Disruption of Telomere Maintenance by Depletion of the MRE11/RAD50/NBS1 Complex in Cells That Use Alternative Lengthening of Telomeres*

Ze-Huai Zhong‡, Wei-Qin Jiang‡, Anthony J. Cesare‡, Axel A. Neumann‡, Renu Wadhwa‡, and Roger R. Reddel§1

From the ‡Cancer Research Unit, Children's Medical Research Institute, Westmead 2145 and the University of Sydney, Sydney 2006, New South Wales, Australia and the §Cell Proliferation Research Group, Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan

Immortalized human cells are able to maintain their telomeres by telomerase or by a recombination-mediated DNA replication mechanism known as alternative lengthening of telomeres (ALT). We showed previously that overexpression of Sp100 protein can suppress ALT and that this was associated with sequestration of the MRE11/RAD50/NBS1 (MRN) recombination protein complex by Sp100. In the present study, we determined whether MRN proteins are required for ALT activity. ALT cells were depleted of MRN proteins by small hairpin RNA-mediated knockdown, which was maintained for up to 100 population doublings. Knockdown of NBS1 had no effect on the level of RAD50 or MRE11, but knockdown of RAD50 also depleted cells of NBS1, and knockdown of MRE11 depleted cells of all three MRN proteins. Depletion of NBS1, with or without depletion of other members of the complex, resulted in inhibition of ALT-mediated telomere maintenance, as evidenced by decreased numbers of ALT-associated promyelocytic leukemia bodies and decreased telomere length. In some clones there was an initial period of rapid shortening followed by stabilization of telomere length, whereas in others there was continuous shortening at a rate within the reported range for normal human somatic cells lacking a telomere maintenance mechanism. In contrast, depletion of NBS1 in telomerase-positive cells did not result in telomere shortening. A recent study showed that NBS1 was required for the formation of extrachromosomal telomeric circles (Compton, S. A., Choi, J. H., Cesare, A. J., Ozgur, S., and Griffith, J. D. (2007) Cancer Res. 67, 1513–1519), also a marker of ALT cells. We conclude that the MRN complex, and especially NBS1, is required for the ALT mechanism.

Telomeres are specialized structures found at the end of chromosomes and, in humans, consist of linear tandem arrays of TTAGGG repeats. Together with their associated binding proteins, telomeres play a critical role in protecting the chromosomes from end degradation and end-to-end fusions and are essential for chromosomal stability and genomic integrity (1). In normal somatic human cells, telomeres shorten by 50–150 bp/cell division as the result of the end replication problem and telomere end processing, which ultimately leads to cellular replicative senescence including permanent withdrawal from the cell cycle (2, 3).

Most cancer cells are able to prevent telomere shortening by activating a telomere maintenance mechanism (4), either telomerase (5) or alternative lengthening of telomeres (ALT)2 (6). Although it has been shown that ALT is a recombination-mediated DNA replication process (7, 8), the molecular details of ALT are yet to be elucidated. One feature of ALT-positive cells, which is in contrast to telomerase positive cells and cells lacking a telomere maintenance mechanism, is the highly heterogeneous length of their telomeres (6). In addition to progressive attrition of telomeres with each cell division, ALT telomeres undergo both rapid elongation and rapid deletion events resulting in telomere lengths that range from very short to extremely long (>50 kb) (8). Another phenotypic marker of ALT cells is the presence of extrachromosomal telomeric circles, which are similar in size to telomeric t-loops and have been proposed to result from homologous recombination events at the telomeres (9, 10). ALT cells also contain a subset of promyelocytic leukemia (PML) nuclear bodies, which are unique to ALT cells and are known as ALT-associated PML bodies (APBs) (11). In addition to the usual constituents of PML bodies including PML and Sp100 protein, APBs contain (TTAGGG)n, DNA and telomere-binding proteins TRF1 and TRF2 (11), hRAP1 (TERF2IP) (12), TIN2 (13), and the DNA repair and recombination MRE11/RAD50/NBS1 (MRN) complex (14, 15).

We have shown previously that the overexpression of Sp100, a constitutive component of PML bodies, suppresses APB formation, causes progressive telomere shortening, and inhibits the rapid changes in telomere length that are characteristic of

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†To whom correspondence should be addressed: Children's Medical Research Institute, 214 Hawkesbury Rd., Westmead, NSW 2145, Australia. Tel.: 61-2-9687-2800; Fax: 61-2-9687-2120; E-mail: reddel@cmri.usyd.edu.au.

2 The abbreviations used are: ALT, alternative lengthening of telomeres; MRN, MRE11/RAD50/NBS1; PML, promyelocytic leukemia; APB, ALT-associated PML bodies; NBS, Nijmegen breakage syndrome; ATLD, Ataxia-telangiectasia-like disorder; shRNA, small hairpin RNA; FISH, fluorescence in situ hybridization; TRF, terminal restriction fragment; PD, population doubling; siRNA, small interfering RNA; AMCA, 7-amino-4-methylcoumarin-3-acetic acid.

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ALT cells. These effects were associated with Sp100-mediated sequestration of the MRN complex (16).

The MRN complex plays a crucial role in maintaining genomic stability (17, 18). NBS1 (Nijmegen breakage syndrome 1) is a key regulator of the enzymatic functions of MRN, initiates the response and localization of the MRN complex following DNA damage (19, 20), and is involved in cell cycle checkpoint control (21–24). In higher vertebrates the MRN complex is required for the repair of double strand breaks by homologous recombination (25). Although the MRN complex is not essential for nonhomologous end joining DNA repair, it has been suggested that it is indirectly involved in nonhomologous end joining activities such as immunoglobulin class switch recombination (26, 27).

RAD50 and MRE11 are highly conserved in evolution (28), whereas NBS1 and its paralogs are found only in eukaryotes and show less homology (25, 29). Hence, it is not surprising that in addition to the formation of the MRN complex, MRE11 and RAD50 are able to associate independently of NBS1. The MRE11/RAD50 complex is found at interphase telomeres in association with TRF1 and TRF2 and is thought to help stabilize telomere t-loop formation. It has been shown that NBS1 joins MRE11 and RAD50 at the telomeres during S phase (14, 15, 30). In ALT-positive cells the MRN complex colocalizes with other recombination factors in APBs (12, 14), which may be sites of recombination-mediated telomere elongation.

Among higher eukaryotes null mutations of MRE11 (31), RAD50 (32, 33) and NBS1 (34, 35) are lethal. In humans, mutations that have been identified in MRN genes are hypomorphic; the encoded proteins are usually truncated and exhibit reduced function. Nijmegen breakage syndrome is the result of hypomorphic NBS1 mutations, which in most cases produce a truncated 70-kDa NBS1 protein (22, 36). Ataxia-telangiectasia-like disorder (ATLD) is caused by the expression of mutant MRE11 (37, 38). Cells from Nijmegen breakage syndrome and ATLD patients exhibit chromosomal instability, hypersensitivity to ionizing radiation, and abnormal S phase checkpoints (37). Nijmegen breakage syndrome cells also exhibit accelerated rate of telomere shortening and reduced capacity for homologous recombination (39, 40). To date, no RAD50-related human syndromes have been identified.

In this study we investigated whether the MRN complex is required for the ALT mechanism. The cells were depleted of MRN proteins by shRNA-mediated knockdown. Knockdown of NBS1 had no effect on the level of RAD50 or MRE11, but knockdown of RAD50 also depleted cells of NBS1, and knockdown of MRE11 depleted cells of all three members of the MRN complex. Each of these treatments resulted in decreased numbers of ALT-associated PML bodies (APBs) and decreased telomere length. In some cases the decrease in telomere length was rapid, but some cells exhibited progressive telomere shortening at a rate (~140 bp/PD) within the range reported for normal human somatic cells that lack an active telomere maintenance mechanism. We also examined the effect of NBS1 knockdown in telomerase-positive cells and found no change in telomere length. We conclude that the MRN complex is required for the ALT mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—The spontaneously immortalized ALT-positive Li-Fraumeni Syndrome fibroblast line, IIICF/c (41), and the human fibrosarcoma-derived telomerase-positive HT1080 cell line were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 50 μg/ml gentamicin in a 5% CO2 humidified atmosphere at 37 °C. Methionine-deficient medium was reconstituted from methionine- and cysteine-deficient Dulbecco’s modified Eagle’s medium (Invitrogen) by adding l-cysteine (48 mg/liter; Invitrogen).

IIICF/c and HT1080 cells were transfected with shRNA constructs using FuGENE 6 (Roche Applied Science) and 5 μg of DNA/10-cm dish and selected with puromycin (Merck), and individual puromycin-resistant colonies were isolated by cloning rings. IIICF/c and HT1080 cells were then passaged continuously in medium containing puromycin (0.2 and 0.4 μg/ml, respectively).

For methionine restriction, the cells were seeded in normal medium and grown for 2 days to ~50% confluence. Before changing to methionine-deficient medium, the cells were washed once with methionine-free medium. Four days later, the cells were fixed and immunostained for the target protein and APBs (as identified by colocalization of TRF1, TRF2, or telomeric DNA with PML bodies) (13).

**Construction of Small Hairpin RNA Plasmids**—shRNA constructs for long term knockdown were designed and then generated by cloning the annealed complementary oligonucleotides into pCIpur vector. Expression of the small hairpin was driven by a human U6 promoter. The effectiveness of each shRNA construct was tested by transient transfection of IIICF/c cells and Western blotting at 48 h post-transfection. The sense strand of each of the constructs is as follows: scrambled control shRNA, 5'-CGCGCTGGGGCTTACGTT-AGCGTGTGCTGTCGGGCTTCAGGT-3'; RAD50 shRNA, 5'-GCCGCAATTTTACAGCGTGTGCTGTCGGGCTTCAGGT-3'; NBS1 shRNA, 5'-GGGAGAATAATGTAATCCAAACGTGTGCTGTCGGGCTTCAGGT-3'; and MRE11 shRNA, 5'-GGGAGAATGTAATCCAAACGTGTGCTGTCGGGCTTCAGGT-3'.

**Antibodies**—The following antibodies were used in this study: mouse anti-NBS1, anti-MRE11, anti-RAD50 (BD Biosciences, San Jose, CA); rabbit anti-actin (Sigma); rabbit anti-NBS1 (Ab-1) (Oncogene Research Products, Boston, MA); mouse anti-RAD50 (GeneTex, San Antonio, TX); rabbit anti-NBS1 (Novus Biologicals, Littleton, CO); goat anti-NBS1 (C-19) and goat anti-PML (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-PML (Chemicon, Temecula, CA); and mouse anti-TRF2 (Upstate Biotechnology, Waltham, MA). Polyclonal anti-TRF1 rabbit serum was raised in-house against a TRF1 peptide (residues 13–35).

**Immunostaining, Telomere Fluorescence in Situ Hybridization (FISH), and Fluorescence Microscopy**—Cells cultured in four-well chamber slides were fixed with 2% paraformaldehyde at room temperature for 15 min and then permeated with
Dependence of ALT on MRN Complex

A panel of 14 clones transfected with NBS1-shRNA (N1–N14) and three clones transfected with empty vector (P1–P3) were isolated following selection with puromycin. A, cell lysates were analyzed by Western blotting at PD28 for NBS1 and actin. The ratio of NBS1 to actin expression is shown for each clone. B, for each of the clones at PD28, lengths of their TRFs were analyzed by Southern blotting using a radiolabeled probe for telomeric DNA. C, telomere length (from Fig. 1B) was measured using Telometric version 1.2 software, and the median telomere length and the NBS1 expression level (from Fig. 1A) were plotted for each of the clones. Of the 13 clones with NBS1 depletion (N1–N13), none exhibited telomere shortening, whereas four clones exhibited minor or no telomere shortening at PD28. N14 did not exhibit significant NBS1 depletion and was grouped with the controls (parental IIICF/c and empty vector controls, P1–P3) for statistical analysis. NBS1-depleted clones had telomeres that were significantly shorter than the controls \((p = 0.039)\). However, the extent of NBS1 depletion in clones that had telomere shortening was not significantly different \((p = 0.55)\) from that in clones where shortening was not apparent at PD28.

FIGURE 1. NBS1 expression and telomere length analysis in IIICF/c ALT cells transfected with NBS1-shRNA. A panel of 14 clones transfected with NBS1-shRNA (N1–N14) and three clones transfected with empty vector (P1–P3) were isolated following selection with puromycin. A, cell lysates were analyzed by Western blotting at PD28 for NBS1 and actin. The ratio of NBS1 to actin expression is shown for each clone. B, for each of the clones at PD28, lengths of their TRFs were analyzed by Southern blotting using a radiolabeled probe for telomeric DNA. C, telomere length (from Fig. 1B) was measured using Telometric version 1.2 software, and the median telomere length and the NBS1 expression level (from Fig. 1A) were plotted for each of the clones. Of the 13 clones with NBS1 depletion (N1–N13), none exhibited telomere shortening, whereas four clones exhibited minor or no telomere shortening at PD28. N14 did not exhibit significant NBS1 depletion and was grouped with the controls (parental IIICF/c and empty vector controls, P1–P3) for statistical analysis. NBS1-depleted clones had telomeres that were significantly shorter than the controls \((p = 0.039)\). However, the extent of NBS1 depletion in clones that had telomere shortening was not significantly different \((p = 0.55)\) from that in clones where shortening was not apparent at PD28.

RESULTS

To determine the requirement of the individual components of the MRN complex for the ALT mechanism, we used shRNA for long term knockdown of MRE11, RAD50, and NBS1 in the IIICF/c ALT cell line. IIICF/c is a spontaneously immortalized Li-Fraumeni syndrome fibroblast cell line in which we have demonstrated that ALT can be suppressed by Sp100-mediated sequestration of the MRN complex (16). Following transfection with shRNA, IIICF/c cells were subjected to puromycin selection, and then individual puromycin-resistant colonies were isolated.

A total of 14 clones expressing NBS1-shRNA (N1–N14) were selected. At PD28, Western analysis was performed to determine the effectiveness of NBS1 knockdown (Fig. 1A). All but one (N14) of the 14 clones were found to have reduced levels of...
NBS1 as compared with the empty vector clones P1–P3. The extent of NBS1 knockdown varied between clones, and in some it was knocked down by more than 95%. There was a small reduction of ∼5–10% in growth rate of NBS1-depleted cells (data not shown), but there were no apparent phenotypic or morphological changes in these cells.

These 14 clones and control cells were analyzed for telomere length by Southern-based TRF analysis at PD28 (Fig. 1B). IIICF/c cells have telomeres with the highly heterogeneous lengths that are typical of ALT. The majority (9 of 13) of the NBS1 knockdown clones had telomeres that were significantly shorter than the parental IIICF/c cells at this early time point (Fig. 1B), but some (4 of 13) did not. This was also confirmed by telomere FISH (Fig. 2 and data not shown). We quantitated telomere length and compared this with the NBS1 expression level (Fig. 1C). The telomeres of the 13 clones in which NBS1 was depleted (clones N1–N13) were significantly shorter (p = 0.039) than those of the control populations (including clone N14, in which NBS1 was not knocked down). When the NBS1 knockdown cells were divided into two groups according to whether there was telomere shortening at PD28, no difference was found in the extent of NBS1 depletion (p = 0.55), although the telomere lengths of these two groups were significantly different (p < 0.001). Thus, there was significant telomere shortening in the NBS1-depleted clones overall, but the NBS1 level did not predict the extent of telomere shortening at this early time point.

To determine whether depletion of NBS1 causes progressive telomere shortening, we examined by TRF analysis the telomere length of one clone that had shortening at PD28 (N4) and one that did not (N11), as well as five control clones (S1–S5) expressing a scrambled control shRNA (Fig. 3A) for up to 77 PD. All of the control clones S1–S5 exhibited telomere length profiles that were indistinguishable from those of the parental IIICF/c cells. In contrast, clones N4 and N11 both displayed significant telomere shortening. As expected, the scrambled shRNA did not alter the levels of MRE11, RAD50, or NBS1 relative to that of the untransfected IIICF/c cells (Fig. 3B). Immunoblotting showed that NBS1 shRNA in clones N4 and N11 effectively knocked down NBS1 expression without affecting MRE11 and RAD50 protein levels (Fig. 3C).

Telomere lengths of the five control clones (S1–S5) and the NBS1-depleted clones (Fig. 3A) were measured and presented graphically (Fig. 3D). All five control clones (S1–S5) showed similar telomere length profiles (Fig. 3D). TRF analysis confirmed that N4 had very short telomeres at the first point of analysis, PD28 (Fig. 3, A and D); we calculated that N4 had lost more than 17 kb of telomeric repeat sequence by PD28 or, on average, 600 bp of telomeric repeats/cell division (Fig. 3D). No further shortening occurred from PD28 to PD77 (Fig. 3A), even though telomerase activity was not detected in these cells by the telomerase repeat amplification protocol assay (data not shown).
Unlike the N4 clone, N11 did not exhibit an initial bulk loss of telomeric DNA, and at the early time point had long heterogeneous telomeres comparable with the parental cells and control transfected clones (Fig. 3, A and D). Detailed analysis of telomere length in N11 showed that there was a continual and gradual erosion of the telomeres with increasing PDs. We calculated the rate of telomere shortening to be 110 to 140 bp/PD, which is comparable with the rate of telomere shortening observed in cells lacking an active telomere maintenance mechanism. Consistent with telomere shortening, there was a substantial reduction in the number of APBs in N4 and N11 compared with the parental IIICF/c and control clones, but the formation of other PML bodies was not perturbed.

Next we transfected IIICF/c cells with a RAD50 shRNA construct. After selection with puromycin, a total of 24 clones were isolated, of which seven clones showed substantial RAD50 knockdown by Western blot analysis (Fig. 6A). Telomere length analysis of these seven clones at PD28 showed that three (R1, R3, and R15) had significantly shortened telomeres that were similar to those in NBS1 knockdown clone N4 (Fig. 6B). The results were confirmed by FISH (Fig. 2 and data not shown). We also plotted telomere length of the seven RAD50 clones and the parental IIICF/c and empty vector control clones (P1–P3), with the level of RAD50 expression. The correlation between RAD50 depletion (together with NBS1 depletion) and telomere shortening was statistically significant, $p = 0.007$ (Fig. 6C).
We directly compared the extent of NBS1 depletion in NBS1 and MRE11 shRNA clones by Western blotting (Fig. 7D). The reduction in NBS1 achieved in NBS1 clones (by NBS1-shRNA) that exhibited the most significant telomere shortening (based on Fig. 1, B and C) were substantially greater than the extent of NBS1 depletion achieved in the MRE11 clones (by MRE11-shRNA). Consequently, even though MRE11 shRNA knocked down all members of the MRN complex, it presumably had less effect on the total amount of MRN complex that was still able to form.

We examined clones M18 and M28 further and observed that MRE11 levels along with NBS1 and RAD50 increased slightly by PD77 (Fig. 7E). Although no initial telomere shortening was observed at PD28, TRF analysis up to PD77 showed substantial, sustained telomere shortening in clone M18 and minor shortening in M28 (Fig. 7F).

**DISCUSSION**

We have shown that the long term depletion of members of the MRN complex in human ALT-positive cells resulted in telomere shortening and a reduction in APBs. Although there was a slight decrease in growth rate, the cells remained viable, there was no increase in genomic instability, and there was no detectable increase in cell death. Compton et al. (44) recently showed that shRNA-mediated depletion of NBS1 in two different ALT cell lines similarly resulted in a slight reduction in growth rate without affecting cellular viability. They reported that NBS1 depletion inhibited the formation of t circles, another marker of ALT. The two studies therefore provide complementary evidence that the MRN complex is required for the ALT phenotype.

Given that most cells within any ALT population, including IIICF/c cells, already contain some very short telomeres (6, 41), it is surprising that telomere shortening caused by MRN complex depletion is compatible with continued proliferation. We propose that incomplete knockdown of MRN permits some residual ALT activity, and as has been demonstrated for recombination-mediated telomere maintenance in yeast (45, 46), this occurs preferentially at the shortest telomeres. However, even though we showed that activation of telomerase did not occur,
we cannot completely exclude the possibility that these cells have activated an unknown telomere maintenance mechanism. Although there is as yet no clear evidence for another ALT mechanism in human cells (47, 48), there is more than one recombination-mediated telomere maintenance mechanism in yeast (46, 49). Compton et al. (44) similarly noted the ability of NBS1-depleted cells to continue to proliferate despite the absence of t circles and concluded that NBS1 and t circles are not required for survival of ALT cells. However, they also suggested the possibility that these cells may contain low levels of t circles that were undetectable by two-dimensional gel analysis and that these cells may possess residual ALT activity.

Interestingly, Compton et al. (44) examined mass cultures of two NBS1-depleted ALT cell lines and saw very little effect of NBS1 depletion on telomere length. In contrast, we found that 13 NBS1-depleted clones had undergone significant telomere shortening by PD28. There was some heterogeneity among the clones, however, with 4 of the 13 clones not showing significant telomere shortening at this time point. We therefore do not regard the data as incompatible, because clonal heterogeneity within mass cultures could mask an effect on telomere length.

We observed two different patterns of telomere shortening in the MRN-depleted cells. Some cells already exhibited significantly shortened telomeres at the first point of analysis. At PD28, the telomeres of clone N4 were 17 kb shorter than the controls. This represents an average loss of ~600 bp of telomeric DNA/cell division, which is significantly greater than the rate of telomere attrition in normal human somatic cells lacking an active telomere maintenance mechanism. Other clones exhibited progressive telomere shortening at a much less rapid rate. Clone N11 did not have obvious telomere shortening by the earliest time point but subsequently exhibited progressive telomere shortening at a rate of ~140 bp/PD, which is similar to the rate of telomere loss observed in normal human somatic cells lacking an active telomere maintenance mechanism.

The reasons for these different patterns of telomere loss are unknown. In previous studies rapid telomere shortening was seen when the ALT mechanism was repressed by fusing ALT cells with telomerase-positive cells (42) but not when MRN was sequestered by overexpression of Sp100 (16). The telomeres of ALT cells undergo both rapid lengthening and rapid deletion events on a background of steady telomere attrition (8); the
rapid lengthening events must be sufficient to counterbalance both the rapid and steady telomere losses. It is possible that in some MRN-depleted clones the rapid lengthening events are preferentially inhibited, and thus rapid deletion of telomeres occurs, whereas in other clones rapid lengthening and rapid shortening are both inhibited, resulting in a slow but progressive shortening. We showed that the extent of NBS1 depletion does not account for these differences, but clonal variations in the levels of other telomere-related proteins could conceivably be responsible.

The contribution of the individual proteins within the MRN complex was difficult to ascertain in these studies because of the effects that depletion of some of these proteins had on levels of one or more of the other components, via mechanisms that are not known. Knockdown of RAD50 by shRNA resulted in depletion of both RAD50 and NBS1, and knockdown of MRE11 depleted all three members of the complex. These observations are consistent with the down-regulation of NBS1 and RAD50 as seen in ATLD patients expressing truncated MRE11 (37). There are no known human RAD50 mutation syndromes, and it is interesting to speculate that hypomorphic RAD50 proteins may result in substantial down-regulation of NBS1 with the combined result being embryonic lethality. There was a statistically significant correlation between RAD50 depletion and shortened telomeres ($p = 0.007$). However, because NBS1 is also depleted in RAD50 clones, it is difficult to determine the contribution of RAD50 depletion on telomere length. We found it very difficult to obtain clones with substantial knockdown of MRE11, and those that were obtained appeared to have the capacity to form more MRN complex than the NBS1 and RAD50 shRNA clones, if it can be assumed that the amount of MRN complex under conditions of depletion is determined by the least abundant component. We showed that NBS1 was more depleted in NBS1 clones than in MRE11 clones. This may explain why MRE11 depletion did not cause the pronounced telomere shortening seen in NBS1 and RAD50 knockdown clones.

We believe that it is highly unlikely that our data can be explained by off target effects of shRNAs for the following reasons. First, in a previous study using the same cell line, siRNAs against each of the three MRN components, each of which targeted different sequences from those targeted by the shRNAs, also caused a reduction in APB formation in transient experiments (13). Furthermore, like the shRNAs used in the current study, siRNAs against RAD50 or MRE11 also reduced RAD50 and NBS1 proteins or all members of the MRN complex, respectively. Second, the data are supported by genetic studies. As noted above, depletion of the entire complex mediated by MRE11 deficiency has been demonstrated in tumor cell lines (50) and in ATLD (37). Third, off target effects can be excluded by targeting different genes in the same pathway. We have done this by disrupting each of the members of the MRN complex, and in each case this resulted in telomere shortening and suppression of APBs. The probability that telomere shortening and suppression of APBs could be caused by the off target effects of three shRNAs targeting completely different sequences in three genes is extremely small.

The lack of telomere shortening in NBS1-depleted telomerase-positive cells implies that the MRN complex has a role in telomere maintenance that is specific for the ALT mechanism, which raises the question of whether the MRN complex would be a useful therapeutic target in the context of ALT-positive tumors. In some clones, >95% knockdown was achieved, and the ALT mechanism was inhibited, but the cells nevertheless survived for many population doublings, and in some cases there was evidence that after a period of shortening the shortened telomeres were eventually maintained. In addition, inhibiting the function of a vital DNA repair complex in normal cells may be problematic. Further work is therefore required to determine the suitability of MRN as a target, or whether it may be more effective to target some other as yet unidentified molecular component of the ALT mechanism for therapeutic purposes.

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