ORIGINAL ARTICLE
Hairpin-end conformation of adeno-associated virus genome determines interactions with DNA-repair pathways

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The palindromic terminal repeats (TRs) of adeno-associated virus (AAV) form DNA hairpins (HPs) are essential for replication and for priming the conversion of single-stranded virion DNA to double strand. In recombinant AAV (rAAV) gene-delivery vectors, they are targets for the DNA-repair pathways leading to circularization, concatemerization and, infrequently, chromosomal integration. We investigated the effect of the TR HP on recombination by comparing specific DNA substrates transfected into wild-type and DNA-repair-deficient cells. DNA molecules with the TR sequences constrained in the T-shaped HP conformation at one or both ends were subject to a loss of gene expression, which was partially relieved in ataxia telangiectasia mutated (ATM−/−) cells. The ATM-dependent effect was mediated by transcriptional silencing of a subset of HP-containing molecules in cis rather than a loss of DNA, and was dependent on the specific T-shaped structure of the HP and not the primary sequence. DNA molecules with simple U-shaped HP ends were unaffected by ATM-dependent silencing. The silenced molecules remained in a linear conformation, in contrast to the expressed molecules, which were circularized. In the absence of ATM activity, this subset remained linear but was actively expressed. DNA molecules with the TR sequence in the open duplex conformation, or without TR sequences, were unaffected by ATM mutation and were predominantly converted to circular forms. A separate HP-specific effect in normal cells resulted in a loss of DNA substrate in the nucleus and was ATM independent. These results suggest that the presence of the HP structure on rAAV vector genomes subjects them to specific, and sometimes unproductive, DNA-repair/recombination pathways.

Gene Therapy (2013) 20, 686–693; doi:10.1038/gt.2012.86; published online 15 November 2012

Keywords: adeno-associated virus (AAV); terminal repeat (TR); hairpin; ataxia telangiectasia mutate (ATM); silencing

INTRODUCTION
One of the defining features of the adeno-associated virus (AAV) genome is the palindromic, GC-rich terminal repeat (TR) at each end of the genome. These stable T-shaped hairpins (HPs) have a vital role in AAV biology, serving as replication origins and priming sites for conversion of single-stranded virion DNA to double-stranded DNA (dsDNA) templates for gene expression. They are the only essential cis-acting components of recombinant AAV (rAAV) gene-delivery vectors. The TR at the 3′ end of the virion DNA serves as primer for DNA synthesis and remains constrained in the HP conformation in the duplex molecule. The TR at the original 5′ end of the genome is thought to be displaced by the elongating replication fork to form a palindromic dsDNA end. Because the TRs form the ends of the linear genome, they are also targets for DNA recombination, typically circularization and concatemerization, but infrequently also chromosomal integration.

The potential for genotoxicity stemming from rAAV vector DNA integration raises the question as to whether the HP structures formed by the TRs are especially recombinogenic compared with other forms of DNA ends.1,2 Nakai et al.3 compared integration of DNA molecules with and without AAV TR sequences by hydrodynamic transfection into mouse liver cells and found little difference between them.3 Although this suggested that the TR palindromes did not promote integration, the DNA molecules used in this experiment were not constrained in the HP structural conformation like those of rAAV vector genomes.

The TR ends of the linear AAV genome are recognized by the host cell as DNA double-strand breaks, and recombination events are mediated by any of the several double-strand break repair pathways. Previous studies have demonstrated the participation of both nonhomologous end-joining and homologous recombination in the circularization and concatemerization of rAAV genomes. The nonhomologous end-joining pathway depends on the activity of DNA-PKcs, a PI3-like kinase that is critical for rAAV recombination in nondividing cells, such as myocytes, though it appears to be less important in hepatocytes and dividing cells.4–8 In contrast, homologous recombination is the dominant recombination pathway in S phase and is orchestrated through the actions of another PI3-like kinase protein, ataxia telangiectasia mutated (ATM).9 A third member of the PI3-like kinase family, ATR (ATM- and rad3-related), is more specifically involved in repairing DNA lesions involving stalled replication forks or significant regions of single-stranded DNA sequence.

We have recently reported that rather than promoting recombination of rAAV ends, wild-type (WT) ATM activity leads to silencing of gene expression from a large fraction of rAAV genomes.10 Further, these molecules remain linear rather than recombining to form circles. In the absence of ATM, these genomes are expressed normally, but remain linear. This suggests that they are committed to a different pathway or compartment from the genomes that are normally expressed and circularized. Similarly, ATR activity led to a smaller degree of silencing of conventional single-strand rAAV (ssAAV) vectors, but not

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Received 1 December 2011; revised 14 September 2012; accepted 17 September 2012; published online 15 November 2012
self-complementary AAV (scAAV) vectors, which do not expose single-stranded DNA in the nucleus.\textsuperscript{11,12} In this study, we test the effect of the TR HP structure on recombination by transfecting cells with linear DNA substrates having covalently closed HP TRs, or fully duplex molecules with or without the palindromic TR sequences. Transfected linear DNA can be circularized by both nonhomologous end-joining and homologous recombination pathways.\textsuperscript{13} We find that a single AAV TR in the HP conformation \textit{in cis} is necessary and sufficient to mediate the previously observed ATM-dependent silencing of gene expression from rAAV genomes. This effect is independent of the primary sequence but requires the specific T-shaped secondary structure of the TR. There is also a separate HP-dependent, ATM-independent, loss of substrate DNA molecules.

RESULTS

TR structure affects the fate of transfected DNA

In order to determine whether the AAV TR HP structures are more likely to undergo recombination than other forms of DNA ends, we constructed three different DNA molecules that contained the TR sequences either in a covalently closed HP conformation (TR\textsubscript{HP}), or an open DNA duplex conformation (TR\textsubscript{+}), or containing no TR sequences (TR\textsubscript{-}/C\textsubscript{0}) (Figures 1a–c). The plain linear molecules were made simply by cutting the vector sequences out of plasmid constructs and filling-in to produce blunt ends, whereas the covalently closed molecules were made using a previously described procedure for creating no-end AAV DNA substrate.\textsuperscript{14} This molecule, containing TR HPs at both ends, has previously been used to reconstitute AAV Rep-dependent DNA replication \textit{in vitro} and yielded all of the predicted products for rolling HP replication.\textsuperscript{15}

To measure circularization, two different green fluorescent protein (GFP) vector configurations were used: one with the intact GFP cassette with an intron in the middle of the coding region (Figure 1, GFP), and a second with a previously described circularization-dependent (CD) arrangement (Figure 1, GFP-CD).\textsuperscript{16} This had the left and right halves of GFP (split through the intron) at the ends of the DNA molecules, such that expression would be possible only after circularization brought the two ends together. This allowed the frequency of recombination between the different end structures to be measured by comparing expression from the intact construct, which is unaffected by recombination, and the CD construct, which is dependent on recombination for expression.

Substrate DNAs were transfected using polyethylenimine, which requires endocytosis and acidification of early endosomes to release the DNA into the cytoplasm before diffusion into the nucleus.\textsuperscript{17,18} The transfections were set up with supercoiled

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\caption{DNA substrates for transfection-based assays. (a–c): Diagrams on left depict vectors with intact GFP-expression cassette under control of the CMV (cytomegalovirus immediate early) promoter. Diagrams on right depict GFP-CD vectors, which require circularization by recombination between the two ends for expression of GFP reporter. GFP-L and GFP-R, left and right halves of GFP separated by human chorionic gonadotropin intron; D-ITR, TR sequence with D region deleted as used to make self-complementary AAV vector; and wt-TR, intact AAV TR sequence. (a) Covalently closed HP conformation (TR\textsubscript{HP}). (b) TR sequence in open duplex conformation (TR\textsubscript{+}) or (c) no TR sequences (TR\textsubscript{-}). (d) Sequence and secondary structure of intact AAV TR in the HP conformation with A, B and C palindromes and D region indicated.}
\end{figure}
plasmid DNA (pSP72) as carrier and supercoiled red-fluorescent protein-coding plasmid (pDSRed2-C1) to normalize transfection efficiency. The molar ratios of the three molecules were first titrated using supercoiled GFP-expressing plasmid, so as to deliver approximately 1–2 molecules of fluorescent protein gene per transfected cell nucleus; that is, approximately half of the transfected cells expressed only green or only red-fluorescent protein and half expressed both. This allowed accurate quantitation of DNA fate because a GFP signal would be the product of a single molecular event. Because the carrier and RFP (red fluorescent protein) plasmids were supercoiled, they were unlikely to interact with the experimental GFP DNA ends or to induce DNA-damage signaling.

After transfection into HeLa cells, the DNA with TRs in the closed HP conformation yielded a lower frequency of GFP expression than DNA with either open duplex TRs or no TR sequences (Figure 2). This was the case whether they were associated with the intact GFP or the CD GFP gene. This surprising result suggested that the presence of the HP structure leads to degradation or silencing.

AAV TR in HP conformation induces an ATM-dependent decrease in gene expression from transfected DNA substrates

We previously described an ATM-dependent loss of functional rAAV genomes in WT fibroblasts compared with ATM−/− fibroblasts transfected either with ssAAV or scAAV vectors.10 A dsDNA molecule with a TR in the HP conformation is an intermediate in the fate of both ssAAV and scAAV genomes, respectively, generated by second-strand DNA synthesis or by self-complementary base pairing. Therefore, we investigated whether the decrease in GFP expression from transfected dsDNA molecules with TRHP ends was ATM dependent.

WT transformed fibroblasts (GM00637 J), and well-characterized transformed ATM−/− fibroblasts (GM05849 E),19 were transfected with the three DNA molecules containing different end structures, carrying either the GFP or GFP-CD expression cassette (Figure 3a). Although the two open-end molecules (TR+ and TR−) were unaffected by the absence of ATM, the number of cells expressing GFP from the HP molecules was increased by twofold in ATM−/− cells compared with WT cells. This was similar to our previously reported effect of ATM on transduction with rAAV vectors, though less pronounced (2.6- and 6.6-fold increases for ssAAV and scAAV, respectively). The transfection-based experiment suggested that the ATM-dependent decrease in gene expression requires the presence of the AAV TRs, specifically, in the HP conformation.

Although expression from the intact GFP-TRHP substrate increased by twofold in ATM−/− cells compared with WT, there was no corresponding increase from the GFP-CD molecules with HP ends, suggesting that the TRHP substrates that became available for expression in the absence of ATM do not circularize. This also parallels the results from our viral transduction-based studies, where the number of cells expressing intact GFP increased in the absence of ATM, whereas the number that expressed GFP from a circularized genome remained the same. In contrast to the HP-containing molecules, ATM deficiency caused a small increase in the circularization of substrates with either TR+ or TR− open-blunt ends, suggesting that ATM is not required for processing these types of ends.

Interestingly, although gene expression in ATM−/− cells transfected with TRHP substrates increased compared with WT, it did not reach the same levels as cells transfected with TR+ or TR− substrates, suggesting that there is an additional TRHP-dependent loss of gene expression that is independent from the ATM pathway (see below).

A single AAV TR in the HP conformation is necessary and sufficient for loss of gene expression

The expected product of conversion of an AAV genome into dsDNA would be a linear molecule with a closed HP TR at one end vector transduction, respectively). The transfection-based experiment suggested that the ATM-dependent decrease in gene expression requires the presence of the AAV TRs, specifically, in the HP conformation.
and an open duplex at the other. To determine whether this structure is subjected to the same ATM-dependent silencing as observed with our double-HP TRHP substrate, we cut off one or both the HPs by restriction enzyme digestion before transfection into WT and ATM−/− fibroblasts (Figure 3b).

Although cutting off one HP from the substrate had no impact on the reduced number of GFP-positive cells, removing both TRs restored its behavior to exactly that of the unmodified DNA fragment that contained no TR sequences (TR−). An increase in the number of GFP-positive cells upon removal of both HPs was observed in both WT and ATM−/− cells, though the effect was significantly greater in WT cells. This indicated that the presence of only one AAV TR in the HP conformation was sufficient for the substrate to interact with an ATM-dependent pathway, leading to a significant loss of expression. Further, the additional increase in GFP-positive cells in the absence of ATM upon removal of the HP ends suggested that there was a second, ATM-independent loss of functional DNA substrate when it was associated with a TR in the HP conformation.

T-shaped TR HP conformation, but not primary sequence, induces ATM-dependent decrease in gene expression

To determine how the secondary structure and/or primary sequence of the TR contribute to the ATM-dependent effect on gene expression, we designed a series of palindromic oligonucleotides with modified primary sequence, but preserving the secondary structure. These HP oligonucleotides were ligated onto both ends of linear TR DNA molecules, generating covalently closed GFP-expressing DNA substrates. Substrate wtTRHP (B + C) contained the T-shaped HP formed by the WT B and C palindromes but lacked the A palindrome portion (Figure 1d). Substrate revTRHP contained the T-shaped TRs with the sequence of the WT AAV TR reversed 5′ to 3′ (not the reverse complement). Substrate TRHP(AT) contains T-shaped TRs with a portion of the GC base pairs substituted with ataxia telangiectasia (AT) such that it is no longer a GC-rich sequence. Substrate Simple TRHP(AT) contains TRs with an AT-rich sequence that fold into a simple U-shaped HP secondary structure.

These substrates, and the previously described TR−, TR+ and TRHP− were transfected into HeLa cells in presence or absence of an ATM inhibitor, which we previously showed to have a similar effect on rAAV transduction as ATM-deficient cells (Figure 4).

Although the two open-end molecules (TR+ and TR−) were unaffected by the presence of the ATM inhibitor, the number of cells expressing GFP from the T-shaped HP molecules (TRHP, wtTRHP (B + C), revTRHP, and TRHP(AT)) was increased by twofold in the presence of the ATM inhibitor. Each of the four T-shaped HP molecules showed similar levels of expression, regardless of the different primary sequence of their TRs, suggesting that it is the specific secondary structure that contributes to the ATM-dependent loss of gene expression.

Interestingly, although there is not an ATM effect on expression from the Simple TRHP(AT) molecule, expression is reduced compared with TR− and TR+ molecules, suggesting that the ATM-independent effect operates on any HP structure.

TRHP-dependent loss of gene expression requires HP in cis, not in trans

The observed deficiency of gene expression from DNA substrates with TR ends in the HP conformation suggested that the molecules were recognized through both ATM-dependent and ATM-independent mechanisms, and silenced, degraded or failed to reach the nucleus (see following section for ATM-independent loss of HP genomes in the nucleus). However, it remained possible that the TR HP structures triggered activation of the DNA-damage signaling pathways leading to a general decrease in cellular gene expression. This could lead to a similar decrease in the number of cells scored as GFP-positive after transfection.

In order to distinguish between the possibilities of the TRHP affecting gene expression in trans, versus strictly cis effects, we tested the effect of the TRHP structure on GFP expression from a separate cotransfected molecule. Human embryonic kidney 293 cells were cotransfected with the GFP-expressing TR− substrate (open duplex ends), and a second substrate carrying an unrelated TR+ substrate (covalently closed HP ends). To determine whether this transfection was performed in triplicate and five different fields (×20 magnification) per transfection were averaged. Graph shows triplicate averages and s.d. Indicated differences were significant (*P < 0.05 and **P = 0.01).

![Figure 4. Effect of HP primary and secondary structure on expression. HeLa cells were transfected with seven GFP-expressing DNA substrates in the presence of 10 μM ATM inhibitor in dimethyl sulfoxide (DMSO) or vehicle only (+ ATM inhibitor and DMSO, respectively). TR−, TR+ and TRHP− are molecules containing open duplex TRs, no TRs or HP TRs, respectively. wtTRHP (B + C) HPs comprising only the B and C palindromes of the AAV TR (see Figure 1d); revTRHP, B and C palindromes with the sequence reversed 5′ to 3′; TRHP(AT), B and C palindromes with GC > AT substitutions; and Simple TRHP(AT), a single palindrome forming a simple U-shaped secondary structure. At 24 h post transfection, GFP-positive cells were counted and normalized to the number of cells expressing cotransfected RFP plasmid. Transfections were performed in triplicate and five different fields (×20 magnification) per transfection were averaged. Graph shows triplicate averages and s.d. Indicated differences were significant (*P < 0.05 and **P = 0.01).](https://example.com/figure4.png)
whether the same mechanism was responsible for the deficiency in gene expression from the transfected DNA molecules with TR\(^{\text{HP}}\) ends, we used quantitative real-time PCR to measure the number of DNA substrate molecules within the nuclei of transfected cells. WT and ATM\(^{-/-}\) fibroblasts were transfected with the three intact GFP substrates (TR\(^{+}\), TR\(^{-}\) and TR\(^{\text{HP}}\)) and were collected at 24 h post transfection. The cells were lysed and low-molecular weight DNA was extracted from nuclear and cytoplasmic fractions for quantitative real-time PCR analysis. Because the covalently closed HP DNA molecules are resistant to PCR amplification (data not shown), all DNA samples were cut with MluI before quantitative real-time PCR to remove the HP ends. Transfection efficiency was normalized by cotransfected supercoiled pCMV\(\beta\) plasmid with each of the DNA substrates and by quantifying using a separate primer/probe set specific for the LacZ gene.

In both WT and ATM\(^{-/-}\) cells, the copy number of TR\(^{\text{HP}}\) substrate in the nucleus decreased by fourfold compared with either TR\(^{+}\) or TR\(^{-}\) substrates (Figure 6). Importantly, there was no difference in TR\(^{\text{HP}}\) copy number between the WT and ATM\(^{-/-}\) cells. This suggests that the ATM-dependent decrease in gene expression from TR\(^{\text{HP}}\) DNA molecules is due to silencing, similar to transduction with rAAV vectors, whereas the ATM-independent mechanism is due to loss of nuclear DNA molecules.

ATM-dependent TR\(^{\text{HP}}\) silencing is saturated at high-dose transfection

We used a competition-based experiment to determine the effect of higher doses of transfected TR molecules on ATM-dependent silencing of HP-containing reporter. The doses of the GFP-reporter DNA molecules were kept constant to facilitate quantitation of GFP-expressing cells, whereas competitor DNA was increased (Figure 7). The competitor for each GFP-expressing DNA substrate contained the LSP-PD-L1 transgene, as above, and the same TR conformation as the molecule with which they compete. The expression of GFP relative to GFP copy number was assayed 24 h post transfection. Each transfection was performed in triplicate. Error bars indicate s.d.

Time course of ATM-dependent effect on rAAV expression

To determine whether the ATM-dependent decrease in gene expression was stable over time, we assessed RNA transcript levels
end open. Importantly, this would be the predicted structure for a rAAV vector genome that has undergone second-strand synthesis, or an scAAV genome that has been released from its capsid and folded into a dsDNA conformation.

The loss of functional genomes with one or two TRs in the HP conformation is partially relieved in cells lacking ATM activity. This is consistent with the effects of ATM mutation or inhibition on transduction from both single-strand rAAV and scAAV vectors, where transduction is increased in ATM-/- cells relative to WT. Despite the increased transduction from rAAV vectors in ATM-/- cells, as measured by GFP expression, the amount of vector DNA in the nucleus, including both linear and circular forms, does not change between the mutant and WT cells. This suggests a silencing effect from interaction with ATM or associated factors, which closely parallels the behavior of transfected DNA with HP ends, where the excess of actively expressing genomes in ATM-/- cells remain linear. Thus, in normal cells, DNA molecules with one or two HP ends can either interact with the ATM-dependent pathway and remain linear and silent, or they become circularized and expressed, presumably through interaction with a different set of recombination factors. In the absence of ATM, the same subset of HP molecules goes through the pathway that leads to circularization and expression, and the remainder that would have interacted with the ATM-dependent pathway are able to express from linear molecules. The time-course analysis shows that the ATM-dependent silencing begins as soon as 3 h post infection and does not change over the subsequent 48 h, suggesting that interaction with ATM is an early event and that viral genomes are not released from silencing through at least the first 48 h after infection.

We have not as yet determined the direct mechanism for ATM-initiated silencing of AAV HP-containing molecules; chromatin modifications are an attractive possibility. It has been observed that ATM-mediated phosphorylation of histone H2AX is associated with reduced transcription in regions undergoing double-strand break repair.20 Also, ubiquitylation of H2A correlates with transcription repression, and E3 ubiquitin ligases RNF8 and RNF168 affect ubiquitylation of H2A on damaged chromosomes in an ATM-dependent manner.21–24 Future work employing immunoprecipitation of specific chromatin components complexed with rAAV vectors, or DNA substrates with the TR in different conformations, may reveal the mechanism at the molecular level.

Although the ATM-dependent effect on gene expression is clearly dependent on the HP end structure, there is a second TR HP-dependent effect that is not related to ATM activity, and results in the loss of DNA molecules, as measured by quantitative real-time PCR. This may be mediated by a specific DNA recombination/repair pathway that recognizes the HP structure and leads to extensive DNA degradation. Alternatively, the loss may be part of a host-cell defense mechanism recognizing HP DNA ends, leading to altered trafficking to the nucleus. As we are transfecting naked DNA, this could occur either in the nucleus or during transport through the cytoplasm. We do not know whether this effect is specific to transfected DNA or causes part of vector DNA ends can undergo recombination without HP ends. Further studies to identify the mechanism of HP-dependent loss of genomes may reveal whether the viral genomes are also affected.

Considering the loss and silencing of DNA molecules with HP ends, evaluating the direct effect of the HP on the frequency of recombination is complex. However, it is clear that the majority of transfected molecules without HP ends express GFP from circularized molecules. Thus, it is probably not accurate to suggest that the AAV HP end is inherently recombinogenic, because simple double-strand DNA ends are at least equally prone to undergo recombination. Rather, recombination involving AAV HP ends is mechanistically different from that of simple DNA ends, with multiple pathways leading to different fates.

**DISCUSSION**

To directly assess whether the AAV TR HP structure is specifically recombinogenic, we tested three different types of defined DNA substrate molecules by transfection into normal and recombinogenic, we tested three different types of defined DNA substrate molecules by transfection into normal and recombina-

**Figure 8.** Effect of ATM on GFP transcript accumulation in rAAV vector-transduced cells. WT fibroblasts were transduced with self-complementary rAAV at a multiplicity of infection of 10 in the presence or absence of 10 μM ATM inhibitor. Gene expression was quantified by reverse transcription PCR using the 2−ΔΔCt method with cellular glyceraldehyde-3-phosphate dehydrogenase as internal standard. The GFP transcript signals at different time points post infection were compared with the uninfected time point (0). Error bars represent s.d., and results are the average of triplicate qPCR assays.
We had previously published a study evaluating the efficiency of recombination between the HP end versus open end in scAAV genomes, using the formation of intermolecular concatamers as a model. These experiments indicated that recombination between two HPs was twice as frequent as recombination between two open ends, and recombination between an open and a HP end was intermediate in frequency. As in the present study, the assay was based on recombination and expression of a split GFP gene. Considering the present results, these concatemerization events probably occurred within the fraction of genomes that do not interact with ATM, and ultimately are expressed and undergo recombination. This suggests that within this compartment, the HP end may be considered recombinogenic, in that it is more likely to join with another DNA end.

Notably, a fourth DNA substrate was planned for this study, but could not be made in our hands. This would have been a dsDNA molecule with two open TRs at each end, and the TR of each strand in the HP conformation. The TRs of AAV are often depicted schematically in this conformation, and it is a critical intermediate step in the replication scheme, which allows priming for the next round of DNA synthesis. We could not find partial denaturation or annealing conditions that would allow the TRs to remain in the HP conformation, instead finding that they always reannealed completely to form simple duplex ends. The determination was determined by susceptibility to digestion with the restriction enzyme AhdI at a site in the apex of the B palindromic (data not shown). This suggests that the double open-HP TR may be maintained only transiently, or must be maintained in the context of host or viral proteins. Further, the blunt-ended, fully duplex TR may best represent the actual conformation of the open end of a duplex AAV genome under physiological conditions.

MATERIALS AND METHODS

Cell lines

The SV40-transformed fibroblast cell lines from a normal individual (WT cells, GM00637 J) and from an AT homozygous patient (AT cells, GM05849 E) were purchased from Coriell Institute (Camden, NJ, USA). WT and AT were cells were grown in MEM Eagle with Earle’s BSS (Lonza BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum. Human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle medium (Gibco, Grand Island, NY, USA).

HeLa cells were grown in Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum. Human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% cosmic calf serum (HyClone, Logan, UT, USA). All cell lines were cultured as monolayer cultures at 37°C in 5% CO2 humidified incubator.

DNA substrates

TR+, TR- and TR0 substrates were excised from plasmids carrying either the intact (scAAV-GFP) or CD (scAAV-GFP-CD) vectors described previously. TR+ substrate was excised by PvuII digestion from both plasmids. TR- substrate was excised by MluI digestion from scAAV-GFP plasmid, or BglII and Sall digestion from scAAV-GFP-CD plasmid, followed by fill-in of 5’ overhangs with Klenow. TR0 has been described previously. Briefly, it was excised by PvuII digestion from both plasmids and incubated for 6 min with exonuclease III to expose single-stranded 5’ termini, which fold into the HP conformation. Subsequently, T4 DNA polymerase and T4 DNA ligase were used to repair the gaps and covalently close the ends of the DNA molecules. Finally, the preparation was digested with excess exonuclease III to remove any molecules containing nicks or gaps (covalently closed HP DNA molecules remained resistant). A single HP was removed from intact GFP-expressing TR+ by KpnI digestion (TR-), and both HPs removed by digestion with MluI (TR0). To generate TR0, a covalently closed DNA substrate with modified TR sequence or structure, the following palindromic oligonucleotides were synthesized: wTR0(b+C): S’-CCGGAGCCGCCGCCGGCAGCAGCTGGCGCA GCGCCGGCGGTTGGGCGGGGGCGCT-3’; revTR0: S’-GGCGTCGGCCGCGGGCCGCG CGCCTGGGGGGCGCGCTGGTGGC-3’; TR1[AT]: S’-CCG GAGGAGGAGGATGAGAACACACATTCATGATGATGATGATGCACTACACTACCTACCT-3’; and Simple TR1[AT]: S’-CCGGAGGAGGAGGATGAGAACACACATTCATACATTCCTACCTACCT-3’.

Each oligonucleotide was boiled and cooled to room temperature, phosphorylated and ligated to the scAAV-GFP plasmid, which was previously digested with MluI to excise the intact GFP cassette without the TR ends. The molar ratio for ligation was 10:1 oligo to plasmid. The ligation reaction was subsequently digested with exonuclease III to eliminate any unligated DNA, and covalently closed substrate of approximately 2200 bp was purified by gel electrophoresis.

DNA molecules used as competitors (PD-L1-TR+, PD-L1-TR- and PD-L1-TR0) were excised from a plasmid (p-trsLSP-PD-L1) in which the expression cassette of the scAAV vector contains a mouse PD-L1 transgene controlled by a LSP, p-trsLSP-PD-L1 was derived from the previously described p-trsLSP-GFP, in which the eGFP-coding sequence was excised and replaced by the mouse PD-L1 transgene. The substrate was excised by PvuII digestion. PD-L1-TR- substrate was excised by BssHII digestion, followed by a fill-in of 5’ overhangs with Klenow. PD-L1-TR0 was excised by PvuII digestion, and covalently closed ends were generated as described above. All DNA substrates were gel purified from low-melting point agarose (using long wavelength (365 nm) ultraviolet illumination to avoid inducing the ultraviolet-damage-repair pathways in recipient cells), and concentrations were normalized by comparing aliquots on ethidium bromide-stained gels (Kodak Gel Logic Imaging system, Woodbridge, CT, USA).

Transfection assays

Cells were seeded on glass cover slips precoated with collagen type I in 24-well plates and transfected using polyethyleneimine (Polysciences, Warrington, PA, USA) as carrier (1.6 μg per well), supercoiled pDSRed2-C1 (Clontech, Mountain View, CA, USA) (17 ng per well) and linear DNA substrate (17 ng/well). Precipitates were added to cells in 24-well plates at 30–35 μl per well. HeLa cells were seeded at 30% confluence on day 1, cotransfected on day 2 and fixed on day 3 with 4% paraformaldehyde in phosphate-buffered saline. Cells on coverslips were mounted on slides, and GFP- and RFP-positive cells were counted under a fluorescent microscope. To assay for the ATM effect in HeLa cells, cells were seeded as described above and treated with 10 μM ATM inhibitor (KU55933; Tocris, Bristol, UK) or 10 μM dimethyl sulfoxide on day 1. Treatment was maintained until cells were collected on day 3.

WT and ATM-/- cells were cotransfected with pSP72 (1.6 μg per well), pdRed2-C1 (12.5 ng per well) and linear DNA substrate (12.5 ng per well) and assayed as described above.

To assay for cis versus trans effects on substrate expression, 293 cells were seeded on day 1 at 30% confluence in 24-well plates. On day 2, cells were cotransfected with pSP72 (1.6 μg per well), supercoiled DSRed-express (3 ng per well), and either linear GFP-TR+ DNA substrate (3 ng per well) or GFP-TR0 DNA substrate (3 ng per well), plus either PD-L1-TR0 (15 ng per well) or PD-L1-TR- DNA substrate (15 ng per well). At 24 h post transfection, cells were fixed and assayed as described above.

For the competition assay, 293 cells were seeded as described above and cotransfected with supercoiled pCMVβ (3 ng per well) to normalize for transfection efficiency, plus constant amounts of GFP-expressing TR+, TR- or TR0 DNA substrate (3 ng per well), decreasing amounts of pSP72 carrier, and increasing amounts of PD-L1-TR+, PD-L1-TR- or PD-L1-TR0 competitor, respectively. Competitors were added at 0, 18, 54, 162, 486 ng per well, whereas pSP72 was added at 1.6, 1.58, 1.55, 1.44 or 1.11 μg per well, respectively. Precipitates were prepared as described above and were added to cells at 34–40 μl per well. Twenty-four hours after transfection, GFP-positive cells were counted under a fluorescent microscope without fixing. To evaluate GFP expression per GFP substrate molecule, nuclear fractions were isolated as described previously, and low-molecular weight DNA was separated by Hirt extraction and digested with MluI to cut off the TR sequences. Copy numbers were quantified using Absolute Blue QPCR ROX Mix on an Applied Biosystems (Carlsbad, CA, USA) Prism 7000 Sequence Detector System. Data were analyzed by absolute quantification, and pCMVβ copy numbers were used to account for transfection efficiency. The primer-probe sequence specific for CMV β was forward primer, 5’-CCGATTTGGGCAAGAAGA-3’; reverse primer, 5’-GGTGTATCGATCGATCGGATCTACATG-3’ (100 nM final concentration); and probe, 5’-6-carboxy-fluorescein-CTTTACTTTTATTATGCGA GCC TAMRA-3’ (100 nM final concentration). Substrate DNAs carrying eGFP expression sequence were quantified using the following primer-probe sequence: forward primer, 5’-AAGCAGCAAGCTTCTCA-3’; reverse primer, 5’-AAGCAGCAAGCTTCTCA-3’.
primer, 5'-TCGTCCTTGAAGAATGGT-3' (300 nM final concentration); and probe, 5'-FAM-CCATGCCCAGGCAAGTTGTACGT- TAMRA-3' (100 nM final concentration).

To evaluate the effect of TR conformation and ATM mutation on DNA substrate copy number, WT and ATM T0/C0 fibroblasts were seeded in six-well plates at 30% confluence on day 1. On day 2, cells were cotransfected with pSG72 (9.6 µg per well), supercoiled pCMV/J (Clontech) plasmid (75 ng per well) and TR+, TR- or TR0 DNA substrate (75 ng per well). Twenty-four hours after transfection, cells were harvested and copy number was quantified by Real Time PCR as described above.

Time-course assay for ATM-dependent silencing
WT fibroblasts were seeded on six-well plates on day 1 at 40% confluence. On day 2, cells were cotransfected with pSP72 (9.6 µg per well), supercoiled pCMV/J (Clontech) plasmid (75 ng per well) and TR- or TR0 DNA substrate (75 ng per well). Twenty-four hours after transfection, cells were harvested and copy number was quantified by Real Time PCR as described above.

Statistical analysis
All statistical analyses were performed using Student's t-test (GraphPad Prism 5 software, GraphPad Software Inc., La Jolla, CA, USA). Error bars indicate mean with s.d.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We thank Kimberly Zaraspe for technical assistance with this research. This work was supported by NIH grant R01 A070244 to DMM.

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