The macroH2A1.2 histone variant links ATRX loss to alternative telomere lengthening

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The growth of telomerase-deficient cancers depends on the alternative lengthening of telomeres (ALT), a homology-directed telomere-maintenance pathway. ALT telomeres exhibit a unique chromatin environment and generally lack the nucleosome remodeler ATRX, pointing to an epigenetic basis for ALT. Recently, we identified a protective role for the ATRX-interacting macroH2A1.2 histone variant during homologous recombination and replication stress (RS). Consistent with an inherent susceptibility to RS, we show that human ALT telomeres are highly enriched for macroH2A1.2. However, in contrast to ATRX-proficient cells, ALT telomeres transiently lose macroH2A1.2 during acute RS to facilitate DNA double-strand break (DSB) formation, a process that is almost completely prevented by ectopic ATRX expression. Telomeric macroH2A1.2 is re-deposited in a DNA damage response (DDR)-dependent manner to promote homologous recombination-associated ALT pathways. Our findings thus identify the dynamic exchange of macroH2A1.2 on chromatin as an epigenetic link among ATRX loss, RS-induced DDR initiation and telomere maintenance via homologous recombination.

Telomere maintenance is essential for the survival of rapidly dividing tumor cells. To achieve this, tumors either re-express telomerase or undergo ALT. The latter is a telomerase-independent mechanism that relies on homology-directed telomere maintenance. ALT occurs in 5–15% of human tumors and is generally associated with poor prognosis1–3. Perhaps the most consistent indicator of ALT is a functional defect in the chromatin remodeler ATRX4,5. Supporting a role for ATRX in ALT, its re-expression was recently shown to suppress ALT hallmarks such as homologous recombination-dependent telomere sister chromatid exchange (T-SCE) through mechanisms that remain to be fully explored6–8. Further supporting a role for chromatin in ALT, loss of the histone chaperone ASF1 resulted in a rapid induction of the ALT phenotype in telomerase-positive cells6. Understanding the mechanistic link between chromatin structure and telomere maintenance pathways may, therefore, provide essential insight into the molecular pathways that regulate the growth of these malignant tumor types.

Chromatin perturbations in ALT cells are thought to act primarily by increasing RS susceptibility, which in turn promotes DSB formation to trigger homologous recombination-dependent telomere lengthening9. How these processes are coordinated is a matter of intense investigation. Of note, ATRX is recruited to chromatin on RS and its depletion aggravates RS-induced replication fork collapse and DSB formation10. Moreover, re-expression of ATRX in ALT cells reduces RS-associated DNA damage, implicating ATRX in the resolution of stalled replication forks11. With respect to chromatin, ATRX has been linked to the incorporation of two histone variants, H3.3 and macroH2A1 (refs. 11–13). We recently identified macroH2A1.2, one of two structurally distinct alternative macroH2A1 splice isoforms, as a mediator of homologous recombination and the replication stress (RS) response. Specifically, macroH2A1.2 promotes the recruitment of the tumor suppressor BRCA1 (refs. 14–16), which has been implicated in repair pathway choice at DSBs and stalled replication forks, where it facilitates homologous recombination as well as break-induced replication (BIR)17–19. BIR involves long-tract, conservative DNA synthesis on DNA break formation and subsequent strand invasion, a process recently found to orchestrate homology-directed telomere maintenance in ALT tumors20. Together, these findings raise the intriguing possibility that ATRX loss may affect ALT by modulating the macroH2A1.2 chromatin landscape at telomeres.

Here, we show that macroH2A1.2 is enriched at telomeres, particularly in ALT cells. Consistent with its role as a homologous recombination mediator, macroH2A1.2 loss results in defective homologous recombination-associated telomere maintenance. Perhaps more importantly, we identify an ATRX-dependent pathway that maintains macroH2A1.2 levels during acute RS, the absence of which accounts for RS-associated DSB formation in ATRX-deficient cells. MacroH2A1.2 thus presents a tightly regulated modulator of both telomere-associated DNA damage formation and its subsequent homology-directed repair, with direct implications for malignant growth.

Results

MacroH2A1.2 is enriched at telomeres and subtelomeric regions. Given the repetitive nature of telomeric DNA and its propensity to form secondary structures, telomeres are particularly difficult to replicate and, thus, intrinsically prone to RS12. We recently identified RS as a driver of macroH2A1.2 accumulation at fragile genomic regions18 and asked if macroH2A1.2 is similarly enriched at and functionally implicated in the maintenance of telomeric DNA. To assess macroH2A1.2 accumulation at chromosome...
ends, we performed macroH2A1.2 chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) using primer sequences against unique subtelomeric genomic loci<sup>21</sup>. Compared with non-fragile control loci, macroH2A1.2 was enriched at subtelomeric chromatin in a total of six cell lines tested. MacroH2A1.2 enrichment was most pronounced in telomerase-negative ALT cell lines, which are particularly susceptible to telomeric RS (Fig. 1a)<sup>2</sup>. Using ChIP followed by dot blot to detect TTAGGG telomere repeat DNA, we confirmed abundant macroH2A1.2 occupancy at telomeres in ALT-positive U2OS cells. The telomere-associated shelterin component TRF2 served as a positive control (Fig. 1b). No enrichment was observed on ChIP with the centromeric CENP-A protein (Supplementary Fig. 1a). Consistent with our previous work<sup>16</sup>, we also observed enrichment of macroH2A1.2 relative to input DNA at non-telomeric Alu repeats, which was, however, approximately tenfold less pronounced than at telomeres (Supplementary Fig. 1b). Together, these findings demonstrate that telomeres and subtelomeric regions are marked by the macroH2A1.2 histone variant.

**MacroH2A1.2 facilitates homologous recombination-dependent telomere maintenance at DSBs.** We next sought to investigate if macroH2A1.2 accumulation at telomeres plays a role in telomere maintenance. Consistent with this notion, RNA interference (RNAi)-mediated depletion of macroH2A1.2 resulted in a reduction in telomere signal intensity in two distinct ALT cell lines as measured by quantitative fluorescent in situ hybridization (Q-FISH) with a telomere-specific DNA probe (Fig. 1c and Supplementary Fig. 1c–f). Short interfering RNAs (siRNA) were directed against two distinct target sequences within the macroH2A1.2-specific exon 6a (si-1.2-1 or si-1.2-2). The efficiency and splice variant specificity of RNAi-mediated macroH2A1.2 depletion was confirmed by western blot (Supplementary Fig. 1c). MacroH2A1.2 depletion in the same two ALT cell lines further resulted in a robust decrease in the formation of extrachromosomal telomere (CCCTAA) DNA circles (C-circles), which are highly specific to ALT cells, provide a responsive marker of ALT activity<sup>22</sup> and have further been linked to elevated telomeric RS<sup>23</sup> (Fig. 1d and Supplementary Fig. 1g). To allow for efficient C-circle formation, cells were analyzed 6–12 days after stable, short hairpin RNA (shRNA)-mediated knockdown; shRNA target sequences were identical to those of siRNAs (Supplementary Table 1). These data demonstrate that macroH2A1.2 contributes to telomere maintenance and the ALT phenotype.

Consistent with macroH2A1.2 being a mediator of homologous recombination at DSBs<sup>18</sup>, its depletion resulted in an approximately twofold reduction in T-SCE in two distinct ALT cell lines (Fig. 2a,b and Supplementary Fig. 2a). T-SCE measures homology-directed...
Fig. 2 | MacroH2A1.2 promotes homology-directed repair at telomeres. a, T-SCE as measured by CO-FISH in U2OS cells in the absence (si-ctrl) or presence of macroH2A1.2 knockdown using two distinct siRNAs, si-1.2-1 and si-1.2-2. Box plots depict percentage of T-SCE events per sister chromatid, *P < 0.05, **P < 0.001, on the basis of Student’s two-tailed t-test. Representative CO-FISH images of a control metaphase chromosome are shown; scale bar, 2 μm. At least 20 metaphases were analyzed per sample. b, T-SCE as in a in GM847 cells. c, Western blot analyses in U2OS cells expressing shRFP, sh-1.2-1 or sh-1.2-2, see Supplementary Dataset 1 for uncorrected blots. d, Telomeric BrdU incorporation at TRF1-FokI-induced DSBs in U2OS cells expressing shRFP (n = 32 cells), sh-1.2-1 (n = 72 cells) or sh-1.2-2 (n = 70 cells). Representative immunofluorescence images are shown; scale bar, 10 μm, ***P < 0.001 by Mann–Whitney U-test. e, Fraction of BrdU+ TRF1-FokI foci from d, values represent mean and s.e.m. from five independent experiments. f, Fraction of γ-H2AX+ TRF1-FokI foci in the presence of the indicated siRNA, values represent mean and s.e.m. from six independent experiments. g, BRCA1 accumulation at TRF1-FokI-induced DSBs in U2OS cells expressing shRFP (n = 77 cells) or sh-1.2-2 (n = 60 cells). Representative immunofluorescence images are shown; scale bar, 10 μm, ***P < 0.001 by Mann–Whitney U-test. h, Fraction of BRCA1+ TRF1-FokI foci from g, values represent mean and s.e.m. from three independent experiments. i, Fraction of BRCA1+ TRF1-FokI foci in U2OS cells expressing si-ctrl or si-1.2-1, values represent mean and s.e.m. (n = 6). j, Fraction of BRCA1+ TRF1-FokI foci in U2OS cells overexpressing macroH2A1.2 or eGFP. e, h, i, *P < 0.05, **P < 0.01, on the basis of Student’s paired, two-tailed t-test.

telomeric gene conversion events via strand-specific labeling of telomere chromatids to allow for the detection of telomere-specific cross-overs by chromosome orientation fluorescence in situ hybridization (CO-FISH)14. To further corroborate a defect in telomeric homologous recombination, we assessed the impact of macroH2A1.2 loss on BIR at telomeres. BIR results in de novo DNA synthesis at sites of DSBs following homology-directed invasion of a broken DNA strand in a manner that is distinct from S phase replication20,25. BIR can be measured via the immunofluorescence-based detection of bromodeoxyuridine (BrdU) incorporation. Using an mCherry–TRF1-FokI fusion protein, which tethers the FokI endonuclease repeats to telomere repeats via the telomeric protein TRF1 and thus allows for telomere-specific DSB induction20,25, we measured BrdU intensity at mCherry–TRF1-FokI foci by immunofluorescence. Depletion of macroH2A1.2 with two distinct shRNAs resulted in a robust decrease in BrdU signal (Fig. 2d,e), indicating that macroH2A1.2 promotes BIR at broken telomeres. TRF1-FokI-induced DSB formation was confirmed by the accumulation of S139-phosphorylated histone H2AX (γ-H2AX) at TRF1-FokI foci and was not significantly altered by macroH2A1.2 depletion (Fig. 2f and Supplementary Fig. 2c). Of note, ALT telomere maintenance has been linked to RS-induced recruitment of BRCA117, which has in turn been implicated in promoting BIR at stalled replication forks18. In line with the fact that macroH2A1.2 promotes BRCA1 recruitment at non-telomeric DSBs14, shRNA-mediated macroH2A1.2 depletion caused a marked decrease in BRCA1 at TRF1-FokI-induced DNA breaks (Fig. 2g,h). Similar results were observed with an independent siRNA (Fig. 2i and Supplementary Fig. 2c,d,e). Conversely, overexpression of macroH2A1.2 resulted in increased BRCA1 accumulation in the same assay (Fig. 2j and Supplementary Fig. 2g). Little γ-H2AX or BRCA1 accumulation was observed with a catalytically dead FokI enzyme (FokI-D450A), demonstrating DSB requirement for efficient DDR activation (Supplementary Fig. 2f). Of note, BRCA1 recruitment to telomeric DSBs remained largely unaltered on depletion of the alternative macroH2A1.1 splice isoform (Supplementary Fig. 2h). Moreover, depletion of macroH2A1.1 did not impair homologous recombination in a reporter assay (Supplementary Fig. 2i). These observations emphasize the existence of unique, splice variant-specific functions of macroH2A1 isoforms, and a detailed dissection of the underlying molecular basis will be an important aspect of future investigations. Together, these findings demonstrate that macroH2A1.2 promotes homologous recombination-mediated telomere maintenance, which is correlated with BRCA1 recruitment to telomeric DSBs.

RS promotes acute macroH2A1.2 loss at telomeres of ATRX-deficient cells. Given the protective role for macroH2A1.2 during RS18, abundant, homologous recombination-permissive macroH2A1.2 levels at ALT telomeres are seemingly at odds with their increased susceptibility to RS. We, thus, asked whether RS may alter macroH2A1.2 occupancy at ALT telomeres to promote a more RS-prone chromatin environment. Consistent with this notion,
expression of the nucleosome remodeler and macroH2A1.2 inter-
this phenomenon is specific to ALT cells (Supplementary Fig. 3c).
Moreover, telomerase-positive tumor cell lines showed
and with a distinct source of RS, the DNA polymerase inhibi-
observed in two additional ALT cell lines (Supplementary Fig. 3c,d)
Fig. 3b,c). RS-induced macroH2A1.2 loss at telomeres was con-
restoration of ATRX almost completely rescued the hydroxyurea-
without ATM or ATR kinase inhibition (ATMi or ATRi). Values represent mean and s.d. from three independent experiments. See Supplementary Fig. 3g
protein inhibitor and replication poison hydroxyurea caused a >tenfold loss of subtelomere-associated macroH2A1.2 relative to its nucleosomal
b, Dot blot for telomeric DNA content in the indicated ChIP or input samples from a. Signal intensities for macroH2A1.2 ChIP were normalized against input, values represent mean and s.d. from three independent experiments. c, ChIP for macroH2A1.2 or H2A in the presence or absence of HU in ALT-negative K562 cells expressing a control shRNA or sh-ATRX. Enrichment relative to histone H2B is shown for the indicated loci. Values represent mean and s.d. from three independent experiments. See Supplementary Fig. 3e for corresponding telomere dot blot.
Conversely, ATRX depletion in ALT-negative K562 cells resulted
distinct subtelomeric loci and telomere-specific dot blot (Fig. 3a,b).
Notably, restoration of ATRX almost completely rescued the hydroxyurea-
induced macroH2A1.2 loss, as determined by both ChIP at distinct subtelomeric loci and telomere-specific dot blot (Fig. 3a,b). Conversely, ATRX depletion in ALT-negative K562 cells resulted in RS-induced loss of subtelomeric and telomeric macroH2A1.2, whereas canonical H2A and H2B histones remained largely unaltered (Fig. 3c and Supplementary Fig. 3c,f). Affinity purification of recombinant histone dimers containing H2B and either H2A or the histone-like region of macroH2A1 (mH2A11–122) using HeLa S3 nuclear extracts. Eluates and input samples were probed for ATRX. e, MacroH2A1.2 ChIP at the indicated loci in the presence or absence of HU with or without ATM or ATR kinase inhibition (ATMi or ATRi). Values represent mean and s.d. from three independent experiments. See Supplementary Fig. 3g for corresponding telomere dot blot. f, MacroH2A1.2 ChIP at the indicated loci, before, during and 6 hours after HU treatment. Values represent mean and s.d. from three independent experiments. For ChIP analyses, all pair-wise comparisons are significant on the basis of Student’s two-tailed t-test (P < 0.05), except when marked NS (not significant). Uncropped gel images are shown in Supplementary Dataset 1.

Fig. 3 | ATRX modulates macroH2A1.2 accumulation at telomeres during RS. a, MacroH2A1.2 ChIP at the indicated subtelomeric loci in the presence or absence of RS in U2OS cells carrying a Dox-inducible ATRX transgene. Cells were either treated with vehicle (control) or Dox (ATRX) before HU treatment. Enrichment relative to histone H2B is shown for the indicated loci, see Supplementary Fig. 3a for H2B ChIP. Values represent mean and s.d. from three independent experiments. b, Dot blot for telomeric DNA content in the indicated ChIP or input samples from a. Signal intensities for macroH2A1.2 ChIP were normalized against input, values represent mean and s.d. from three independent experiments. c, ChIP for macroH2A1.2 or H2A in the presence or absence of HU in ALT-negative K562 cells expressing a control shRNA or sh-ATRX. Enrichment relative to histone H2B is shown for the indicated loci. Values represent mean and s.d. from three independent experiments. See Supplementary Fig. 3e for corresponding telomere dot blot.
d, Affinity purification of recombinant histone dimers containing H2B and either H2A or the histone-like region of macroH2A1 (mH2A11–122) using HeLa S3 nuclear extracts. Eluates and input samples were probed for ATRX. e, MacroH2A1.2 ChIP at the indicated loci in the presence or absence of HU with or without ATM or ATR kinase inhibition (ATMi or ATRi). Values represent mean and s.d. from three independent experiments. See Supplementary Fig. 3g for corresponding telomere dot blot. f, MacroH2A1.2 ChIP at the indicated loci, before, during and 6 hours after HU treatment. Values represent mean and s.d. from three independent experiments. For ChIP analyses, all pair-wise comparisons are significant on the basis of Student’s two-tailed t-test (P < 0.05), except when marked NS (not significant). Uncropped gel images are shown in Supplementary Dataset 1.

exposure of U2OS cells to low levels of the ribonucleotide reductase
inhibitor and replication poison hydroxyurea caused a >tenfold loss of subtelomere-associated macroH2A1.2 relative to its nucleosomal binding partner H2B, by ChIP analysis (Fig. 3a). H2B levels were not substantially affected by RS or ATRX expression (Supplementary Fig. 3a). RS-induced macroH2A1.2 loss at telomeres was confirmed by telomere-specific dot blot (Fig. 3b). Similar results were observed in two additional ALT cell lines (Supplementary Fig. 3c,d) and with a distinct source of RS, the DNA polymerase inhibitor aphidicolin (Aph) (Supplementary Fig. 3b). No major macroH2A1.2 loss was detected at a non-fragile control locus, pointing to an RS-specific change in chromatin (Fig. 3a and Supplementary Fig. 3b,c). Moreover, telomerase-positive tumor cell lines showed little to no RS-induced telomeric macroH2A1.2 loss, suggesting that this phenomenon is specific to ALT cells (Supplementary Fig. 3c). Given that most ALT cell lines, including those tested here, lack expression of the nucleosome remodeler and macroH2A1.2 inter-
ATRA(4,12,28), we asked if the absence of ATRX is functionally linked to RS-induced macroH2A1.2 loss at telomeres. To do so, we took advantage of a doxycycline (Dox)-inducible system for ATRX re-expression in U2OS cells, which was shown to repress several hallmarks of ALT through yet to be defined mechanisms(8). Notably, restoration of ATRX almost completely rescued the hydroxyurea-
induced macroH2A1.2 loss, as determined by both ChIP at distinct subtelomeric loci and telomere-specific dot blot (Fig. 3a,b). Conversely, ATRX depletion in ALT-negative K562 cells resulted in RS-induced loss of subtelomeric and telomeric macroH2A1.2, whereas canonical H2A and H2B histones remained largely unaltered (Fig. 3c and Supplementary Fig. 3c,f). Affinity purification of recombinant histone dimers containing H2B and either H2A or the histone-like region of macroH2A1 (mH2A11–122) revealed that ATRX preferentially interacts with the histone-domain of macroH2A1, supporting a role for ATRX in macroH2A1 variant-specific nucleosome remodeling (Fig. 3d). ATRX deficiency does not, however, generally interfere with macroH2A1.2 accumulation at telomeres, as macroH2A1.2 is abundant at these genomic loci in the absence of RS both in ALT-positive cell lines and ATRX-depleted K562 cells (Fig. 3a–c and Supplementary Fig. 3c–f). Together, these
observations point to a unique, RS-specific role for ATRX in macroH2A1.2 maintenance at telomeres that is distinct from its impact on chromatin in the absence of damage.

DNA damage signaling and ATRX coordinate RS-associated macroH2A1.2 remodeling. Given the abundance of macroH2A1.2 at ALT telomeres, we reasoned that mechanisms must exist to counteract RS-induced macroH2A1.2 loss. In ATRX-proficient cells, macroH2A1.2 accumulation at sites of RS is controlled by DDR signaling. We, thus, sought to determine whether the DDR similarly affects macroH2A1.2 levels at ALT telomeres. Consistent with this notion, inhibition of ATM- or ATR-signaling significantly exacerbated macroH2A1.2 loss during hydroxyurea treatment in ALT cells, indicating that DDR signaling counteracts RS-induced macroH2A1.2 depletion in ATRX-deficient cells. Comparatively minor effects were observed at a non-fragile, non-telomeric control locus, consistent with fragile site-specific macroH2A1.2 reorganization described previously. In agreement with dynamic macroH2A1.2 depletion and re-accumulation at sites of DSBs, we observed a robust recovery of telomeric macroH2A1.2 levels 6 hours after hydroxyurea removal. In light of these findings, we propose that macroH2A1.2 deposition at sites of Rs involves both ATRX-dependent chromatin remodeling and DDR activation, resulting in transient, RS-specific macroH2A1.2 loss in ATRX-deficient cells.

ATRX prevents macroH2A1.2 loss and concomitant excessive DNA damage. We next sought to determine the impact of RS-associated macroH2A1.2 loss on telomeric DNA break formation. We have previously shown that macroH2A1.2 depletion results in a significant increase in fragile site-associated DNA damage in ATRX-proficient K562 cells. The latter is also the case for subtelomeric loci, which show a marked increase in $\gamma$-H2AX by ChIP in macroH2A1.2-deficient cells (Fig. 4a and Supplementary Fig. 4a). MacroH2A1.2 ChIP confirmed CRISPR-Cas9-mediated macroH2A1.2 gene inactivation (Supplementary Fig. 4a). We next asked if RS-induced macroH2A1.2 loss in ALT cells is similarly related to DSB formation. To do so, we assessed $\gamma$-H2AX induction in U2OS cells on RS in the absence or presence of ATRX re-expression, to allow for or prevent RS-associated macroH2A1.2 loss, respectively. ATRX induction was able to suppress DNA damage at ALT telomeres in response to RS, as determined by $\gamma$-H2AX ChIP qPCR at subtelomeric loci and by immunofluorescence for telomere dysfunction-induced foci (TIF) (Fig. 4b,c). TIFs are defined as DNA damage-bearing telomeres, which are detected on $\gamma$-H2AX immunofluorescence with telomere-FISH. To determine whether the protective effect of ATRX is dependent on macroH2A1.2, we re-expressed ATRX in the presence or absence of macroH2A1.2 knockdown followed by a quantification of TIF formation. In contrast to macroH2A1.2-proficient cells, ATRX re-expression did not significantly reduce TIFs in

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**Fig. 4 | ATRX and macroH2A1.2 cooperate to protect from RS-induced telomere damage.** a. ChIP for $\gamma$-H2AX at the indicated loci in K562 cells in the presence (wild type, WT) or absence of macroH2A1.2 (1.2 CRISPR-KO). Samples were normalized to untreated WT samples, values represent mean and s.d. from three independent experiments. See Supplementary Fig. 4a for macroH2A1.2 ChIP. b. $\gamma$-H2AX ChIP at the indicated loci in U2OS cells treated with vehicle (DMSO) or HU in the presence or absence of ATRX re-expression. Values represent mean and s.d. from three independent experiments. c. Frequency of TIFs in U2OS cells with ATRX induction in the presence or absence of Aph, TIFs were defined on the basis of colocalization of $\gamma$-H2AX (red) and TRF2 (green), a representative image from Aph-treated, shRFP expressing U2OS cells is shown; scale bar, 5 μm. Box plots depict the number of TIFs per cell. N: number of cells. ***P < 1 × 10<sup>-8</sup> by Mann–Whitney test, one of two independent experiments is shown. d. Model linking ATRX and macroH2A1.2 to ALT telomere maintenance. ATRX-dependent macroH2A1.2 retention at stalled replication forks protects from excessive DNA damage in ALT-negative cells. In ALT-positive cells, ATRX deficiency leads to macroH2A1.2 loss and DSBs in response to RS, which triggers DDR-dependent macroH2A1.2 re-deposition to facilitate homologous recombination. In the absence of RS, lack of ATRX has little effect on telomeric macroH2A1.2 levels, pointing to DNA damage-induced modulation of ATRX function, which may involve ATRX phosphorylation in S phase (see Discussion).
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...of macroH2A1.2-depleted cells exposed to RS, supporting the notion that ATRX counteracts RS-induced DNA damage by preventing macroH2A1.2 loss at telomeres (Fig. 4c). ATRX deficiency thus allows for increased RS at ALT telomeres via acute chromatin reorganization involving the transient depletion of macroH2A1.2. Underlining the potential relevance of these findings for ALT tumor growth, stable depletion of macroH2A1.2 caused a progressive, proliferative defect in two independent ALT cell lines (Supplementary Fig. 4b–d), whereas no growth defect was observed in two telomerase-positive and ALT-negative tumor cell lines (Supplementary Fig. 4e,f).

Discussion

Here, we identify macroH2A1.2 as a telomere-enriched chromatin component that links ATRX loss to homologous recombination-dependent telomere maintenance. Our findings are consistent with the following model. By promoting acute loss of macroH2A1.2 during RS, the absence of ATRX causes increased DSB formation to initiate ALT. A dynamic, DDR-dependent re-deposition of macroH2A1.2 at DSBs allows for macroH2A1.2 restoration at telomeres to facilitate homology-directed repair and, thus, promote the execution of ALT (Fig. 4d). Together, these findings provide a mechanistic rationale for ATRX loss in tumors that rely on ALT and identify macroH2A1.2 as a potential therapeutic target for the manipulation of ALT tumor growth.

Changes in telomeric chromatin have previously been implicated in ALT-associated telomere maintenance. The nucleosome remodeling and histone deacetylation complex was shown to promote remodeling of telomeric chromatin to create an environment that facilitates T-SCE

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**Methods**

Cell lines, viral infection and drug treatments. U2OS (American Type Culture Collection, ATCC, female), SaOs-2 (ATCC, female), GM(00847) (Coriell, male), HT1080 (ATCC, male), HeLa (ATCC, female) and HEK-293T cells (gift from the Broad Institute) were cultured in DMEM (Gibco) with 10% FBS (Gibco), with primary antibodies, followed by Alexa Fluor-labeled secondary antibody with 0.5% Triton X-100, and blocked with 3% BSA. Cells were immunostained were induced for ATRX expression with Dox for 24 h, followed by 1 mM Aph detection of TIFs. (Supplementary Fig. 2f).

Enrichment detected on expression of catalytic dead TRF1-FokI-D450A in asynchronous cells. Enrichment of γ-TRF2 (Novus Biologicals, IMG-124A), secondary antibody for western blotting were: α-H2B (Millipore, 07-2270), α-macroH2A1.1 (Cell Signaling, 12455s), α-macroH2A1.2 (Cell Signaling, IMG-124A), secondary antibodies were goat anti-mouse or goat anti-rabbit IgG (H+L) coupled to Alexa Fluor 488 or 647 (Life Technologies).

**Detection of TIFs.** Control and macroH2A1.2 knockdown U2OS-ACTRX cells were induced for ATRX expression with Dox for 24 h, following Dox induction, TRF1-FokI was stabilized and translocated to the nucleus using 1 µM Shield-1 (Takara Clontech) and 1 µM 4-hydroxytamoxifen (Sigma-Aldrich) for 2 h. Cells were then fixed in PBS + 2% parafomaldehyde, followed by permeabilization in PBS + 0.5% Triton X-100. Cells were subsequently blocked in 3% BSA in PBS + 0.5% Triton X-100. For BeDu immunofluorescence, U2OS TRF1-FokI cells were pulsed with 100 µM BeDu (BD Biosciences) along with Shield-1 and 4-hydroxytamoxifen for 2 h. Cells were then fixed in PBS + 4% parafomaldehyde, followed by permeabilization in PBS + 0.5% Triton X-100. Cells were denatured in 300 U ml⁻¹ DNAse (Roche) in 1× reaction buffer (20 mM Tris-HCl (pH 8.4), 2 mM MgCl₂, 50 mM KCl in PBS) for 25 min at 37 °C in a humidified chamber. Primary and secondary antibody stainings were carried out in PBS-T. Confocal z-stacks were acquired using a Zeiss LSM 780 microscope and a x40 oil objective (numerical aperture, 1.4). Images were displayed and analyzed as maximum intensity projections. Analysis of BeDu⁺ foci was limited to non-S phase cells, as defined by lack of RNA BeD staining, which was performed in asynchronous cells. Enrichment of γ-H2AX, BCA1 or BeDu was determined by measuring the fluorescence intensity at mCherry-labelled TRF1 foci relative to total nuclear intensity. Foci were defined as BeDu⁺, BCA1⁺ or γ-H2AX⁺, when the signal intensity at TRF1 foci was more than twofold greater that total nuclear signal intensity. This threshold was determined on the basis of background enrichment detected on expression of catalytic dead TRF1-FokI-D450A (Supplementary Fig. 2f).

**Telomere length measurement.** Q-FISH analysis was carried out as described previously, metaphases were collected 72 h post siRNA transfection. In brief, cells were incubated with 0.1 µg ml⁻¹ colcemid for 2 h at 37 °C, collected and immediately incubated in 75 mM KCl for 15–20 min at 37 °C, fixed in ice-cold methanol and glacial acetic acid (3:1) and dropped onto glass slides. Metaphase spreads were hybridized with 5'-end biotinylated PNA (GCCGGGCCGGCGCGGCCGGCGC; Invitrogen) and Cy3-labeled PNA (CCCTAA) (0.5 µg ml⁻¹). Panegane), washed and mounted with ProLong Gold antifade reagent (Invitrogen). Images were captured using Cytovision software (Applied Imaging) on a fluorescence microscope (Axio2; Carl Zeiss), followed by quantification of fluorescence signals of individual telomeres using Image or the TFL-Telo software (GE Healthcare Life Sciences).

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**Western blotting.** Cells were lysed with RIPA lysis buffer (150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS, 25 mM Tris-HCl pH 7.5) with protease inhibitor cocktail (Roche cComplete) and phosphatase inhibitors (Calbiochem). The protein samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Thermo Scientific) and detected with the indicated antibodies.

**ChIP and dot blot.** Cells were treated as indicated, crosslinked with 1% formaldehyde in culture media for 10 min, followed by quenching with 125 mM glycine. Cells were then permeabilized in swelling buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40). Nuclei were resuspended in MNase digestion buffer (10 mM Tris pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) and 0.8 U µl⁻¹ MNase (Thermo Scientific) was added for 30–45 min at 37 °C. The reaction was stopped by adding 50 mM EDTA, followed by centrifugation. The nuclear pellets were resuspended in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% SDS, 10%glycerol, 0.5% IGEPAL CA-630 and 0.5% N-lauroylsarcosine. lysates were sonicated briefly to disrupt nuclear membranes using an ultrasonicator with water bath (Bioruptor, Diagenode). Diluted lysates were incubated overnight at 4 °C with the indicated antibodies after addition of 1% Triton X-100. Immunoprecipitations were performed using protein A/G magnetic beads (Pierce, 10% v/v). Eluted DNA was purified with QIAquick PCR purification kit (Qiagen) according to the manufacturer instructions. Purified CHIP DNA was analyzed by qPCR using a LightCycler 480 II (Roche); see Supplementary Table 2 for primer sequences. All ChIP samples were normalized to input. In ChIP–PCR quantification, the standard comparative cycle threshold method was used to measure the amount of DNA. Where indicated, input-corrected CHIP DNA was used as a control normalization. For dot blot analyses, CHIP DNA was denatured using 0.5 M NaOH and 1.5 M NaCl and equal volume samples were loaded onto a Hybond N + nitrocellulose membrane (GE Biosciences) using the Bio-Dot apparatus (Bio-Rad). Unless noted otherwise, volumes loaded for immunoprecipitation samples were generally greater than volumes loaded for input samples. Signal intensity was normalized to input and expressed as arbitrary units. Membranes were washed once with denaturing buffer and wash 3x SSC, followed by UV-crosslinking (UV Stratalinker 1800, Stratagene) and blocking with 5x Denhardt’s solution (Thermo Scientific) for 1 h at 37 °C. Hybridization with TelC-Biotin (Biotin-PEG-CCTAACTACCTAAAC-TTACG, 37 °C, 30 min) was performed at 37 °C overnight. Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Scientific, catalog no. 89880) was used for signal detection and images were acquired using ChemiDox XRS with Image Lab software (Bio-Rad). Signal intensities were quantified with ImageQuant TL software (GE Healthcare Life Sciences).

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membrane (GE Biosciences) using the Bio-Dot apparatus (Bio-Rad) and UV-crosslinked, followed by TelC-Biotin hybridization and signal detection as described above.

**Histone dimer affinity assay.** Human H2A and macroH2A1-122 were cloned into a pET30-based vector harboring an amino (N)-terminal, TEV protease-cleavable histidine tag and a carboxy (C)-terminal FLAG tag. Human histone H2B was cloned into a pET30-based vector harboring an N-terminal HRV 3C protease-cleavable histidine tag. The recombinant histones were separately expressed in BL21 (DE3) *Escherichia coli* cells and purified under denaturing conditions as previously described. During the purification and renaturation into histone dimers the N-terminal histidine tags are proteolytically cleaved (TEV obtained from Promega, catalog no. V610A and HRV 3CN obtained from Pierce, catalog no. 1896490) yielding histones with native, ‘scarless’, N-terminal tails. Refolded H2A-H2B dimers or macroH2A1-122-H2B dimers (2.5 μg) were incubated with Anti-FLAG M2 affinity gel (A2220, Sigma) equilibrated in binding buffer (20 mM HEPES (pH 7.9), 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% Tween-20, 0.5 mM DTT, 0.1 mg ml⁻¹ BSA) with agitation at 4 °C. The histone-bound beads were then incubated with 250 μg of HeLa S3 nuclear extract for 2 h in nuclear extract-binding buffer (20 mM HEPES (pH 7.9), 450 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% Tween-20, 0.5 mM DTT) with agitation at 4 °C. The beads were then washed three times in nuclear extract-binding buffer before elution of the histone dimer-bound complexes with elution buffer (20 mM HEPES (pH 7.9), 50 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% Tween-20, 0.5 mM DTT, 200 μg ml⁻¹ FLAG peptide (F3290, Sigma)). The eluted material was separated by SDS-PAGE with a nuclear extract standard curve, transferred to nitrocellulose and immunoblotted.

**Statistics and reproducibility.** Statistical comparisons between replicate experiments were performed on the basis of Student’s two-tailed t-test; paired t-tests where used for paired datasets. The Mann–Whitney U-test was used for comparison of datasets that do not assume a normal distribution. One-way analysis of variance (ANOVA) was used for comparison of Q-FISH datasets. Relevant statistical tests are indicated in the figure legends. Statistical significance was set to *P* ≤ 0.05. The number of independent replicates is listed for each experiment, sample sizes are indicated where appropriate. For box plots, bottom and top hinges denote first and third quartile, respectively, whiskers extend to 1.5x the interquartile range and lines represent the median.

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

**Data availability**
Raw data and image files are available upon request.

**References**

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Reporting Summary

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☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
☐ Clearly defined error bars
 State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection
Immunofluorescence data were acquired using Zen imaging software (Zeiss Instruments).

Data analysis
Image J (https://imagej.net/Fiji; Schindelin J, 2012) was used to measure telomere signal intensity. The results were confirmed by TEL-Telo software (https://www.flinqbox.com/public/project/502; Zijlmans JM, 1997). Statistical analysis was performed using R.

References:
Schindelin, J., Arganda-Carreras, I., Frise, E. et al. (2012), ”Fiji: an open-source platform for biological-image analysis”, Nature methods 9(7): 676-682, PMID 22743772, doi:10.1038/nmeth.2019
Zijlmans JM, Martens UM, Poon SS, Raap AK, Tanke HJ, Ward RK, Lansdorp PM. (1997). Proc Natl Acad Sci USA 94: 7423–7428.

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- A list of figures that have associated raw data
- A description of any restrictions on data availability

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
We did not compute statistical analyses to predetermine sample sizes prior to performing experiments. However, our sample sizes are similar to those published elsewhere. We describe sample sizes in greater detail in the Methods section of the paper.

Data exclusions
No samples were excluded. Within each fixed sample, dead cells and mitotic cells (identified by DAPI staining) were excluded.

Replication
All the described findings were reliably reproduced.

Randomization
The cells, metaphases or foci quantified in each experiment were randomly sampled from the total population of cells.

Blinding
We assured reliable and unbiased data quantification by having different investigators quantify experiments independently. Analyses were done on randomized samples. TRF1-FokI foci were detected using imageJ automatic thresholding (Renyi Entropy) and particle detection. TIF foci were analyzed blinded.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a Involved in the study
- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

n/a Involved in the study
- ChiP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

Antibodies used see below

Validation

1. macroH2A1.2 (clone 14G7, Millipore MA8851), validated for WB and ICC by manufacturer, validated for ChIP in Kim et al., Mol Cell 2018, 69(1), validated for WB in Figure S1C
2. phospho-S139-H2AX (clone JBW301, Millipore 05-636), validated for ChIP, IF and WB as indicated on manufacturer’s information
3. phospho-S139-H2AX (Abcam ab11174), validated for IF by the manufacturer.
4. H2B (Abcam ab52484), validated for ChIP by manufacturer.
5. H2A (Abcam ab18255), validated for ChIP by manufacturer.
6. TRF2 (clone 4A794 15, Novus Biologicals NB100-56506, IMG-124A), validated for IF as indicated on manufacturer's website.
7. BRCA1 (clone D-9, Santa Cruz sc-6954), validated for IF as indicated on manufacturer's website.
8. ATRX (clone D1N2E, Cell signaling 14820s), validated for WB by the manufacturer.
9. GAPDH (clone 6C5, Santa Cruz sc-32233), validate for WB by the manufacturer.
10. H2AX (Abcam ab20669), validated for ChIP by manufacturer.
11. BrdU (clone 3D4, BD Biosciences 555627), validated for IF by manufacturer.
12. normal mouse IgG (Millipore 12-371).
13. macroH2A1.1 (Cell signaling, 12455s). Validated for WB in Fig. S1C.
14. CENP-A (Abcam, ab13939). Validated for ChIP by manufacturer.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
U2OS (American Type Culture Collection, ATCC, female)
SaOS-2 (ATCC, female)
GM(00)847 (Coriell, male)
HT1080 (ATCC, male),
Hela (ATCC, female)
K562 cells (ATCC, female)
U2OS-ATRX, from R. I. G.
U2OS-TRF1-FokI and U2OS-TRF1-FokI-D450A, gift from Roger Greenberg, University of Pennsylvania
HEK-293T cells, gift from The Broad Institute, Cambridge, MA

Authentication
Transgenic cell lines were authenticated based on transgene expression.
Cell lines purchased from ATCC or Coriell were authenticated by the vendor.

Mycoplasma contamination
All cell lines were negative for mycoplasma

Commonly misidentified lines
(See ICLAC register)
HEK-293T cells, commonly used for virus production.