adar1-null mice17,18 and also the pathogenesis of Aicardi-Goutières syndrome (AGS; AGS1-7 subgroups known), a severe human autoimmune disease against endogenous nucleic acids14-16. Mutations of seven genes, including RNaseH2A (AGS4), RNaseH2B (AGS2), RNaseH2C (AGS3), and ADAR1 (AGS6), have been identified in association with AGS, and ten AGS6 mutations of ADAR1 have been reported so far19. Finally, this ADAR1p150-mediated suppression of IFN signaling also represses tumor responsiveness to immune checkpoint blockade20, revealing the pro-oncogenic ADAR1p150 function. Analysis of The Cancer Genome Atlas database revealed elevated ADAR1 expression and A-to-I editing levels in almost all types of cancers21,22, indicating that this pro-oncogenic ADAR1p150 function helps cancer cells suppress inflammatory responses and thus avoid host immunosurveillance20. In contrast to the recent advance in the knowledge of ADAR1p150 functions, the biological functions of the nuclear-localized ADAR1p110, other than its involvement in editing of intronic Alu dsRNAs23, have remained mostly unknown.

 Newly transcribed RNA usually dissociates from its template DNA strand immediately after transcription, but occasionally it forms a stable RNA:DNA hybrid, which consequently leaves the sense DNA in a single-stranded form. This structure, called an R-loop, often spans 100–2000 bp and causes abortive transcription and instability of the genome24,25. Several mechanisms are known to suppress the formation of R-loops, for example, degradation of RNA strands of RNA:DNA hybrids by RNase H126 and RNase H2,27,28 and unwinding of RNA:DNA hybrids by helicases such as DEAH-box helicase 9 (DHX9)29 and senataxin (SETX)30. Human diseases including amyotrophic lateral sclerosis type 4, ataxia-ocular apraxia type 2, and AGS are caused by the accumulation of R-loops due to deficiency in one of those suppression mechanisms31,32. Telomere end regions consisting of repetitive Alu and LINE elements are also known to be naturally prone to the formation of R-loops33,34, which in turn causes telomere instability and perhaps underlies carcinogenesis of certain cancers31,35,36. The canonical hexameric repeat sequence of the telomeric G-strand (sense strand) is TTAGGG35,37. Interestingly, detection of widespread telomeric variant repeats such as TCAGGG and TTGGGG has been reported in cancer cells38-40. Mutations/variants (nucleotides) of telomeric canonical repeat DNA sequence TTAGGG (antisense sequence CCCCCTA) detected in cancer cells such as TTGGGG (CCCCCAA) and TCAGGG (CCTCGGA) are emphasized by underlining. Their RNA sequence versions are UUUUGGG (CCCCCAA) and UCAGGG (CCCUAGA). In addition, adenosine residues to be edited by ADAR1 were also emphasized by underlining: TTAGGG (RNA sequence UUAGGG).

In this study, we found an important role for the ADAR1p110 isoform in resolution specifically of the R-loops formed at telomeric repeat regions. ADAR1p110 edits both the RNA and the DNA strands of telomeric repeat RNA:DNA hybrids containing mismatched base pairs formed between canonical and variant repeats. ADAR1p110-mediated editing of A-C-mismatched base pairs, which converts them to I:C-matched base pairs, is required for degradation of the RNA strands of telomeric repeat RNA:DNA hybrids by RNase H2. We found that RNase H2 is incapable of resolving mismatch-containing telomeric RNA:DNA hybrids by itself. This newly found ADAR1p110 role in suppression of telomeric R-loops seems to be essential for the continued proliferation of telomerase-reactivated cancer cells with accumulated variant telomeric repeats, revealing yet another pro-oncogenic function of ADAR1.

**Results**

**Depletion of ADAR1 results in telomere DNA damage, telomere abnormalities, and mitotic arrest.** We recently made several observations that indicated the involvement of ADAR1 in the maintenance of telomere stability and mitosis. First, significantly increased telomere abnormality, such as telomere fusions, was detected with Adar1-null mouse embryonic fibroblast (MEF) cells derived from Adar1-null mouse embryos18 (Supplementary Fig. 1a, b). In contrast, no significant telomere abnormality was detected with Adar2-null MEF cells (Supplementary Fig. 1c) derived from Adar2-null mouse embryos41. Chromosome orientation fluorescence in situ hybridization (CO-FISH) analysis revealed significantly increased involvement of leading strands in telomere fusions detected in Adar1-null MEF cells (Supplementary Fig. 1d), indicating that ADAR1 is involved in the mechanism that maintains the integrity of the telomere leading strands. Detection of significantly increased telomere dysfunction-induced foci (TIF) revealed by telomere FISH and yH2AX immunostaining suggested accumulation of DNA damage, if not exclusively, mainly at telomeres in Adar1-null MEF cells (Supplementary Fig. 1e), which must be closely related to the increased telomere fusions (Supplementary Fig. 1b, c). Second, time-lapse imaging of HeLa cells undergoing ADAR1 gene knockdown revealed many aberrantly shaped nuclei (Fig. 1a and Supplementary Fig. 2a) that appeared to be arrested during mitosis, and these aberrant cells eventually died, most likely by apoptosis (Supplementary Movies 1 and 2). Close examination revealed the presence of increased bridged nuclei, micronuclei, and multinuclei in ADAR1-depleted cells (Fig. 1b and Supplementary Fig. 2a). Significantly increased TIFs were detected also in ADAR1-depleted HeLa cells (Fig. 1c). Ectopic expression of Adar1p110-WT but not Adar1-E912A, a mutant without catalytic activity,6 (Supplementary Fig. 2b), suppressed induction of TIFs in ADAR1-depleted cells (Supplementary Fig. 3), demonstrating the importance of ADAR1-mediated A-to-I editing activity in the maintenance of telomere stability. Western blotting analysis revealed significantly upregulated DNA damage markers such as phosphorylated DNA-dependent protein kinase catalytic subunit (DNA-PKcs), yH2AX, and phosphorylated RPA32 in ADAR1-depleted HeLa cells (Fig. 1d). In addition, elevated levels of CCNB1 and histone H3 phosphorylated at serine 10 as well as decreased expression of phosphorylated CDC2 indicated that ADAR1-depleted cells were arrested during mitosis (Fig. 1d).
These results together suggest that ADAR1 deficiency causes aberrant mitotic arrest with extensive DNA damage at telomeres.

**Accumulation of R-loops specifically at telomeric repeat regions in ADAR1-depleted cells.** One of the DNA damage markers increased in ADAR1-depleted cells was replication protein A 32 kDa subunit (RPA32 or RPA2) (Fig. 1d). Phosphorylated RPA32 binds to single-stranded DNAs (ssDNAs) and, thus, is an effective marker for R-loops. We reasoned that ADAR1 depletion might result in the accumulation of R-loops, which could cause DNA damage, telomere abnormalities, and mitotic arrest. Dot blot analysis using the S9.6 antibody specific to RNA:DNA hybrids revealed that ADAR1 depletion indeed resulted in significantly increased the formation of RNA:DNA hybrids (Fig. 2a and Supplementary Fig. 2a). Treatment with *Escherichia coli*-RNase H, which digests RNA strands of RNA:DNA hybrids, eliminated dot blot signals, confirming specific detection of RNA:DNA hybrids (Fig. 2b). ADAR2 depletion had almost no effect on the formation of RNA:DNA hybrids (Fig. 2c and Supplementary Fig. 2a), indicating that the R-loop regulatory function is specific to ADAR1.
**Fig. 1 ADAR1 depletion resulted in abnormalities of the nucleus and upregulation of DNA damage pathway and cell cycle marker genes.**

a HeLa cells were first transfected with siControl or siADAR1 (siADAR1-1) for 72 h and then treated with CellLight Tubulin-GFP. Nuclei were visualized by staining of DNA with SiR-DNA reagent. Representative images taken from real-time videos (Supplementary Movies S1 and 2) are presented. Scale bar, 50 μm. b The frequency of abnormalities of the nucleus (nucleoplasmic bridge, micronuclei, and multinuclei) was estimated by examining at least 200 individual HeLa cells treated with siControl or siADAR1-1 RNAs. Values are mean ± standard error (n = 3, biologically independent samples) with significant differences by two-tailed Student’s t test indicated, **P < 0.01. Scale bar, 10 μm. c Telomere DNA damages in ADAR1-depleted cells. Telomere FISH and immunostaining for γH2AX revealed significantly increased telomere dysfunction-induced foci (TIF, indicated by yellow arrowheads), suggesting the causative relevance of the telomeric repeat DNA damage to chromosome abnormality detected in ADAR1-depleted HeLa cells. At least 200 individual HeLa cells treated with siControl or siADAR1-1 RNAs were examined. HeLa cells with one or more TIFs were counted as TIF-positive cells. Values are mean ± SD (n = 3, biologically independent samples) with significant differences by two-tailed Student’s t test indicated, **P < 0.01. Scale bar, 10 μm. d Western blotting analysis was done using total cell extracts from HeLa cells treated with siControl or two separate siADAR1 (siADAR1-1 and -2) RNAs for 72 h. Protein molecular weight markers are presented in Source Data file. e The R-loop structure consisting of an RNA:DNA hybrid formed between the RNA strand newly transcribed by RNA polymerase II and the template DNA strand with the single-stranded antisense DNA bound with ssDNA-binding protein RPA as well as major regulators are schematically shown.

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**Fig. 2 A-to-I editing activity of ADAR1p110, not ADAR1p150 or ADAR2, is required for suppression of R-loops.**

a–e Dot blot analysis for RNA:DNA hybrids was conducted using control oligos (a) or genomic DNA (b–e). a The S9.6 antibody recognized specifically RNA:DNA but not DNA:DNA or RNA:RNA oligo duplex controls. b, c Increased RNA:DNA hybrids were detected only in ADAR1-depleted but not in ADAR2-depleted HeLa cells. b The S9.6 antibody signals were abolished by E. coli-RNase H treatment, confirming specific detection of RNA:DNA hybrids. d Comparison of RNA:DNA hybrid levels between depletion of ADAR1 versus depletion of known R-loop regulators. e Increased RNA:DNA hybrid formation resulting from depletion of endogenous ADAR1 was rescued by infection of ADAR1p110-WT (wild type) but not by infection of ADAR1p110-E912A deamination defective mutant or ADAR1p150-WT. c–e Data are mean ± SD (n = 3, biological replicates); significant differences were identified by two-tailed Student’s t tests: *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant. All individual experimental data values and exact P values are presented in Source Data file.
they all are capable of resolving already formed R-loop structures (Fig. 1e). Depletion of these R-loop regulators, either by single knockdown or combination knockdown of RNase H1 and RNase H2, except for RNase H1 single knockdown (see “Discussion”), resulted in significant accumulation of RNA:DNA hybrids in HeLa cells (Fig. 2d and Supplementary Fig. 2c). Importantly, the amount of RNA:DNA hybrid formed in ADAR1-depleted cells was equivalent to or even more than those formed upon depletion of these known regulators (Fig. 2d). Using various ADAR1-expressing lentivirus systems, we conducted rescue experiments: repression of RNA:DNA hybrids formed in ADAR1-depleted HeLa cells. As expected, ectopic expression of FLAG-tagged ADAR1p110-WT (wild type) efficiently suppressed the formation of RNA:DNA hybrids (Fig. 2e). In contrast, the ADAR1p110-E912A could not rescue despite being expressed at a higher level than that of endogenous ADAR1p110 (Fig. 2e and Supplementary Fig. 2b). Furthermore, ADAR1p150-WT did not suppress the formation of RNA:DNA hybrids (Fig. 2e and Supplementary Fig. 2b). These results demonstrated that A-to-I editing activity of ADAR1p110, but not ADAR1p150, is required for suppression of R-loops.

Widespread detection of telomeric variant repeats in ALT and non-ALT cancer cells. Variant telomeric repeats such as TCAAGG and TTGGGG are detected in the telomeres of cancer cells including HeLa cells. In some cases, the proportion of variant repeats almost equals that of canonical repeats. Cancer cells maintain telomere length either by the non-canonical telomere extension mechanism, known as the alternative lengthening of telomeres (ALT), or by reactivating telomerase in ALT-negative cancer cells such as HeLa cells (non-ALT cells). Telomeric variant repeats are amplified by homologous recombination between telomeric repeats in ALT cells, and also by a currently unknown mechanism in non-ALT cells. Using telomeric canonical TTAGGG and variant TCAAGG or TTGGGG repeat-specific probes capable of distinguishing a single-nucleotide mismatch (Supplementary Fig. 4a, b), we detected indeed significant amounts of telomeric variant TCAAGG and TTGGGG repeats in four non-ALT cell lines, including HeLa cells, as well as three ALT cell lines, whereas the amount of telomeric variant repeats was much less in two primary fibroblast cell lines examined (Fig. 5a, b).

Accumulation of RNA:DNA hybrids containing telomeric variant repeats in ADAR1-depleted cells. Although ADAR1 does edit A:U base pairs of completely matched dsRNAs, A–C mismatched base pairs present in naturally occurring dsRNAs such as inverted Alu dsRNAs are, in fact, the favored ADAR1 target sites. Detection of TCAAGG and TTGGGG variant repeats surrounded by TTGGG canonical repeats within a stretch of telomeric sequence has been reported in HeLa cells. We hypothesized that RNA:DNA hybrids containing A–C-mismatched base pairs could arise in two ways: first, from slipped binding of telomeric repeat-containing RNAs (TERRAs) derived from a stretch of TCAAGG variant repeats (UCAGGG) to the C-strand of canonical TTAGGG repeats (CCCTAA) (Fig. 6a); second, binding of TERRA RNAs derived from canonical TTAGGG repeats (UAAAGG) to the C-strand of TTGGGG variant repeats (CCCGAA) (Fig. 6b). In particular, TTGGGG variant repeats (both DNA and RNA strands) are prone to the formation of a G-quadruplex structure, which then causes frequent formation of R-loops. G-quadruplex formation of the G-strand TERRAs carrying this particular variant (Fig. 6b, UUGGGG repeats highlighted in orange) is expected to leave its C-strand DNA single stranded, which in turn may cause more frequent slipped hybridization with the canonical repeat G-strand TERRAs (Fig. 6b). Second, there would be an alternative type of pairing,
namely in trans formation of RNA:DNA hybrids, which has been previously implicated for telomeric repeat sequences\textsuperscript{25,57}. Thus, in trans hybridization of telomeric repeat RNAs transcribed from one loci either with canonical or variant repeats to C-strand DNAs containing variant or canonical repeats from the other loci, respectively, could also result in telomeric repeat RNA:DNA hybrids containing A–C-mismatched base pairs (Supplementary Fig. 5a, b). G-strand TERRA RNAs containing variant T\textsuperscript{CAGGG} (U\textsuperscript{CAGGG}) and canonical T\textsuperscript{TAGGG} (UUAGGG) repeat sequences are expected to accumulate in C–A (Fig. 6a and Supplementary Fig. 5a) and A–C (Fig. 6b and Supplementary Fig. 5b) mismatch-containing RNA:DNA hybrids, respectively. Similarly, C-strand DNAs containing canonical T\textsuperscript{TAGGG} (CCCTAA) and variant TT\textsuperscript{GGGG} (CCC\textsuperscript{CAA}) repeat sequences are anticipated to be present in C–A (Fig. 6a and Supplementary Fig. 5a) and A–C (Fig. 6b and Supplementary Fig. 5b) mismatch-containing RNA:
ADAR1p110 edits A–C-mismatched base pairs of telomeric RNA:DNA hybrids formed between canonical and variant repeats. To this end, we prepared additional telomeric repeat duplex substrates containing A–C-mismatched base pairs and tested them in vitro editing with ADAR1p110. ADAR1p110 again edited very efficiently six adenosine residues of telomeric repeat dsRNA containing A–C mismatches, as expected (Fig. 7a, top). In contrast, ADAR1p110 editing of C-strand adenosines of A–C-mismatched dsRNA was less efficient, perhaps due to the fact that two adenosines are not in the most favored U:A top). In contrast, ADAR1p110 editing of C-strand adenosines of repeat dsRNA containing A–C mismatches in telomeric repeat RNA:DNA hybrids was conducted using ADAR1p110-WT recombinant protein. In vitro editing assay for completely matched telomeric repeat dsRNA hybrids using ADAR1p110-WT recombinant protein. No significant levels of editing for matched RNA:DNA hybrids were detected. b, c PCR products (RT-PCR-amplified RNA strands and PCR-amplified DNA strands) were subjected to Sanger sequencing. The editing frequency was estimated as the % ratio of the guanosine (black) peak over the sum of guanosine and adenosine (green) peaks of the sequencing chromatograms. Editing frequency estimated for three independent experiments (n = 3, technical replicates) is presented in Supplemental information (Supplementary Data 2 and Source Data file).

Editing of A–C mismatches in telomeric repeat RNA:DNA hybrids facilitates their resolution by RNase H2. In order to obtain insight into the mechanism by which ADAR1p110-mediated editing of telomeric repeat RNA:DNA hybrids contribute to resolution of telomeric R-loops, we looked for candidate cofactors of ADAR1p110 required for removal of RNA strands from telomeric RNA:DNA hybrids. We conducted immunoprecipitation experiments using HEK293T cells stably expressing FLAG-ADAR1p110-WT. We found that FLAG-ADAR1p110-WT co-immunoprecipitated with RNase H2A and H2C subunits but not with RNase H1 (Fig. 8a, upper panels), revealing the close association of ADAR1p110 with RNase H2 subunits. Similar experiments with FLAG-ADAR2 revealed no association of ADAR2 with RNase H1 or RNase H2 subunits (Supplementary Fig. 7). The ADAR1p110 interaction with RNase H2 subunits was lost when FLAG-ADAR1p110-EAA dsRNA-binding defective mutant was used as the bait (Fig. 8a, upper panels). Finally, we conducted a reciprocal pull-down experiment using FLAG-tagged RNase H2A as the bait. FLAG-RNase H2A pulled down endogenous ADAR1p110 (Fig. 8a, lower panels). Furthermore, both FLAG-ADAR1p110-WT and FLAG-RNase H2A pulled down TRF2, a telomere-binding protein and a member of the Shelterin complex (33,34). These results together indicate that ADAR1p110 interacts with RNase H2, but not with RNase H1, on the telomeric RNA:DNA hybrids, and possibly collaborates in dissociating telomeric RNA:DNA hybrids containing mismatched A–C base pairs.

Accordingly, we prepared telomeric repeat RNA:DNA hybrids containing different numbers of A–C-mismatched base pairs as well as I:C-matched RNA:DNA hybrids (mimicking A-to-I-edited hybrids), which were then subjected to in vitro assay using purified human recombinant RNase H1 and RNase H2A/2B/2C complex proteins. To our surprise, RNase H1 digested RNA strands of telomeric repeat RNA:DNA hybrids regardless of the number of A–C-mismatched base pairs (Fig. 8b, upper panels and...
In contrast, we found that digestion by RNase H2 is very sensitive to the presence of A-C-mismatched base pairs. The RNA strand of the RNA:DNA hybrids containing six A-C-mismatched base pairs was almost completely resistant to digestion by RNase H2 (Fig. 8b, lower panels and 8c, right). As A-C-mismatched base pairs are replaced with matched I:C base pairs, the RNA:DNA hybrids became more permissive to RNase H2-mediated digestion (Fig. 8c, right): more efficient digestion for hybrids with more I:C-matched base pairs (Fig. 8d and Supplementary Fig. 8).

**Fig. 5 Detection of telomeric variant repeats in ALT-positive and non-ALT cancer cell lines.** a Dot blot hybridization analysis for genomic DNA samples was conducted using three separate telomeric repeat probes capable of distinguishing a single-nucleotide mismatch (Supplementary Fig. 4a, b). In addition to canonical TTAGGG signals, varying amounts of TCAGGG and TTGGGG variant repeat signals were detected in both ALT and non-ALT cancer cell lines, but not in primary human fibroblast cells. b Quantitation of canonical and variant telomeric repeats was done by comparing dot blot signals of genomic DNA and canonical and variant repeat-specific control oligos. Three independent dot blot hybridization analyses were performed. Data are mean ± SD (n = 3, biological replicates). All individual experimental data values are presented in Source Data file.
Fig. 6 Increased telomeric RNA:DNA hybrids containing variant repeats in ADAR1-depleted cells. Formation of telomeric repeat RNA:DNA hybrids containing A–C mismatches by in cis slipped hybridization (a, b). a) TERRA RNAs transcribed from the region containing four TCAGGG (green) variant repeats surrounded by TTAGGG (gray) canonical repeats form an RNA:DNA hybrid containing four C–A mismatches by in cis slipped hybridization to the C-strand DNA containing canonical TTAGGG (CCCTAA) repeats. b) TERRA RNAs transcribed from the region containing four TTGGGG (orange) variant repeats surrounded by TTAGGG (gray) canonical repeats form an RNA:DNA hybrid containing four A–C mismatches by in cis slipped hybridization to the C-strand DNA containing TTGGGG (CCC CAA) variant repeats. c, d) Detection of increased RNA:DNA hybrids containing TCAGGG and TTGGGG variant repeats in ADAR1-depleted HeLa cells. DRIP products were examined for G-strand RNAs of UCAGGG variant and UUAGGG canonical repeats by dot blot analysis using high-affinity LNA-oligonucleotide probes capable of distinguishing a single-nucleotide mismatch (Supplementary Fig. 4c). Similarly, DRIP products were examined for C-strand DNAs of TTAGGG (CCCTAA) canonical and TTGGGG (CCC CAA) variant repeats using LNA-oligonucleotide probes capable of distinguishing a single-nucleotide mismatch (Supplementary Fig. 4d). c, d) Dot blot signals were abolished by E. coli-RNase H treatment prior to DRIP. The significance of the increase in RNA:DNA hybrids containing telomeric canonical and variant repeats (RNA and DNA strands) was confirmed by conducting three independent dot blot hybridization analysis of DRIP products. Data are mean ± SD (n = 3, biological replicates); significant differences were identified by two-tailed Student’s t tests: *P < 0.05, **P < 0.01, ***P < 0.001. All individual experimental data values and exact P values are presented in Source Data file.
ADAR1 depletion leads to the accumulation of RNA:DNA hybrids only in non-ALT cancer cells. Our FLAG-ADAR1p110 immunoprecipitation experiments revealed the association of ADAR1p110 with RNase H2 subunits, but not with RNase H1 (Fig. 8a). Efficient resolution of telomeric RNA:DNA hybrids by RNase H1 has been reported specifically with ALT activity positive cancer cell lines (ALT cells)45. Interestingly, the same study also reported that RNase H1 had no or very little effects on resolution of telomeric RNA:DNA hybrids in non-ALT cell lines45, indicating different mechanisms used for regulation of telomeric R-loops in ALT versus non-ALT cancer cells. Accordingly, we investigated the effects of ADAR1 depletion on accumulation of RNA:DNA hybrids in both ALT and non-ALT cancer cells. Interestingly, we found that ADAR1p110 expression levels are much higher in non-ALT cancer cells as compared to those in ALT cancer cells and primary fibroblast cells (Fig. 10a). Furthermore, the interaction of ADAR1p110 with RNase H2A was detected only in non-ALT cancer cells, but not in ALT cancer cells and primary fibroblast cells (Fig. 10b). Interestingly, ADAR1 depletion resulted in the upregulation of M-phase-specific marker genes (Fig. 1d), perhaps indicating an M-phase-specific ADAR1p110 function in suppression of telomeric R-loops. Accordingly, we analyzed the interaction of ADAR1p110 and RNase H2 by F-ADAR1p110 co-immunoprecipitation (co-IP) experiments in cells synchronized to the M phase. First, we noticed that RNase H2A expression levels increased significantly, >2-fold, at the M phase (Fig. 10c, compare unsynchronized and M-phase extract lanes), while endogenous ADAR1p110 levels unchanged (Fig. 10c, compare unsynchronized and M-phase extract vector-only lanes). Most importantly, we found that the ADAR1p110–RNase H2A interaction increased >3-fold in M-phase synchronized cells as compared to unsynchronized cells (Fig. 10c, FLAG-IP lanes). These results perhaps indicate a special need in non-ALT cancer cells for two-step removal of RNA strands of telomeric RNA:DNA hybrids accumulated specifically around late G2 to M phase: first correction of mismatched base pairs by the nuclear ADAR1p110 and then for degradation of RNA strands by RNase H2 (Fig. 8d and Supplementary Fig. 8). Taken together, our results indicate a differential role played by RNase H1 and RNase H2 in resolution of R-loops accumulated in ALT and non-ALT cancer cells, respectively, due to their different activity in degrading mismatch-containing telomeric RNA:DNA hybrids (Supplementary Fig. 10).

**Discussion**

Several regulators of telomeric R-loops such as 5' to 3' exonuclease, Rat1p49, flap endonuclease 160, and RNase H1 and...
H245,59,61 have been reported. In this study, we identified ADAR1p110 as a major regulator of telomeric R-loops specifically in cancer cells. Variant telomeric repeats such as TCGGG and TTGGGG repeats are widespread in both ALT and non-ALT cancer cell lines38,39 (Fig. 5). Because of these variant repeats, cancer cells encounter a unique problem in solving telomeric RNA:DNA hybrids containing mismatched base pairs. Hybridization of TERRA molecules carrying the G-strand UCCCGG variant sequences to the C-strand DNA carrying the canonical CCCTAA (antisense of TTAGGG) repeats

**Fig. 8** Editing of A–C mismatches in telomeric repeat RNA:DNA hybrids facilitates RNase H2 cleavage of RNA strands. a F-ADAR1p110-WT recombinant protein was copurified with endogenous RNase H2 subunits H2A and H2C, but not with RNase H1. No RNase H2 association detected with a dsRNA-binding defective mutant F-ADAR1p110-EAA (upper panels). Similarly, FLAG-RNase H2A pulled down endogenous ADAR1p110 (lower panels). Note that both FLAG-ADAR1p110 and FLAG-RNase H2A pulled down endogenous TRF2, a telomere-binding protein and a member of the Shelterin complex, supporting our hypothesis that ADAR1 and RNase H2 collaborate together to resolve telomeric R-loops. Protein molecular weight markers are presented in Source Data file. b In vitro assay for digestion of 5′-32P-labeled RNA strands of telomeric repeat RNA:DNA hybrids by RNase H1 or RNase H2A/2B/2C complexes. RNase H2 could not degrade the RNA strands of telomeric repeat RNA:DNA hybrids containing six A–C mismatches, but began digesting RNA strands of RNA:DNA hybrids containing four A–C mismatches. Replacement of all A–C mismatches with I:C-matched base pairs resulted in efficient digestion of RNA strands by RNase H2. RNase H1 degraded RNA strands regardless of the number of A–C mismatches. c Time course of digestion of RNA strands of telomeric repeat RNA:DNA hybrids with varying numbers of A–C mismatches by RNase H1 or RNase H2. Data are mean ± SD (n = 4, technical replicates); significant differences were identified by two-tailed Student’s t tests: *P < 0.05, ***P < 0.001, n.s., not significant. All individual experimental data values and exact P values are presented in Supplementary Data 3. d A-to-I editing of A–C mismatches to I:C base pairs in RNA:DNA hybrids facilitates digestion of RNA strands by RNase H2.
(Fig. 6a and Supplementary Fig. 5a) or hybridization of TERRA molecules carrying the G-strand canonical UUAGGG sequence to the C-strand DNA carrying the CCCCAA (antisense of TTGGGG) variant sequence (Fig. 6b and Supplementary Fig. 5b), either by slipped hybridization or in \textit{trans} hybridization, would result in the formation of telomeric repeat RNA-DNA hybrids containing C–Ao or A–C mismatches, respectively. The possibility of R-loop formation induced by mismatches between nascent RNA and DNA sequences has been previously pointed out\cite{52}. 

**Fig. 9 ADAR1 regulates accumulation of telomeric R-loops only in non-ALT cells.** a Detection of increased RNA-DNA hybrids only in non-ALT cells. Genomic DNA samples (0.25 µg) collected from various cells treated with siControl or siADAR1-1 RNAs for 72 h were examined by dot blot assay using the S9.6 antibody. Data are mean ± SD (n = 3, biological replicates); significant differences were identified by two-tailed Student’s t tests: *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant. b DRIP products from select cell lines were further subjected to dot blot analysis for the C-strand telomeric repeat DNA using the canonical telomeric repeat (TTAGGG) specific probe (Supplementary Fig. 4a). Telomeric repeat RNA-DNA hybrids were detected only in HEK293T and HCT116 non-ALT cancer cell lines. Data are mean ± SD (n = 3, biological replicates); significant differences were identified by two-tailed Student’s t tests: *P < 0.05, **P < 0.01, n.s., not significant. a, b All individual experimental data values and exact \( P \) values are presented in Source Data file.
We found that ADAR1p110 could edit efficiently A–C-mismatched adenosines of both RNA and DNA strands within telomeric RNA:DNA hybrids and convert them to I:C-matched Watson–Crick base pairs. Interestingly, telomeric variant repeats have been reported to expand during ALT-mediated inter- and/or intra-telomeric recombination in ALT cells, and by a currently unknown mechanism in non-ALT cells. An interesting possibility arises upon A-to-I editing of these mismatched A–C base pairs to I:C-matched base pairs: replication of A-to-I-edited C-strand DNA could generate more variant telomeric TCAGGG repeats (Supplementary Fig. 8, bottom), perhaps explaining the reported expansion of this variant repeat in cancer cells. Most importantly, we found that A-to-I editing of A–C mismatches within RNA:DNA hybrids is critical for efficient digestion of RNA strands by RNase H2, and consequent resolution of telomeric R-loops (Fig. 8d and Supplementary Fig. 8), because RNase H2, unlike RNase H1, is incapable of digesting RNA strands of mismatch-containing RNA:DNA hybrids. Our findings on association of ADAR1p110 with RNase H2, but not with RNase H1 in living cells (Fig. 8a), together with elevated expression of RNase H2A subunit (Fig. 10a), especially at the M phase (Fig. 10c), further confirms collaboration between ADAR1p110 and RNase H2 in resolving specifically telomeric RNA:DNA hybrids containing A–C mismatches, perhaps at late G2 to M phase, in non-ALT cancer cells (Supplementary Fig. 10).

We currently do not know the exact reason why a specific requirement of ADAR1p110 for suppression of telomeric R-loops is restricted to non-ALT cells (Fig. 9a, b and Supplementary Fig. 10), as widespread presence of telomeric variant repeats has been detected in both ALT and non-ALT cancer cells (Fig. 5). The cell cycle-dependent function of RNase H2, specifically at G2–M phase, in resolution of R-loops, has been reported also in yeast. In contrast, RNase H1 has been reported to act through the entire cell cycle in yeast. We confirmed elevated expression of RNase H2 and its interaction with ADAR1p110 specifically at the M phase in non-ALT cancer cells (Fig. 10c). Most interestingly, an association of RNase H1 with telomeric RNA:DNA hybrids at strong R-loop-forming loci has been reported only in ALT cancer cells with overly elevated TERRA expression levels (Supplementary Fig. 10). Association of RNase H1 to telomeric R-loops may also be further facilitated by more frequent binding of RPA32 to the single-stranded G-strand DNA specifically in ALT cells. RPA32 has been shown to enhance the association of RNase H1 and R-loops (Supplementary Fig. 10, left). Knockdown of RNase H1 in HeLa cells (non-ALT cells) indeed did not lead to a significant increase of R-loops (Fig. 2d), in agreement with the previous report: RNase H1-dependent resolution of telomeric R-loops occurs only in ALT cells (Supplementary Fig. 10, left). TERRA expression levels seem to be another factor that differentiates non-ALT cells from ALT cells. In non-ALT HeLa cells, expression of TERRA is regulated in a cell cycle-dependent manner: lower in S and early G2, but increasing toward the end of G2 and M phase. Furthermore, TERRA expression levels remain low in non-ALT cells such as HeLa and HEK293T, and thus unable to support strong R-loop-forming loci and recruit RNase H1 (Supplementary Fig. 10, right). Instead, RNase H2 together with ADAR1p110 is recruited to such not so
strong R-loop-forming loci during G2–M phase, and RNase H2 and ADAR1p110 cooperatively resolve telomeric repeat RNA:DNA hybrids containing mismatched base pairs (Supplementary Fig. 1, right). Clearly, many issues remain to be resolved with regard to why and how two mechanisms are differentially employed for resolution of telomeric R-loops in ALT and non-ALT cancer cells.

Telomere abnormalities such as telomere losses and telomere leading-strand-mediated fusions, most likely caused by unresolved telomeric R-loops, were detected in ADAR1-depleted cells (Supplementary Fig. 1). Although we have no direct evidence to specify the exact timing, telomeric RNA:DNA hybrids may form and accumulate from the late S phase through M phase in ADAR1-depleted HeLa cells. Unresolved telomeric RNA:DNA hybrids as well as prolonged exposure of single-stranded G-strands most likely lead to double-stranded DNA breaks and eventually to telomere losses and fusions. Apparently, the telomerase activated in non-ALT cells does not mend the shortened telomere ends. Non-homologous end joining and DNA-PKcs participate in telomere end-capping, exclusively producing telomeres generated by leading-strand synthesis in non-ALT cancer cells.\(^{66,67}\) This end-capping process does not seem to function efficiently in ADAR1-depleted cells. It has been reported that unresolved and persistent R-loops interfere with the DNA double-strand break (DSB) repair mechanism.\(^{68}\) Although factors involved in DSB repair, such as DNA-PKcs and \(\gamma\)-H2AX, are activated in ADAR1-depleted cells (Fig. 1d), the DSB repair mechanism may be hindered due to the presence of unresolved RNA:DNA hybrids, which could also contribute to the telomere abnormalities detected. Additional and specific studies will be required to address these issues.

Recent elegant studies by Nicholas Haining and his colleagues\(^{20}\) revealed the possibility that ADAR1 inhibitors could restore MDAS-MAVS-IFN signaling and inflammatory responses in tumors and resurrect their response to therapy utilizing immune checkpoint blockade. However, our studies presented here suggest another possibility: elimination of ADAR1 and/or suppression of its A-to-I editing activity would lead to the accumulation of telomeric repeat R-loops and consequent genome instability and apoptosis particularly in non-ALT and telomerase-positive cancers, which are, in fact, 70–80% of all types of cancers.\(^{45}\) We predict that ADAR1 inhibitors would be very effective therapeutics for cancer treatment because they will interfere with two completely different pro-oncogenic ADAR1 functions: suppression of MDAS-MAVS-IFN signaling by the cytoplasmic ADAR1p150 and maintenance of telomere stability in telomere-reactivated cancer cells by the nuclear ADAR1p110.

Methods

**Cell lines and cell culture reagents.** HeLa human ovarian carcinoma (ATCC CCL-2), HEK293T human embryonic kidney (ATCC CRL-11268), HCT116 human colon carcinoma (ATCC CCL-247), H460 human fibrosarcoma (ATCC CCL-121), U2OS human osteosarcoma (ATCC HTB-96), WI38-V13A human fibroblasts (ATCC CCL-75.1), Saos2 human osteosarcoma (ATCC HTB-85), WI38 lung fibroblasts (ATCC CCL-75), and IMR90 lung fibroblast cells (ATCC CCL-186) were used in this study. Adar1\(^{−/−}\) MEF cells and isogenic control cells were established from Adar1\(^{−/−}\)/− and Adar2\(^{−/−}\)/−/− MEF cells and isogenic control cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gemini), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere with 5% CO\(_2\), U2OS, WI38-V13A, and WI38 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

**Plasmid construction.** An Ncol restriction site was added to the multi-cloning site ( MCS) of CSII-EF-MCS-IREs-purumycin-resistant gene (puro) by inserting the new MCS site oligonucleotide into Norl–BamHI-digested CSII-EF-MCS-IREs-puro vector\(^{70,71}\). CSII-EF-FLAG-ADAR1p110-WT-IREs-puro, CSII-EF-FLAG-ADAR1p110-E912A-IREs-puro, or CSII-EF-FLAG-ADAR1p110p3XFLAG-IREs-puro overexpression vectors in HeLa cells was prepared by PCR cloning using pX3FLAG-CMV10-ADAR1p110-WT, pX3FLAG-CMV10-ADAR1p110-E912A, or pX3FLAG-CMV10-ADAR1p110p3XFLAG-IREs-puro, respectively.\(^{69}\) The FLAG-ADAR1p110 PCR products were amplified using primers CSII-FLAG-p3XFLAG-10-F and CSII-FLAG-p3XFLAG-10-R. The PCR products were digested with Norl and BamHI and then inserted into CSII-EF-MCS-IREs-puro. The FLAG-ADAR1p150 PCR products were amplified using primers CSII-p3XFLAG-150-F and CSII-p3XFLAG-150-R. The PCR products were digested with Norl and Nhel and then inserted into CSII-EF-MCS-IREs-puro. CSII-EF-FLAG-RNaseH2A was prepared by PCR cloning using pcDNA3.1 RNaseH2A plasmid.\(^{72}\) The FLAG-RNaseH2A PCR products were amplified using primers CSII-FLAG-F and CSII-FLAG-R and cloned into CSII-FLAG-RNaseH2A-F and CSII-FLAG-RNaseH2A-R. The PCR products were digested with Norl and BamHI and then inserted into CSII-EF-MCS-IREs-puro. CSII lentivirus vector was a kind gift from Hiroyuki Miyoshi and Toru Nakano. pET28-FLAG-RNaseH2A used for recombinant protein purification was prepared by PCR cloning using pCI-NheRNaseH2A plasmid. The PCR products were amplified using primers pET28-FLAG-RNaseH2A-F and FLAG-RNaseH2A-R, digested with Xhel and Xhol, and inserted into pET28 vector.\(^{73}\) pET28-His-RNaseH2A and pET15-His-RNaseH2A/2/C were kind gifts from Marcin Nowotny. Oligo DNAs used for plasmid construction are listed in Supplementary Data 1.

**Gene knockdown.** Gene knockdown experiments were done by RNA interference methods using Lipofectamine RNAiMax (Life Technologies) or HiperFect at a final short interfering RNA (siRNA) concentration of 1, 2, or 5 nM. All siRNAs used in this study are listed in Supplementary Data 1.

**Lenti virus infection.** HEK293FT cells (5–6 × 10\(^5\)) incubated in a 1 cm dish for a confluency of 80% were transfected with the following three plasmids using Lipofectamine 3000: 17 μg of CSII-EF-FLAG-ADAR1 or CSII-EF-FLAG-RNaseH2A plasmid, 10 μg of pCAG-HIVgpr (GAG-POL DNA), and 10 μg of the vesicular stomatitis virus G (VSV-G) envelope plasmid pCMV-VSV-G. After 48 h incubation, the cell culture supernatant was filtered with a 0.45 μm filter and concentrated by Lenti-X concentrator (Clontech). The lentiviral pellet was resuspended in fresh culture medium containing 8 μg/ml of polybrene (Sigma) and added to 1 × 10\(^5\) cells cultured in a 6-well plate. Infected cells were then incubated with puromycin (1 μg/ml) for 48 h post infection for antibiotic selection. The extent of infection of CSII-EF-FLAG-ADAR1 or CSII-EF-FLAG-RNaseH2A was evaluated by western blotting analysis and immunostaining with anti-FLAG 12C antibody. ADAR1 rescue experiments required exogenous FLAG-ADAR1 expression in every cell and were carried out using early passage cells (spassage 6).

**Immunofluorescence staining.** Transfection of siRNAs (siADAR1-1) into HeLa cells at 1 nM concentration was carried out as described above. After incubation for 24 h, the culture medium was replaced with a fresh medium containing CellLight Tubulin-GFP and BacMam 2.0 (Thermo Fisher Scientific). Nuclei were stained with SI-R-DNA reagent (Cytoskeleton) at 0.25 μM for 6 h. Cells were cultured on Ibidi μDish 3.5 cm. After 72 h, cells were fixed with 4% paraformaldehyde and soaked in Dulbecco’s PBS. Microscopic images were obtained by using a Leica TCS SP5 DMI6000 CS Confocal Microscope and LAS X software (Leica), equipped with ultraviolet 405 diode, Argon, DPS3561, and HeNe594 lasers. Fluorescent images were captured with a 40x lens for满分 frame. For quantification, the following wavelength settings were used: Tubulin-GFP (Ex 488 nm/Em 498–630 nm) and SI-R-DNA reagent (Ex 647 nm/Em 657–800 nm). Nuclear morphological analysis was performed using 4',6-diamidino-2-phenylindole (DAPI)-stained HeLa cells.

**Immunoblot analysis.** Cell lysates were prepared in Laemmli buffer containing benzamidine nuclease (Sigma), complete EDA-free proteinase inhibitor cocktail (Roche), and PhosStop phosphatase inhibitor cocktail (Roche) and size-fractionated by 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were blotted to Immobilon-P nylon membrane (Millipore). Membranes were blocked with 10% Blocker BSA (bovine serum albumin) buffer (Thermo Fisher Scientific) and incubated with the primary antibodies overnight at 4 °C. After incubation with each appropriate secondary antibody, bands were detected with ECL (GE Healthcare) using X-ray films. Antibodies were diluted in 10%...
Blocker BSA buffer (Thermo Fisher Scientific). Antibodies used in this study are listed in Supplementary Data 4.

Dot blot analysis of genomic DNA. Cells were treated with sRNA for 72 h in a 10 cm dish, detached from the dish surface with TrypLE Express Enzyme, and harvested by centrifugation. After PBS wash, genomic DNA was purified using QIAGEN Blood & Cell Culture DNA Midi Kit. Briefly, the cell pellet was resuspended in buffer C1. After repeated buffer C1 wash and removal of the cell debris, the nuclear pellet was resuspended in buffer G2 (without RNase A) and treated with 2 μg of proteinase K at 50 °C for 60 min. The nuclear fraction was applied to a buffer QBT-treated QIAGEN Genomic-tip and washed twice with buffer QC. Genomic DNA was eluted with buffer QF and precipitated with 2-propanol. The DNA pellet was washed twice with 80% ethanol and air-dried. Genomic DNA was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubated overnight at 4 °C.

Genomic DNA was diluted in 100 μl of 6X saline sodium citrate (SSC) and spotted onto a Hybond N+ (GE Healthcare) using a Bio-Dot Applicator (#1706545, Bio-Rad). The membrane was cross-linked with ultraviolet (UV) (0.24 J) and blocked with 5% non-fat dry milk (LabScientifics) in PBS with 0.1% Tween-20 (PBST) for 1 h and then with Superblock buffer (Thermo Fisher Scientific) for 1 h at room temperature. The membrane was incubated with S9.6 antibody (Sigma) at 0.1 μg/ml in Superblock buffer overnight at 4 °C. After washing four times with PBST, dot signals were detected with ECL (GE Healthcare) using X-ray films. For the treatment with RNA H, 1 μg of genomic DNA was preincubated with 2 μl of cDNA (Invitrogen) (NEB) at 2 h at 37 °C. DNA:DNA, RNA: RNA, or RNA:DNA oligo duplex controls were annealed in buffer containing 10 mM Tris-HCl (pH 7.6) and 50 mM NaCl at 80 °C for 5 min, followed by slow cooling to room temperature. Oligonucleotides were used as controls are listed in Supplementary Data 1.

DNA:RNA hybrid immunoprecipitation. Fifty micrograms of genomic DNA prepared as described above was diluted in 250 μl of sonication buffer and sonicated using Bioruptor (Diagonode) (20 cycles at high power, 30 s ON/60 s OFF) or Sonicator W-220 (Heat Systems Ultrasonics) (20 cycles at 1x3.5, 10 s ON/40 s OFF). During sonication, samples were kept on ice to prevent DNA degradation. Sonicated genomic DNA was mixed with purified donkey anti-mouse IgG secondary antibody (Jackson Immuno Research) (0.04 μg/ml) at 0.1 μg/ml in Superblock buffer 1 h at room temperature. After washing four times with PBST, dot signals were detected with ECL (GE Healthcare) using X-ray films. For the treatment with RNA H, 1 μg of genomic DNA was preincubated with 2 μl of cDNA (Invitrogen) (NEB) at 2 h at 37 °C. DNA:DNA, RNA: RNA, or RNA:DNA oligo duplex controls were annealed in buffer containing 10 mM Tris-HCl (pH 7.6) and 50 mM NaCl at 80 °C for 5 min, followed by slow cooling to room temperature. Oligonucleotides were used as controls are listed in Supplementary Data 1.

Dot blot analysis of DRIP products. DRIP products or whole genomic DNA (10 μl) were mixed with 15 μl of 0.13 N NaOH/3.3 mM EDTA solution and incubated at 90 °C for 10 min. The denatured DRIP products were diluted in 100 μl of 6X SSC and spotted onto a Hybond N+ using Bio-Dot Apparatus. The membrane was cross-linked with UV (0.24 J) and pre-hybridized with ULTRAhyb Ultrastreisn Hybridization Buffer (Invitrogen) overnight at 42 °C. 5'-32P-labeled DNA or LNA-oligonucleotide probe was added to the hybridization buffer and incubated overnight at 42 °C. RNase H treatment was overnight at 37 °C in 0.1X SSC/ 0.1% SDS solution for 15 min at 42 °C or at 50 °C. A fraction of DRIP samples (5%) was spotted as an input control onto the membrane. Hybridized probe signals were detected using Typhoon RGB Imager (GE Healthcare, Amersham Typhoon Control software). Oligonucleotides used as probe and washing temperatures are also listed in Supplementary Data 1. Consensus α-Alu, Alu, and LINE1 probes were hybridized and washed at 42 °C. Using these less stringent hybridization and washing conditions, these probes target variations known to exist within subfamily members of each repetitive element. In particular, the Alu consensus probe is 44 nucleotides, so it can recognize all Alu subfamilies, except Alu that is missing the 3′ region. Therefore, consensus Alu and LINE1 probes recognize ~11% and 18% of the human genome, respectively.

RNA strand analysis of DRIP products. Fifty micrograms of genomic DNA prepared as described above was diluted in 250 μl of sonication buffer and sonicated using Sonicator W-220 (20 cycles at Lyv3.5, 10 s ON/40 s OFF). During sonication, samples were kept cold very carefully. A fraction of the genomic DNA sample (90%, 225 μl) was used for immunoprecipitation with the S9.6 antibody (Sigma) or Kerafast), and the remaining fraction (10%, 25 μl) was used as the input control. Protein A beads (Dynabeads Protein A, Invitrogen) were preincubated with 25 μl of proteinase K (Roche) in the presence of 160 U of RNasin Plus inhibitor (Promega). Sonicated genomic DNA was diluted in 100 μl of 1X RNase H (NEB) for 2 h at 37 °C. DNA:DNA, RNA:RNA, or RNA:DNA oligo duplex controls were annealed in buffer containing 10 mM Tris-HCl (pH 7.6) and 50 mM NaCl at 80 °C for 5 min, followed by slow cooling to room temperature. Oligonucleotides were used as controls are listed in Supplementary Data 1.

Preparation of duplex substrates. Sense or antisense oligonucleotides of telomere sequences were purchased from DTG and Dharmacon. The 5′ ends of RNA and DNA strands to be analyzed were biotinylated. Sense and antisense oligonucleotides were annealed in annealing buffer (10 μM Tris-HCl pH 7.5, 50 mM NaCl) to prepare perfectly matched or mismatched dsRNAs or RNA:DNA hybrids, which were used as substrates for in vitro editing assay.

Preparation of recombinant ADAR1 proteins. All procedures were carried out at 4°C. HAT-ADAR1p110-WT-, FLAG-ADAR1p110-WT-, or HA-ADAR1p110- EAA-expressing 96-well plates were purchased with baculovirus. The cells were washed with PBS and resuspended in Tris+ buffer (250 mM Tris pH 7.8, 1 mM dithiothreitol (DTT), 0.6 mM mersalen (MMS), proteinase inhibitor cocktail). The cells were sonicated and debris was removed by centrifugation. The supernatant (cell extract) was diluted with an equal volume of 2x TGK buffer
Deoxycholate, 0.1% SDS, proteinase inhibitor cocktail, PhosStop, RNasin Plus (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium with 0.3% formaldehyde in PBS containing 1 mM DTT at room temperature for Protein co-IP chromatograms were analyzed by CodonCode Aligner (CodonCode Corporation). Platinum Transcriptionase (Thermo Fisher Scienti-
for 5 h, the cells were washed three times with PBS at 45 °C for 10 min. Finally, DNA was counterstained with DAPI. The slides were mounted with ProLong Gold (Thermo Fisher Scientific). Microscopic images were obtained by using a Leica TCS SP5 DMi6000 CS Confocal Microscope (Leica). Fluorescent images were captured with a ×63.0 lens by LAS X software (Leica). Ectopic expression of FLAG-ADAR1p110 was evaluated by immunostaining with anti-FLAG M2 antibody.

**Chromosome orientation-FISH.** Cells were cultured in a medium containing a 3:1 ratio of 5′-bromo-2′-deoxyuridine (BrdU, Sigma)/5′-bromo-2′-deoxyurycytidine (BrdC, Sigma) at a total final concentration of 10 μM during the final 24 h. Colcemid addition led to the accumulation of mitotic cells. Cultures were trypsinized (BrdC, Sigma) at a total

stained with 0.5 μg/ml Hoechst 33258 (Sigma) in 2× SSC for 15 min at room temperature. Slides were then exposed to 365 nm UV light for 25 min. The BrdU/BrdC-substituted DNA strands were digested with 3 U/μl exonuclease III (Promega) in a buffer supplied by the manufacturer (50 mM Tris-HCl, 5 mM MgCl₂, and 5 mM dithiothreitol, pH 8.0) for 10 min at room temperature. An additional denaturation was performed in 70% formamide, 2× SSC at 70 °C for 1 min, followed by dehydration in a cold ethanol series (70, 85, and 100%). The stranded chromosomal target DNA for hybridization of single-stranded probes.

**Time-lapse imaging.** HeLa cells were treated with CellLight Tubulin-GFP and BacMam 2.0 (Thermo Fisher Scientific) for 12 h before siRNA transfection. Transfection of siRNAs into HeLa cells at 1 nM concentration was carried out as described above. Nuclei were visualized by staining of DNA with Sir-DNA reagent (Cytoskeleton) (0.25 μM) for 6 h. Cells were cultured in CellView 3.5 cm glass-bottomed dishes (Greiner). Time-lapse images were obtained by using a Leica TCS SP5 DMi6000 CS Confocal Microscope between 48 and 72 h post transfection.

**Statistics and reproducibility.** All experiments were performed at least twice or more independent times with similar results. Image quantitation was done using Image J or ImageQuant software (GE Healthcare). Data were analyzed using Microsoft Excel (Microsoft Corporation) and were presented as means ± SD or SEM. Two-tailed t tests were conducted where the minimum level of significance was P < 0.05.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data supporting the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

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