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Sicong Zeng
Washington University School of Medicine in St. Louis
Qin Yang
Washington University School of Medicine in St. Louis

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Extra View

The MUS81 endonuclease is essential for telomerase negative cell proliferation

Sicong Zeng† and Qin Yang*

Department of Radiation Oncology; Washington University School of Medicine; St. Louis, MO USA

†Present address: Institute of Reproduction and Stem Cell Engineering; Central South University; Changsha, Hunan China

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A substantial number of human tumors (~10%) are telomerase negative, and cells in such tumors have been proposed to maintain telomere length by the alternative lengthening of telomeres (ALT) pathway. Although details of the molecular mechanism of ALT are largely unknown, previous studies have shown that telomere homologous recombination (HR) is implicated in the ALT pathway. MUS81 is a DNA structure-specific recombination endonuclease and functions on aberrant DNA replication and recombination. Recently, we demonstrate that MUS81 plays a key role in the maintenance of telomeres in ALT cells (Zeng, et al. Nature Cell Biology, 2009). The MUS81 endonuclease specifically localizes to ALT-associated promyelocytic leukemia nuclear bodies (APBs) and interacts with telomeres in ALT cells. Depletion of MUS81 leads to reduced telomere recombination resulting in the growth arrest of ALT cells. The endonuclease activity of MUS81, regulated by its binding partner TRF2, is found to be essential for telomere post-replicative recombination. This study provides the first direct evidence that MUS81 specifically functions on ALT recombination-based cell survival. The specific function of MUS81 on the ALT pathway provides a potential powerful diagnostic marker and a therapeutic target for ALT tumors.

Telomere Maintenance and Telomere Recombination

Telomeres are specialized structures at chromosome ends consisting of tandem repetitive DNA sequences [(TTAGGG)n in humans] and associated proteins, which are necessary for telomere function. The structure and function of telomeres are regulated by a multiprotein complex termed "shelterin,"[2,3] which allows cells to distinguish natural chromosome ends from DNA double-strand breaks and maintains telomere length and stability. Progressive telomere shortening in normal cells during DNA replication leads eventually to a permanent halt of cell division referred to as replicative senescence. This end replication problem of human telomeres has received particular attention due to its implications in ageing and cancer. Most human tumor cells acquire indefinite replicative capacity to escape from the normal proliferative limitations through maintaining their telomeres, either by telomerase[4] or by an alternative lengthening of telomeres (ALT) mechanism.[5,6]

Telomerase-negative cancer cells maintain their telomeres via the alternative lengthening of telomeres (ALT) pathway. Although a growing body of evidence demonstrates that the ALT mechanism is a post-replicative telomere recombination process, molecular details of this pathway are largely unknown.[7-10] In particular, the highly significant increase in the number of post-replicative telomere exchanges demonstrates that the ALT pathway is HR-dependent.[9,11] The increased telomere recombination is ALT-specific and extends the proliferative life of ALT cells.[12] A growing body of evidence hints that HR proteins and telomere associated proteins play a primary role in the ALT pathway. These proteins co-localize with telomeric (TTAGGG)n repeats at nuclear substructures known as ALT-associated promyelocytic leukemia (PML) nuclear bodies (APBs), which are present only in ALT cells.[6,13-15] Imaging of live cells has revealed that an individual telomere may move in and out of contact with an APB,[16] suggesting possible on-going telomere extension in ALT cells. Additionally, APBs are enriched during the G_2 phase of the cell cycle when HR is most active and ALT activity is likely occurring.[17-19] APBs preferentially accumulate linear extrachromosomal telomeric DNA, which may provide DNA substrates for telomere recombination.[20] ALT has only been detected in abnormal situations, such as tumor-derived cell lines or cell lines immortalized in vitro. It seems likely that ALT occurs due to a dysfunction in the highly regulated mechanisms controlling telomere recombination.

Activities of MUS81 in ALT Cells

MUS81, a highly conserved DNA structure-specific endonuclease, is required for the survival of cells undergoing aberrant

*Correspondence to: Qin Yang; Division of Radiation and Cancer Biology; Department of Radiation Oncology; Washington University School of Medicine; 4511 Forest Park; St. Louis, MO 63108 USA; Tel.: 314.747.5445; Fax: 314.362.9790; Email: qyang@wustl.edu

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replication and recombination.[21-24] MUS81 functions as an endonuclease by cleaving intact and nicked Holliday junctions, replication forks, 3' flap and D-loop substrates which are preferred for DNA replication and recombination.[21,22,24-27] RNA interference experiments show that MUS81 is required for mitotic recombination in somatic human cells.[21] MUS81 homozygote and heterozygote knockout mice have a predisposition to develop cancer[28] and show sensitivity to agents that stall replication forks, such as hydroxyurea, methyl methane sulfonate and mitomycin C.[28,29] Because MUS81 functions on aberrant DNA replication and recombination, it is a candidate "ALT protein", which might be required for abnormal telomere recombination in ALT cells.

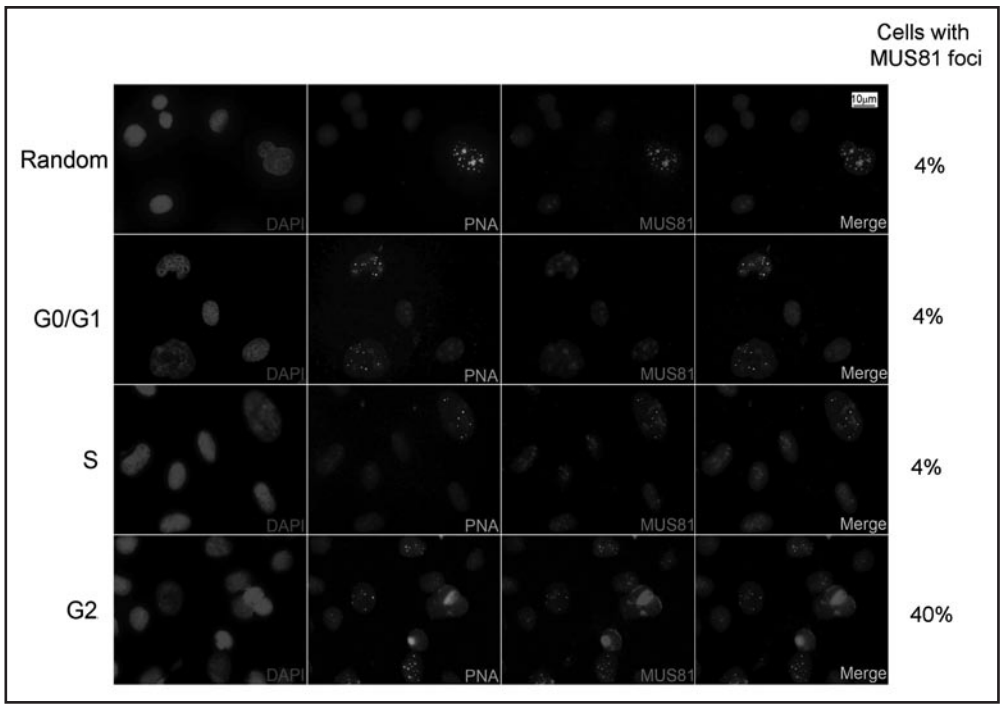

Figure 1. Cell cycle-dependent MUS81 foci formation in ALT cells. U2OS cells (ALT cells) were blocked by double thymidine treatment and released from 5 hours (S phase) or 13 hours with an additional block by Hoechst 33342 (G_2 phase). G_0/G_1 cells were obtained by methionine restriction treatment for 4 days. FACS analysis was conducted to confirm cell cycle phases (data not shown). Cells were stained with immunofluoresence combined with telomeric DNA-FISH. Bright foci staining of FITC-labeled PNA probe (telomeric C-strand oligo) indicated APBs MUS81 proteins were stained by MUS81 antibody and an Alexa Fluor 568-conjugated anti-mouse IgG (Molecular Probes). The merged images showed that MUS81 co-localized with telomeric DNA in APBs. The percentage of cells with co-localization of MUS81 foci with telomeric DNA (PNA probe) is shown. At least 200 cells were scored, and results were summarized from three independent experiments.

In our recent study,[1] we have demonstrated that the MUS81 endonuclease specifically localizes to APBs and associates with telomeric DNA in ALT cells, which is enriched during G_2 phase of the cell cycle (Fig. 1). Knockdown of MUS81 results in reduction of ALT specific-telomere recombination leading to proliferation arrest of ALT cells. Furthermore, proliferation of ALT cell requires the endonuclease activity of MUS81, and the interaction of MUS81 with TRF2 regulates this enzymatic activity to maintain telomere recombination. Thus, our results indicate that MUS81 is essential for ALT cell viability by ensuring efficient telomere recombination.

Role of MUS81 in Telomere Recombination

We find that MUS81 contributes to ALT cell survival by promoting telomere recombination.[1] In yeast, MUS81 is required for the survival of cells undergoing aberrant replication and recombination.[22,24] However, the biological functions of MUS81 in human cells are largely unknown. A variety of models predict the formation and resolution of different DNA structures during homologous recombination.[21-24] These structures include 3' flaps, forks, D-loops, nicked, gapped and intact Holliday junctions. Recent studies have indicated that these structures are possible in vivo substrates of MUS81.[30-32] Our results show that cleavage of telomere recombination substrates by MUS81 may be necessary for ALT cells survival, directly linking MUS81 endonuclease activity with abnormal telomere recombination in ALT cells. In addition, we present evidence indicating that telomere maintenance in the ALT pathway is a post-replicative recombination process, which is essential for ALT cell survival. MUS81 does not regulate telomere maintenance in non-ALT cells, in agreement with the hypothesis that MUS81 only functions on abnormal DNA recombination, such as in ALT cells with a high frequency of telomere recombination. Our studies also provide clues for studying functions of MUS81 in other biological processes, for example, in DNA damage and repair pathways.

We carefully examine telomere length after depletion of MUS81 by using Q-FISH and TRF assays and do not observe significant telomere length changes. One possibility is that telomere recombination leads to unequal sister chromatid exchange (SCE) at telomeres, which is responsible for rapid telomere elongation and shortening events. Before mitosis, telomere recombination leads to exchanges of equal amounts of telomeric DNA or results in a net gain of telomere sequences in one chromatid and a net loss in the other. Thus, the average changes of telomere length are not significantly altered. The Q-FISH and TRF assays could only detect the overall changes of telomere length and could not address on the specific chromosomes. This interpretation is consistent with a current model, which indicates maintenance of telomeres by unequal telomere SCE.[12] Because telomeres are repetitive

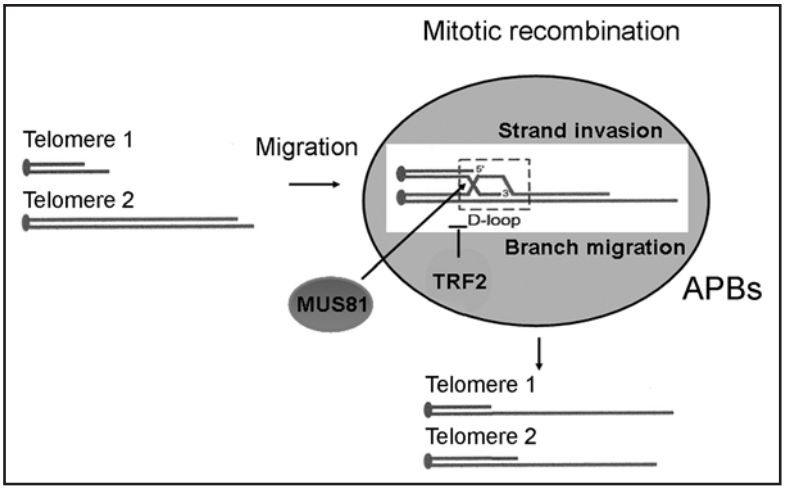

Figure 2. Proposed model for telomere processing in ALT cells. Telomeres move into APBs for processing recombination in G_2 phase of ALT cells. MUS81 is recruited into recombination sites to cleave the recombination structure, which is inhibited by TRF2. Elongated telomeres move out from APBs into next phase of the cell cycle.

sequences, sister chromatids can find sites of homology anywhere to align two telomere strands asymmetrically. Telomere recombination results in a gain or loss between two telomeres. The daughter cell inheriting chromosomes with elongated telomeres would bypass the limits on proliferative potential. Our data indicate that MUS81 enhances telomere recombination to maintain the ALT cell proliferation without significant alteration of overall telomere length, supporting this model.

We do not observe obvious chromosome end-to-end fusion in ALT cells after depletion of MUS81 (data not shown). These results are consistent with the fact that knockdown of MUS81 do not induce the formation of telomere dysfunctional induced-foci (data not shown), suggesting that MUS81 does not affect telomere end protection in ALT cells. Furthermore, MUS81 depletion do not change TRF2 binding on telomeres by the ChIP assay (data not shown), supporting this conclusion. Although we observe increased telomere loss on some chromosome ends in the MUS81-depleted ALT cells, it is possible that enough telomere sequences are still present in these chromosome ends to ensure chromosome end protection.

APBs for Telomere Maintenance

APBs are a hallmark of ALT cells and are usually found in <5% of cells within an asynchronously growing ALT cell population. Although the function of APBs is not well understood, they have been proposed to serve as depots for storage of nuclear factors and provide a platform for telomere maintenance in ALT cells.[14,33] The fact that APBs largely increase in G_2 phase of the cell cycle and that HR is active in this phase has led to the conclusion that they function on the maintenance of telomeres by recombination. We find that MUS81 specifically localizes to APBs and is enriched in G_2 phase cells (Fig. 1), supporting the notion that APBs in G_2 phase are the sites for telomere recombination. MUS81 localizes to nucleoli, but is not present in PML bodies in human telomerase-positive cells,[34] suggesting that MUS81 is recruited into APBs in ALT cells. These results support a model that activation of the ALT pathway is through recruiting "ALT proteins" to telomeres.[14]

A recent study reports that the proportion of ALT cells with APBs is increased following methionine starvation.[15] These induced APBs contain TRF1, TRF2, TIN2, RAP1, the PML protein, the MRN complex, 53BP1, SP100 and telomeric DNA. We have also observed induction of APBs containing telomeric DNA upon methionine restriction. However, MUS81 is not present in these induced APBs in G_0/G_1 phase cells (data no shown), indicating that it might be a specific effect of the methionine starvation on MUS81 cellular metabolism/localization. These results also suggest that APBs may have different functions in different phases of the cell cycle. APBs in G_2 phase cells may help to conduct telomere recombination. Unlike other recombination and telomere associated proteins present in APBs, MUS81 to APBs is only enriched in G_2 phase cells. This implies that MUS81 specifically functions on the ALT pathway, and not on global telomere maintenance. These results also implicate that the localization of MUS81 in ALT might be a unique marker for ALT tumors.

Interaction of TRF2 and MUS81 in the ALT Pathway

TRF2 is thought to be a suppressor for telomere recombination,[3,10] although the mechanism by which TRF2 governs telomere recombination is unclear. We find that TRF2 forms a complex with MUS81, inhibits the endonuclease activity of MUS81 by suppressing MUS81 binding to the DNA substrates, and mediates T-SCE through interaction with MUS81.[1] These findings, together with the fact that the MUS81 enzymatic activity is required for telomere recombination and ALT cell viability, support a model that TRF2 suppresses telomere recombination through regulation of the endonuclease activity of MUS81 in the ALT pathway (Fig. 2). We observe that there is no significant change of telomere circle (t-circle formation) after depletion of MUS81 (data not shown), suggesting that t-circles may be not involved in MUS81-mediated ALT cell survival. However, because a lower level of t-circles may be insufficient to detect, we could not rule out the role MUS81 on t-circle formation. TRF2 mediates t-loop formation and disruption of t-loop by $TRF2^{\Delta B}$ mutant generates t-circles.[10,35,36] MUS81 does not interact with $TRF2^{\Delta B}$ mutant, implicating that the MUS81 endonuclease may be involved in the disruption of t-loop, which is mediated by TRF2.

The levels of MUS81 protein in G_2 phase cells do not change,[1] suggesting that the recruitment of MUS81 to APBs is subjected to regulation in ALT cells. TRF2 and MUS81 interaction suggests that TRF2 may regulate MUS81 access to APBs, since TRF2 affects APBs formation in G_0/G_1 cells.[15] To test this hypothesis, we examine the localization of MUS81 in APBs after depletion of TRF2. However, depletion of TRF2 does not change APB formation and localization of MUS81 to APBs at G_2 phase in ALT cells (data not shown), excluding that TRF2 regulates the

recruitment of MUS81 to APBs. Further investigation will be needed to clarify which protein is involved in the recruitment of MUS81 into APBs.

MUS81 for Telomere Maintenance in ALT Cancer Cells

Our observations demonstrate that MUS81 endonuclease is specifically involved in telomere recombination and in the maintenance of ALT cell survival.[1] We propose a model where telomeres move into APBs for processing recombination based telomere maintenance after DNA replication during G_2 phase of the cell cycle. MUS81 is recruited into recombination sites to cleave the recombination structure, for example, D-loop (Fig. 2). TRF2 negatively regulates the MUS81 enzymatic activity to balance this process. Then, telomeres that completed recombination move out from APBs into next step of the cell cycle. In this manner, ALT cells maintain their telomeres and extend the proliferative lifespan.

The telomere maintenance mechanism is essential for the indefinite proliferation of cancer cells. Understanding the specific requirements of ALT is key to developing diagnostic tools and therapies that target this pathway. Inhibiting telomerase activity in non-ALT tumors may activate the ALT pathway, suggesting that suppression of ALT activity is important for most tumors. MUS81, which is specifically present in APBs and is essential for the ALT cell survival, will provide a powerful diagnostic marker and a putative therapeutic target for ALT tumors.

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References

1. Zeng S, Xiang T, Pandita TK, et al. Telomere recombination requires the MUS81 endonuclease. Nat Cell Biol 2009; 11:616-23.
2. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev 2005; 19:2100-10.
3. Palm W, de LT. How shelterin protects mammalian telomeres. Annu Rev Genet 2008; 42:301-34.
4. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985; 43:405-13.
5. Bryan TM, Englezou A, la-Pozza L, et al. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med 1997; 3:1271-4.
6. Bryan TM, Englezou A, Gupta J, et al. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J 1995; 14:4240-8.
7. Dunham MA, Neumann AA, Fasching CL, et al. Telomere maintenance by recombination in human cells. Nat Genet 2000; 26:447-50.
8. Cesare AJ, Reddel RR. Telomere uncapping and alternative lengthening of telomeres. Mech Ageing Dev 2008; 129:99-108.
9. Londono-Vallejo JA, Der-Sarkissian H, Cazes L, et al. Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. Cancer Res 2004; 64:2324-7.
10. Wang RC, Smogorzewska A, de Lange T. Homologous recombination generates T-loop-sized deletions at human telomeres. Cell 2004; 119:355-68.
11. Cesare AJ, Reddel RR. Telomere uncapping and alternative lengthening of telomeres. Mech Ageing Dev 2008; 129:99-108.
12. Bailey SM, Brenneman MA, Goodwin EH. Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. Nucleic Acids Res 2004; 32:3743-51.
13. Yeager TR, Neumann AA, Englezou A, et al. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res 1999; 59:4175-9.
14. Muntoni A, Reddel RR. The first molecular details of ALT in human tumor cells. Hum Mol Genet 2005; 14:191-6.
15. Jiang WQ, Zhong ZH, Henson JD, et al. Identification of candidate alternative lengthening of telomeres genes by methionine restriction and RNA interference. Oncogene 2007; 26:4635-47.
16. Molenaar C, Wiesmeijer K, Verwoerd NP, et al. Visualizing telomere dynamics in living mammalian cells using PNA probes. EMBO J 2003; 22:6631-41.
17. Wu G, Lee WH, Chen PL. NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G_2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres. J Biol Chem 2000; 275:30618-22.
18. Grobelny JV, Godwin AK, Broccoli D. ALT-associated PML bodies are present in viable cells and are enriched in cells in the G(2)/M phase of the cell cycle. J Cell Sci 2000; 113:4577-85.
19. Yang Q. Cellular senescence, telomere recombination and maintenance. Cytogenet Genome Res 2008; 122:211-8.
20. Fasching CL, Neumann AA, Muntoni A, et al. DNA damage induces alternative lengthening of telomeres (ALT) associated promyelocytic leukemia bodies that preferentially associate with linear telomeric DNA. Cancer Res 2007; 67:7072-7.
21. Blais V, Gao H, Elwell CA, et al. RNA interference inhibition of Mus81 reduces mitotic recombination in human cells. Mol Biol Cell 2004; 15:552-62.
22. Osman F, Whitby MC. Exploring the roles of Mus81-Eme1/Mms4 at perturbed replication forks. DNA Repair (Amst) 2007; 6:1004-17.
23. Heyer WD. Recombination: Holliday junction resolution and crossover formation. Curr Biol 2004; 14:56-8.
24. Hollingsworth NM, Brill SJ. The Mus81 solution to resolution: generating meiotic crossovers without Holliday junctions. Genes Dev 2004; 18:117-25.
25. Ehmsen KT, Heyer WD. *Saccharomyces cerevisiae* Mus81-Mms4 is a catalytic, DNA structure-selective endonuclease. Nucleic Acids Res 2008; 36:2182-95.
26. Chen XB, Melchionna R, Denis CM, et al. Human Mus81-associated endonuclease cleaves Holliday junctions in vitro. Mol Cell 2001; 8:1117-27.
27. Boddy MN, Gaillard PH, McDonald WH, et al. Mus81-Eme1 are essential components of a Holliday junction resolvase. Cell 2001; 107:537-48.
28. McPherson JP, Lemmers B, Chahwan R, et al. Involvement of mammalian Mus81 in genome integrity and tumor suppression. Science 2004; 304:1822-6.
29. Dendouga N, Gao H, Moechars D, et al. Disruption of murine Mus81 increases genomic instability and DNA damage sensitivity but does not promote tumorigenesis. Mol Cell Biol 2005; 25:7569-79.
30. Osman F, Dixon J, Doe CL, et al. Generating crossovers by resolution of nicked Holliday junctions: a role for Mus81-Eme1 in meiosis. Mol Cell 2003; 12:761-74.
31. Whitby MC. Junctions on the road to cancer. Nat Struct Mol Biol 2004; 11:693-5.
32. Gaillard PH, Noguchi E, Shanahan P, et al. The endogenous Mus81-Eme1 complex resolves Holliday junctions by a nick and counternick mechanism. Mol Cell 2003; 12:747-59.
33. Cesare AJ, Reddel RR. Telomere uncapping and alternative lengthening of telomeres. Mech Ageing Dev 2008; 129:99-108.
34. Gao H, Chen XB, McGowan CH. Mus81 endonuclease localizes to nucleoli and to regions of DNA damage in human S-phase cells. Mol Biol Cell 2003; 14:4826-34.
35. Griffith JD, Comeau L, Rosenfield S, et al. Mammalian telomeres end in a large duplex loop. Cell 1999; 97:503-14.
36. Stansel RM, de Lange T, Griffith JD. T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. EMBO J 2001; 20:5532-40.