2020

Defining RAD54 function in the alternative lengthening of telomeres pathway

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

DEFINING RAD54 FUNCTION IN THE ALTERNATIVE LENGTHENING OF TELOMERES PATHWAY

by

KATHERINE TERRANOVA

B.A., Boston College, 2016

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

Approved by

First Reader ____

Rachel Litman Flynn, Ph.D.
Assistant Professor of Pharmacology and Medicine

Second Reader ____

Vickery Trinkaus-Randall, Ph.D.
Associate Professor of Biochemistry and Ophthalmology

ACKNOWLEDGMENTS

Thank you to Dr. Rachel Flynn and Dr. Emily Mason-Osann for allowing me to participate in your research and for taking the time to teach me about the experimental research process. I especially want to acknowledge the efforts of Dr. Mason-Osann in designing the experiments and implementing those that were beyond my capabilities. I am very grateful to have had this opportunity to learn from you.

DEFINING RAD54 FUNCTION IN THE ALTERNATIVE LENGTHENING OF TELOMERES PATHWAY

KATHERINE TERRANOVA

ABSTRACT

Background: Telomeres are the DNA sequences at the end of chromosomes that are made up of highly repetitive sequences to protect the ends of chromosomes from damage. Telomeres shorten with each round of DNA replication eventually causing cellular senescence. Many cancer cells are able to overcome or evade senescence by elongating their telomeres. Alternative lengthening of telomeres (ALT) is a recombination based mechanism used by cancer cells to maintain telomere length. Evidence supports that unresolved replication stress at telomeric DNA promotes telomere elongation. Given the role of RAD54 in recombination genome wide, we were interested in investigating whether RAD54 is contributing to ALT telomere maintenance.

Objective: To define the role of RAD54 in ALT telomere maintenance.

Methods: Several different known ALT cell lines and non-ALT cell lines were examined using wet-lab techniques. Combined immunofluorescence and DNA FISH (IF-FISH) was used to visualize co-localization between RAD54 and telomeres, siRNA was used to deplete specific mRNA, and western blots were used to confirm these knockdowns.

Results: IF-FISH showed enrichment of RAD54 at the telomeres in ALT cells as compared to non-ALT cells. There was a decrease in incorporation of the synthetic nucleotide EdU in the absence of RAD54, indicating a decrease in DNA synthesis. No change was seen in the recruitment of RAD51, a recombinase, to telomeres in the absence of RAD54. There was a significant increase in MUS81 colocalization to ALT telomeres in the absence of RAD54 and an increase in the number of ultra-fine anaphase bridges.

Conclusions: Using combined immunofluorescence and DNA FISH, we found enrichment of RAD54 at the telomeres in ALT cells as compared to non-ALT cell lines. Furthermore, RAD54 is predominantly found at ALT telomeres in ALT-associated promyelocytic leukemia (PML) bodies (APBs), which are nuclear condensates containing telomeres and many repair proteins. APBs are thought to be sites of active recombination, mediated by the recombinase RAD51. We can monitor recombination using EdU incorporation events at telomeres. RAD54 promotes DNA synthesis events at ALT telomeres, as measured through EdU incorporation. No change was found in RAD51 recruitment to telomeres in the absence of RAD54, indicating that RAD54 is not required for RAD51 mediated synapsis. By over-expressing RAD54 mutants, we found that sites of DNA synthesis, that are thought to be elongation events at ALT telomeres, are dependent on the ATPase and branch migration activities of RAD54. These results suggest that RAD54 is promoting telomere elongation by mediating the migration of the branched DNA structures formed at telomeres during recombination. When RAD54 is depleted, we found an increase in recruitment of the nuclease MUS81 to ALT telomeres, suggesting that in the absence of branch migration, ALT telomeres are cleaved to resolve recombination intermediates. Together, these data demonstrate a crucial role for RAD54 in promoting ALT mediated telomere elongation through resolving homologous recombination intermediates via branch migration.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALT .... Alternative Lengthening of Telomeres

APB .... ALT-associated PML Bodies

ATP .... Adenosine Triphosphate

ATRX .... Alpha-Thalassemia/Mental Retardation, X-linked

BIR .... Break Induced Replication

BSA .... Bovine Serum Albumin

BLM .... Branch Migration Enzyme

BTR .... BLM, TOP31, RMI1/2 Complex

CAL72 .... Osteosarcoma Cell Line

cDNA .... Complementary DNA

$CuSO_4$ .... Copper Sulfate

DAPI .... 4′,6-diamidino-2-phenylindole

DAXX .... Death Domain Associated Protein

DMEM .... Dulbecco's Modified Eagle Medium

DPBS .... Dulbecco's Phosphate-buffered Saline

dsDNA .... Double Stranded Deoxyribonucleic Acid

ECTR .... Extrachromosomal Telomeric Repeats DNA

EdU .... Synthetic Nucleotide 5-ethynyl-2'-deoxyuridine

EME1 .... Essential Meiotic Structure-Specific Endonuclease 1

ERCC1 .... DNA Excision Repair Protein 1

FBS .... Fetal Bovine Serum

G292 .... Osteosarcoma Cell Line G-292

H3.3 .... Histone H3 Replacement Variant

HeLa LT .... Henrietta Lacks Long Telomeres Immortal Cell Line

HRP .... Horseradish Peroxidase

HuO9 .... Osteosarcoma Cell Line

IF-FISH ..... Combined Immunofluorescence and DNA Fluorescence in situ Hybridization

$MgCl_2$ .... Magnesium Chloride

MRE11 .... Double-strand Break Repair Protein, part of RAD52 Epistasis Group

MUS81 .... Human Endonuclease

Na_2HPO_4 .... Disodium Phosphate

NaCl .... Sodium Chloride

NOS .... Osteosarcoma Cell Line

NP40 .... nonyl phenoxypolyethoxylethanol

PBG .... PBS, BSA, Fish Gelatin

PBS .... Phosphate-buffered Saline

pDEST-SFB .... Gateway Destination Vector

PIPES .... piperazine-N,N′-bis(2-ethanesulfonic acid)

PML .... Promyeloyctic Leukemia

POT1 .... Protection of Telomeres 1

PVDF .... Polyvinylidene difluoride

RAD50 .... DNA Repair Protein, part of RAD52 Epistasis Group

RAD51 .... Eukaryote Protein that plays a role in Homologous Recombination

RAD52 ....Human Protein with a role in DNA Repair

RAD54 ....DNA Repair and Recombination Protein, part of RAD52 Epistasis Group

RAD55 .... DNA Repair Protein, part of RAD52 Epistasis Group

RAD57 .... DNA Repair Protein, part of RAD52 Epistasis Group

RAD59 .... DNA Repair Protein, part of RAD52 Epistasis Group

RAP1... Ras-associated Protein 1

RDH54/TID1 .... DNA Repair Protein, part of RAD52 Epistasis Group

RMI1/2...RecQ-Mediated Genome Instability Protein 1 and 2

RPA..Replication Factor A

RPMI.. Roswell Park Memorial Institute

SaOS2 ....Osteosarcoma Cell Line

SDS-PAGE .... sodium dodecyl sulphate-polyacrylamide gel electrophoresis

siRNA ....Small Interfering Ribonucleic Acid

SJSA1..Osteosarcoma Cell Line

SLX1-4...Proteins involved in DNA Repair

SMX.. SLX1-4, MUS81-EME1, XPF-ERCC1 Complex

SSC ....Saline-Sodium Citrate buffer

SWI2/SNF2.. SWItch/Sucrose Non-Fermentable

TBS-T .... Tris-buffered Saline with Tween

TIN2.. TRF1-interacting Nuclear Factor 2

TMM...Telomere Maintenance Mechanism

TOP3A ....DNA Topoisomerase 3 Alpha

TPP1..Tripeptidyl Peptidase 1 human gene

TRF1 ....Telomeric Repeat Binding Factor 1

TRF2 ....Telomeric Repeat Binding Factor 2

T-SCE .... Telomere-Sister Chromatid Exchanges

U20S .... Human Bone Osteosarcoma Epithelial cell line

XRS2.. DNA Repair Protein, part of RAD52 Epistasis Group

INTRODUCTION

Telomeres are the DNA sequences at the end of chromosomes. Telomeric DNA is composed of TTAGGG hexameric repeats that function to protect the ends of chromosomes from DNA damage. Telomeres in humans are 10-15 kilobases (kb) and in mice are 20-50 kb of TTAGGG repeats (Martinez and Blasco, 2015). The structure of telomeres includes the G-strand overhang, a 300-400 nucleotide 3' overhang, the telomere loop (T-loop), and displacement loop (D-loop) (Martinez and Blasco, 2015). The T-loop is formed when the G-strand overhang invades the double-stranded DNA region of the telomere. The D-loop is formed at the invasion site (Martinez and Blasco, 2015). These structures are crucial for chromosomal protection from DNA repair activities and degradation.

The shelterin protein complex is composed of telomeric DNA binding factors TRF1 and TRF2, the TRF2-interacting protein RAP1, TIN2 bridging factor, POT1 binding protein and its direct interactor TPP1. The shelterin complex is another important aspect of the telomere (Zhao et al., 2019). The shelterin complex mediates the formation of the T-loop by binding with sequence specificity. The T-loop prevents the recognition of the 3' end of the telomere as being a single strand or double strand DNA break, which protects the telomere from DNA damage response (DDR) proteins instructing the cell to stop growing (Zhao et al., 2019).

Each time a cell divides 25-200 bases are lost from the ends of telomeres due to the "end replication problem". The eukaryotic nuclear genome is packaged into discrete linear chromosomes. The linear configuration, in conjunction with semiconservative DNA replication, prevents the ends of chromosomes from being replicated. With continued cell division, sequence is lost from the end of the linear chromosomes, and thus without a telomere maintenance mechanism, telomeres shorten with each round of replication (Cesare and Redell, 2010). Typically, critically short or dysfunctional telomeres induce cellular senescence and contribute to cellular aging. Cancer cells are able to bypass cellular senescence and become immortal by promoting telomere elongation. Thus, one of the hallmarks of cancer is replicative immortality (Hanahan, 2011).

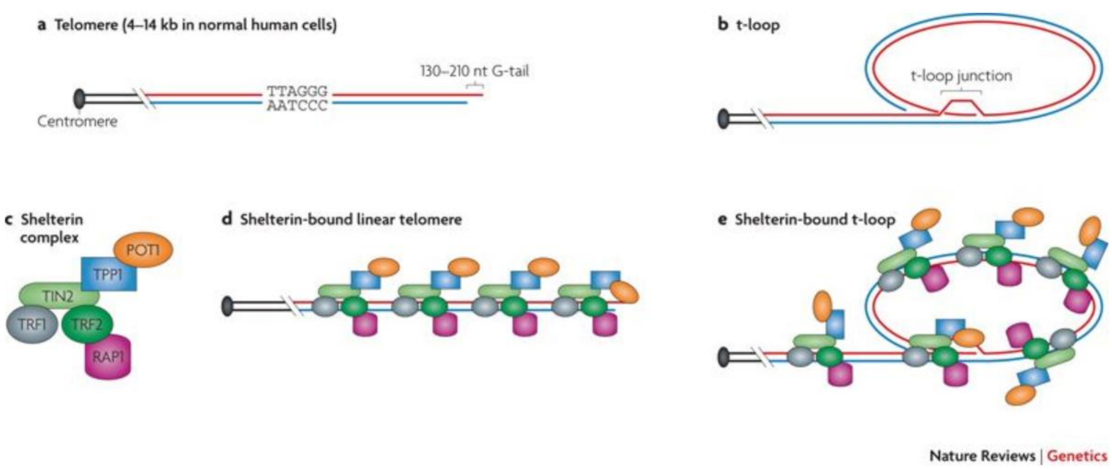


Figure 1. Structure of Telomeres. Figure 1 shows the structure of telomeres. **a.** Repeat DNA sequence (5′-TTAGGG-3′)n of telomeres, which are mostly double stranded besides the G-tail. **b.** Formation of the T-loop from the telomere folding back on itself and the single-stranded terminus invading duplex telomeric DNA. **c**. Telomeric DNA is bound by the six-subunit shelterin complex. **d,e .** Two shelterin proteins (telomeric repeat-binding factor 1 (TRF1) and TRF2) bind directly to double-stranded telomeric DNA, and one (protection of telomeres 1 (POT1)) binds single-stranded telomeric DNA directly. POT1, TPP, TRF1-interacting nuclear protein 2 (TIN2) and RAP1 also interact indirectly with double-stranded telomeric DNA through their interactions with other shelterin proteins. Taken from (Cesare and Redell, 2010).

There are two major telomere maintenance mechanisms (TMM): reactivation of the enzyme telomerase or activation of the Alternative Lengthening of Telomeres (ALT) pathway (De Vitis et al. 2018). ALT is active in only a small subset of tumors, less than 4% of all human cancers, but is prevalent in specific cancer types, especially osteosarcoma and glioblastoma (Heaphy et al., 2011). The prognosis for these types of cancers is especially poor, and treatment options have remained stagnant for over 30 years (Heaphy et al., 2011). There are some therapies in development for cancers that use the telomerase pathway, but there are no known targets for ALT cancer treatments (Flynn et al., 2015). Thus, the study of the ALT pathway is important to identify specifically targeted cancer therapies.

Telomerase is an enzyme that maintains telomere length in the vast majority of cancers. Telomerase elongates telomeres via reverse transcriptase activity by complexing with other proteins and a functional RNA to make a ribonucleoprotein enzyme complex (Jafri et al., 2016). The gene hTERT encodes telomerase while the gene hTERC encodes the functional RNA. In somatic cells hTERC is constitutively expressed, but hTERT is usually silent and the cells do not achieve replicative immortality. However, in ~90% of human cancers there is a significant increase in hTERT expression allowing cells to bypass senescence and achieve proliferative immortality (Jafri et al., 2016).

The other 5-10% of cancers maintain telomere length via the telomerase-independent ALT mechanism. The ALT phenotype is characterized by persistent DNA damage, heterogeneous telomere length, ALT-associated PML bodies (APBs) and C-rich extrachromosomal telomeric repeats (C-circles). ALT is a recombination based pathway that promotes telomere elongation via break-induced replication (BIR). DNA damage occurs spontaneously every day in human cells to a certain extent, but ALT cells have been shown to have large amounts of intrinsic DNA damage leading to chromatin instability (Cesare et al., 2009). This leads to persistent DNA damage responses at ALT telomeres that can be repaired via homologous recombination (Liu et al., 2018). In homologous recombination repair, a damaged DNA molecule invades an undamaged DNA molecule of similar sequence and DNA is resynthesized using the undamaged strand as a template (Wright and Heyer, 2014).

BIR is homologous recombination that is used as an efficient way to repair single-ended double strand breaks (Malkova and Ira, 2013). BIR works through a 3' ssDNA overhang invading a homologous DNA molecule followed by DNA synthesis. This DNA synthesis is promoted by DNA polymerases Pol η and Pol δ - proteins normally involved in DNA synthesis. In eukaryotes, the protein RAD51 mediates single strand invasion and formation of a displacement loop (D-loop) that initiates BIR. A Holliday junction (HJ) is formed from single end invasion of the 3' end. The migrating D-loop carries out BIR and then branch migration of an unresolved Holliday junction promotes displacement of a newly synthesized strand (Malkova and Ira, 2013). ALT cells synthesize new telomeric DNA via BIR using the telomere itself as a DNA template, a telomere on a sister chromatid or even a telomere on another chromosome or an extrachromosomal telomeric repeat (ECTR). As a result of this, ALT cells have long, heterogeneous telomeres (Lawlor et al., 2019).

We are researching the ALT pathway to determine which genes play a role in ALT activity. Research suggests that ALT is dependent on homologous recombination (Cesare and Reddel, 2010). In ALT, the telomeric 3' overhang invades other telomeric DNA and uses it as a template for DNA replication to extend the telomere (Cesare and Reddel, 2010). In ALT cells, there are proteins that are required for ALT-mediated telomere elongation and proteins that are required for preventing telomere loss (Cesare and Redell, 2010). Telomere elongation is dependent on homologous recombination in response to double-stranded DNA breaks (DSBs).

The ALT mechanism does not function in normal cells due to factors that prevent ALT from maintaining the lengths of their telomeres. When normal cells are fused with ALT cells, the ALT mechanism is repressed in the resulting hybrids, demonstrating that ALT might result from loss of a normal function (Cesare and Reddel, 2010). The proteins known to be required for ALT activity are most likely present in normal cells, but the mechanism through which the ALT activity is prevented in normal cells is unknown (Cesare and Reddel, 2010).

The loss of function of two genes, ATRX and DAXX, are strongly associated with ALT activity (Heaphy et al., 2011 b). In a study of pancreatic neuroendocrine tumors, 100% of tumors with ATRX or DAXX gene mutations were ALT-positive (Heaphy et al., 2011 b). ALT only occurs in a small subset of tumors, but when the study was expanded to include different tumor types, there was consistent correlation between inactivation of ATRX or DAXX and the ALT phenotype (Heaphy et al., 2011 b). ATRX and DAXX encode components of the H3.3 histone chaperone complex. DAXX is a multifunctional protein with roles in cell death pathways, H3.3 deposition and transcriptional regulation (Yost et al., 2019). It has been shown that if wild type DAXX is introduced into an osteosarcoma cell line it suppresses the ALT phenotype, but when the DAXX is withdrawn, the ALT phenotype is restored (Yost et al., 2019). This demonstrates that continued deficiency in DAXX is necessary for ALT to be maintained in this system. Similarly, expression of wild type ATRX suppresses ALT and is associated with rapid shortening of telomeres (Clynes et al., 2015). ATRX is a chromatin remodeling factor and its repression of ALT is dependent on DAXX (Clynes et al., 2015). The loss of ATRX leads to a loss of H3.3 incorporation, which causes defective telomere chromatinization. This causes the replication fork to stall and collapse, leading to HR and telomere synthesis at ALT telomeres (Clynes et al., 2015).

Mutations in the ATRX/DAXX chromatin remodeling complex and histone H3.3 complex correlate with features of ALT in tumors and thus, might contribute to activation of the ALT pathway (Lovejoy et al., 2012). The exact function of ATRX/DAXX and H3.3 at telomeres is not defined, but it is clear that ATRX deficiency leads to increased ALT activity as there is diminished ATRX in 90% of ALT cell lines (Lovejoy et al., 2012). This research on the ALT pathway confirms that knockdown of ATRX is associated with the ALT pathway, but is not enough to establish ALT, and suggests that there must be a multistep process that requires additional genetic changes to fully activate the ALT pathway.

Homologous recombination is thought to be the elongation method used by ALT. Homologous recombination occurs over several hundred base pairs from the exchange of

DNA between sequences of perfect or near perfect homology (Symington, 2002). Double-stranded DNA breaks, caused by replication fork collapse or exposure to other forms of DNA-damage, are repaired via homologous recombination. There are thought to be three stages to homologous recombination: pre-synapsis, synapsis, and post-synapsis (Tan et al., 2003).

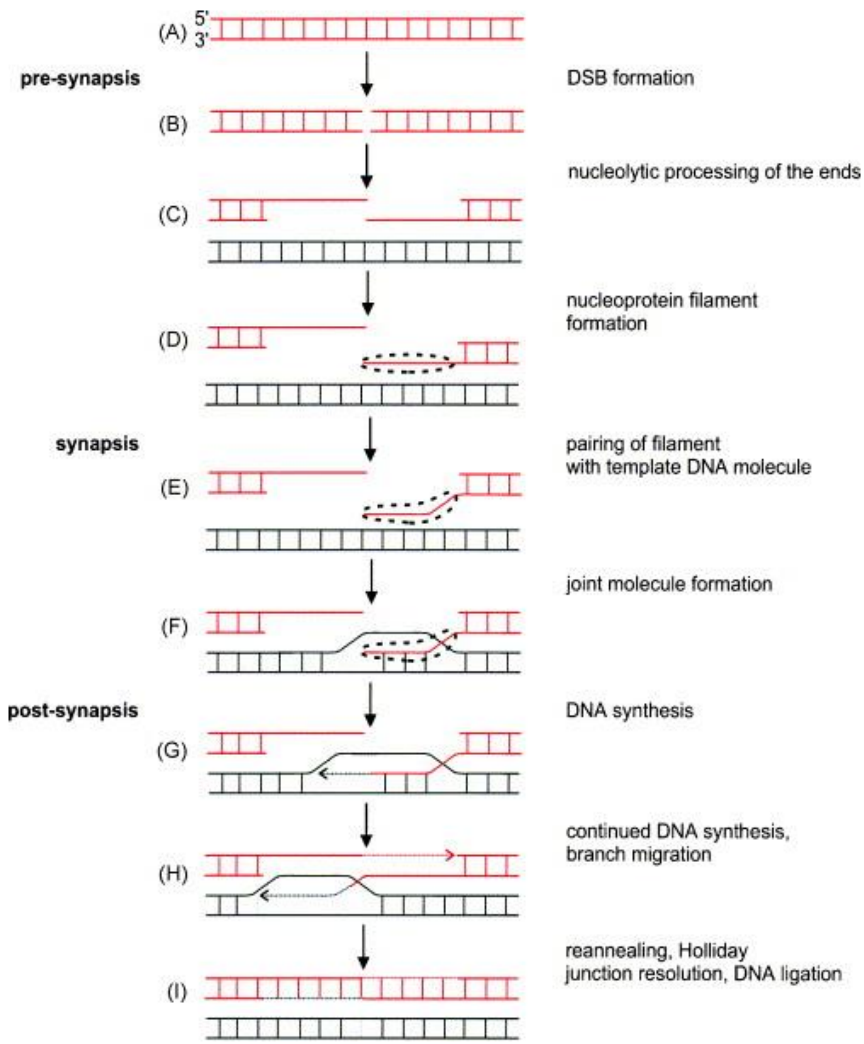


Figure 2. Figure 2 shows a diagram of a DNA double-strand break repaired via homologous recombination. The pathway is divided into three steps, pre-synapsis (A–D), synapsis (E and F), and post-synapsis (G–I). As depicted, intact DNA (A) suffers a double-strand break (B). The broken DNA ends are processed to produce single-stranded 3′ overhangs (C). A RAD51 nucleoprotein filament is formed on the single-stranded DNA tails with the help of accessory

proteins (D). During synapsis, the RAD51 nucleoprotein filament can pair with homologous sequences in the template DNA (E). This results in joint molecule formation where the single-stranded end of the broken DNA is base paired to one strand of the template (F). During post-synapsis, the broken single-stranded end can prime DNA synthesis to recover any DNA sequences lost (G). Branch migration, Holliday junction resolution and ligation restore two complete copies of the DNA spanning the original break (H and I). Taken from (Tan et al., 2003).

Another group of proteins that are involved in the ALT pathway due to their role in homologous recombination is the RAD52 epistasis group. The RAD52 protein group consists of RAD50, RAD51, RAD52, RAD54, RDH54/TID1, RAD55, RAD57, RAD59, MRE11, and XRS2 (Symington, 2002). RAD54 is a member of the RAD52 epistasis group and is placed within the SWI2/SNF2 family of DNA-dependent ATPases (Tan et al., 2003). RAD51 is the central protein in homologous recombination since it mediates homologous pairing and DNA strand exchange between the damaged and undamaged DNA strands (Agarwal et al., 2011). Several other RAD52 epistasis group proteins, such as RAD54, are involved as mediator and accessory proteins to assist RAD51 in various stages of homologous recombination (Agarwal et al., 2011). The ATPase activity of RAD54 is triggered by dsDNA. RAD54 plays a role in each of the three stages of homologous recombination. During pre-synapsis RAD54 interacts with RAD51 and stabilizes the RAD51 single-stranded DNA interaction (Tan et al., 2003). RAD54 is thought to stabilize RAD51 independent of its ATPase activity, increasing the efficiency of the homology search (Tavares et al., 2019). During synapsis, RAD54's ATPase activity plays a role in joint molecule formation (Tan et al., 2003). In reactions lacking RAD54, but still containing RAD51, no D-loops were formed demonstrating that RAD54 is likely required to form RAD51-mediated synaptic complexes in the damage incurred from this experimental set-up (Tavares et al., 2019). During post-synapsis, RAD54 plays a role in branch migration, which we propose aids in resolving recombination intermediates in the ALT mechanism. There have been other studies that have shown that RAD54 is not required for RAD51-mediated D-loop formation. RAD51 is not essential for BIR at ALT telomeres since RAD52 can also function to promote homologous pairing (Min et al., 2017). Whether RAD51 or RAD52-mediated, a D-loop is still formed, which serves as an integral part of BIR. The D-loop is extended through telomere synthesis and then needs to be resolved to complete the telomere elongation event.

Branch migration promotes DNA synthesis at ALT telomeres. Branch migration is also responsible for resolving joint molecule D-loops. Studies have shown that RAD54 catalyzes branch migration of Holliday junctions (Goyal et al., 2018). RAD54 has been shown to promote branch migration with much greater efficiency than other known DNA damage repair proteins, such as BLM or RECQ1 (Goyal et al., 2018). DNA synthesis from branch migration causes telomere elongation, thus, defining the enzymes involved in branch migration at ALT telomeres could help identify targets for inhibiting TMM in ALT cancers.

ALT and telomerase TMM pathways may both be present in certain cancers, and potentially a third, unknown mechanism known as "ever-shorter telomeres" may be present in some aggressive cancer types such as neuroblastomas (Dagg et al., 2017). The tumors seen in these aggressive cancers initially have long telomeres which proliferate for over 200 population doublings with ever shorter telomeres (Dagg et al., 2017). These findings indicate that oncogenesis may not require the prevention of telomere shortening.

This has been echoed by other findings in which the inhibition of telomerase or ALT alone for the treatment of some tumors led to treatment failure, suggesting the activation of a third pathway (Qiang et al., 2014). Other research shows that TMMs are not mutually exclusive in some tumors (Gocha et al., 2013). In order to maximize therapeutic effect against tumors, it would be necessary to determine which type or types of TMM are present.

RAD54 has the potential to be a good target for ALT cancer therapies because it is nonessential for cell viability (Ivanov and Haber, 1997). Cells are still viable without RAD54, but RAD54 has been shown to be essential for homologous recombination (Ivanov and Haber, 1997). Most DDR proteins are essential for cell viability, which means if they are targeted for inhibition the effects would be detrimental to healthy cells instead of just to cancer cells. The goal of this thesis is to detect how RAD54 is necessary for the ALT mechanism of telomere elongation to potentially identify it as a therapeutic target in ALT cancer cells.

SPECIFIC AIMS

In order to determine the role of RAD54 in the ALT pathway, the specific aims of this study were:

1. To determine if RAD54 localizes to ALT telomeres and to sites of active telomere elongation.
2. To define the translocase role of RAD54 in ALT activity.
3. To determine the involvement of RAD54 in resolving recombination intermediates at ALT telomeres.

METHODS

Antibodies

The following is a list of the antibodies used where noted in the descriptions below. MUS81 (Santa Cruz sc53382), PML (Santa Cruz sc-5621), PML (Santa Cruz sc-966), RAD51 (Santa Cruz sc-8349, IF), RAD51 (Abcam ab176458, western blot), RAD54 (Santa Cruz sc-374598), TRF1 (Millipore 04-638), TRF2 (Millipore 05-521), Tubulin (Cell Signaling Technology 2125S).

Culturing the ALT and non-ALT cell lines

SaOS2 cells were grown in Roswell Park Memorial Institute (RPMI-1640) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. U2OS and HeLa-LT cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% penicillin/streptomycin. HuO9 and SJSA1 cells were cultured in RPMI-1640 containing 5% FBS, 1% penicillin/streptomycin, and 1% sodium pyruvate. CAL72 cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12) with 10% FBS and 1% penicillin/streptomycin. All cell lines were grown at 37°C in a humidified incubator with 5% CO_2. Cells were split every 2-4 days to carry the cell line and to grow cells in multi-well plates for experiments. Cells were plated in 10 cm plates, 15 cm plates or 6-well plates. Cells were counted and seeded at 100,000 cells/well, 150,000 cells/well or 500,000 cells/well based on the time in culture, the doubling time of the cells, and the intended experiment.

Tissue Culture

All steps took place in a sterile tissue culture hood. First the cell culture media was aspirated from the plate and cells were washed in Dulbecco's Phosphate-buffered Saline (DPBS). After the addition of 1.5 mL of trypsin, cells were placed in an incubator for 3-5 minutes. The trypsin treated cells were removed from the incubator and 5 mL of cell culture media were added to the plates. The cells were collected into tubes and centrifuged at 1250 rpm for 3 minutes at 20°C forming visible cell pellets. The old media was aspirated off of the pellets and cells were re-suspended in fresh media and seeded onto new plates at a density one-half to one-eighth of their original density.

RAD54 Enrichment at ALT Telomeres Experiments

Combined IF and DNA FISH

Cells were washed twice with PBS for 5 minutes each. The cells were then treated with cytobuffer (100 mM NaCl, 300 mM sucrose, 3mM $MgCl_2$, 10 mM PIPES pH 7, 0.1% Triton X-100) for 7 minutes at 4°C. Cells were next rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. To permeabilize the cells, 0.5% NP40 in PBS was used for 10 minutes at room temperature. Following a PBS rinse, cells were blocked for 1 hour at room temperature in PBG (0.5% BSA, 0.2% Fish gelatin, PBS) and then incubated with RAD54 (Santa Cruz sc-374598) primary antibody diluted 1:50 in PBG overnight at 4°C. The cells were washed three times with PBS for 5 minutes each at room temperature, and then incubated with Goat anti-Mouse IgG (H+L) Secondary Antibody Alexa Fluor® 488 conjugate diluted 1:250 in PBG for 45 minutes at room temperature in the dark. After this incubation the cells were washed three times with PBS at room temperature for 5 minutes each and then fixed with paraformaldehyde in PBS for 10 minutes at room temperature. Cells were rinsed with PBS and then digested with RNaseA 200 μg/mL in 2X SSC for 30 minutes at 37°C. A series of ethanol washes (70%, 85%, 100%) for 2 minutes each at room temperature were used to dehydrate the cells. The coverslips were then dried at 37°C for 20 minutes. Telomere probe (PNA-(CCTAA)4 Tel-Cy3) diluted 1:750 in hybridization buffer (70% formamide, 0.25% blocking reagent [Roche], 10mM Tris pH 7.5, 4.1 mM Na_2HPO_4, 1.25 mM $MgCl_2$, 0.45 mM citric acid) was added to glass slides and coverslips were flipped onto it. Slides were denatured at 85°C for 3 minutes and then placed in a humidified chamber at 37°C overnight. Coverslips were washed with 2X SSC and formamide mixed 1:1 three times for 5 minutes each at 37°C, and then three times in just 2X SSC at 37°C. One μg/mL DAPI (4′,6-diamidino-2-phenylindole) 1:1000 was added into 2X SSC for a final 10-minute wash at room temperature. Coverslips were mounted on glass slides using vectashield mounting medium.

Five different known ALT cell lines (SaOS2, U2OS, NOS, HuO9, Cal72) were stained as well as two non-ALT cell lines (SJSA1, HeLa LT) for comparison. Stained cells were imaged under the microscope. Analyses of images were performed by manually counting at least 100 cells per cell line and counting a cell as positive if it contained at least one co-localization between RAD54 and telomeric DNA foci.

IF-FISH for PML

The same combined IF and DNA FISH methods used to detect RAD54 were used when both RAD54 and PML were being detected. Rabbit polyclonal PML antibody diluted 1:500 in PBG was added to the cells for 45 minutes at room temperature following overnight incubation with RAD54 at a 1:50 dilution in PBG and three five-minute washes with PBS. Two ALT cell lines (SaOS2, HuO9) were stained for both RAD54 and PML. Cells were counted as positive when at least one RAD54 foci aligned with a PML foci and telomere foci.

RAD54 Knockdown Experiments

siRNA

RAD54 was depleted via reverse-transfection with siRNA. To transfect the cells, media was aspirated from the wells and the cells were washed in DPBS. Cells were then incubated with 300 μL trypsin for 5 minutes in a 37°C incubator. Media was added to stop the trypsin and the cells were transferred to a conical tube and centrifuged for 3 minutes at 1250 rpm. Next cells were re-suspended in media and then they were counted using a hemocytometer under the microscope. Cell counts were made to be 200,000 cells/well in a volume of 1500 μL. Transfection was done using Lipofectamine RNAiMax reagent in Opti-MEM reduced serum media. SiRAD54 was added to 490 μL Opti-MEM with 5 μL Lipofectamine RNAiMax to a final concentration of 20 nM and allowed to incubate for 15 minutes at room temperature before being added to the cells.

Transfected cells were seeded into 6-well plates and incubated for 48 hours before being used for experiments.

Western Blot Analysis

To confirm knockdown of RAD54, western blot analyses were used. To collect cells for western blot, first the media was aspirated and cells were washed in DPBS. Cells were then incubated in trypsin for 5 minutes in a 37°C incubator. After trypsin incubation, media was added and cells were centrifuged for 3 minutes at 1250 rpm. The cell pellets were re-suspended in PBS and spun down for 3 minutes at 2000 rpm. Finally, cells were re-suspended in 35-40 μL of 2X sample buffer. When examining RAD54 knockdown 10% gels were prepared. Cell samples were lysed for 15 minutes at 95°C before being loaded in the gel. Seven μL of protein ladder was added in the first lane of the gel and 10 μL of the samples were added in the next lanes. Soluble protein lysates were run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with running buffer in an electrophoresis apparatus and transferred onto Polyvinylidene difluoride (PVDF) membranes with transfer buffer using a wet transfer system. The membranes were activated briefly in methanol prior to the transfer. Once transferred, the membrane was blocked in TBS-T (1X tris-buffered saline, 0.1% Tween-20) containing 5% non-fat dry milk for 20-60 minutes. The membrane was cut under 75 kDa, and under 50 kDa to separate the different sections. The top part of the membrane was incubated overnight at 4°C in RAD54 primary antibody diluted in 5% milk in TBS-T at a dilution of 1:1000. The middle section was incubated in Tubulin antibody diluted in 5% milk in

TBST-T 1:1000 overnight at 4°C and the bottom section of the membrane was incubated in RAD51 primary antibody diluted in 5% milk in TBS-T 1:1000 overnight at 4°C. Following overnight incubation, the membrane was washed 3 times in TBS-T for 5 minutes each at room temperature. Primary antibody was detected using horseradish peroxidase (HRP) conjugated secondary antibody, either rabbit or mouse anti-human IgG diluted in 5% milk in TBS-T 1:8000 and incubated for 1 hour at room temperature. Following this 1-hour incubation, the membranes were washed 3 times for 5 minutes each with TBS-T at room temperature and then visualized using enhanced chemiluminescence reagents from BioRad. A developing solution was prepared using 1mL of Western Lighting Plus ECL oxidizing reagent plus and 1 mL of Western Lighting Plus ECL enhanced luminol reagent plus and dispersed over the membrane prior to imaging.

RAD54 Promotes ALT Activity

C-Circle Assays

To determine if RAD54 had an impact on the quantity of C-rich extra-chromosomal telomeric repeats (C-circles) a C-circle assay was performed. First genomic DNA was purified using QiaAMP DNA mini kit, following manufacturer's instructions. DNA was digested overnight with Alu1 and Mbo1 restriction enzymes. The next day the DNA was purified using Qiagen PCR purification kit. A nanodrop spectrophotometer was used to quantify diluted DNA. DNA was diluted using 80 ng of gDNA in 25 µL of 1X Φ29 buffer (NEB) containing BSA (NEB; 0.08 mg/mL), 0.1%

Tween, 0.25 mM each dATP, dGTP, dTTP. Samples were incubated in the presence (+Φ29) or absence (-Φ29) of 7.5 U Φ29 polymerase (NEB) at 30°C for 8 hours, then at 65°C for 20 minutes.

The amplification products were boiled in 10X SSC and next run through a BioRad vaccum dot blot manifold onto Hybond N+ membrane. The membrane was UV crosslinked for 35 seconds at 125J and then pre-incubated for 1 hour at 50°C in Ultra-Hyb hybridization buffer (Ambion). Roche's DIG oligonucleotide 3'-end labeling kit was used according to manufacturer's directions to label the telomere probe $(CCCTAA)_4$ with digoxigenin. The labeled probe was diluted 1:1000 into hybridization buffer. The membrane was incubated overnight at 50°C. The next day the membrane was washed twice in 2X SSC with 0.1% SDS for 5 minutes each at room temperature and then twice in 0.5X SSC with 0.1% SDS for 15 minutes each at 50°C. To prepare and develop the membrane Roche's DIG Wash and Block Buffer set, Roche's anti-DIG-AP and Roche's CDP-Star were used according to manufacturer's instructions. The membrane was developed using BioRad chemiluminescent imager (Mason-Osann et al., 2020).

RAD54 Rescue Experiments

We received RAD54-WT plasmid as a gift from Dr. Markus Lobrich. This plasmid was then modified to introduce K189R, S49E, and silent siRNA resistance mutations using the InFusion cloning technique. The gene insert was also moved into an pDEST-SFB backbone. HuO9 cells were forward transfected with 2 μg empty vector (EV), RAD54-WT, RAD54-K189R, or S49E construct using Fugene 6 transfection reagent. Then cDNA was mixed with 5 µL Fugene 6 in 150 µL of Opti-MEM media and incubated at room temperature for 20 minutes before being added to cell culture media. Cells were plated 16-24 hours prior to Fugene transfection. After 24 hours of the Fugene transfection, cells were reverse transfected with 20 nM siRAD54#2 using the same protocol described in the RAD54 knockdown experiments.

Click-It EdU Incorporation with combined IF-FISH

HuO9 or SaOS2 cells were grown on coverslips and reverse transfected with 20 nM siRAD54#2. Forty-eight hours after reverse transfection with siRAD54 cells were pulsed with 10 µM EdU in a 6-well plate with DMEM media and left to incubate at 37°C for 1.5 hours. Immediately following the 1.5 hours, the coverslips were stained for combined IF-FISH. First the coverslips were washed twice with PBS for 5 minutes each time. Then the cells were treated with cytobuffer (100 mM NaCl, 300 mM sucrose, 3mM MgCl2, 10 mM PIPES pH 7, 0.1% Triton X-100) for 7 minutes at 4°C. Cells were rinsed with PBS and then fixed with 4% paraformaldehyde for 10 minutes at room temperature. Coverslips were washed with PBS and then permeabilized with 0.5% NP40 in PBS for 10 minutes at room temperature. Cells were washed twice with PBS for 5 minutes each and then labeled using the Click-It reaction. 500 µL of Click-It reaction mixture (100 mM TRIS pH 8.5, 1 mM $CuSO_4$, 10 µM AlexafFluor 488-azide, 100 mM ascorbic acid - added last) were used for each coverslip. The reaction mixture was added directly to the cells and left to stain for 30 minutes at room temperature protected from the light. Coverslips were washed three times with 1X TBS + 0.2% Triton X-100 at room temperature. Then cells were digested with RNase 200 μg/mL in 2X SSC for 30 minutes at 37°C. A series of ethanol washes (70%, 85%, 100%) for 2 minutes each at room temperature were used to dehydrate the cells. The coverslips were then dried at 37°C for 20 minutes. Telomere probe (PNA-$(CCTAA)_4$ Tel-Cy3) diluted 1:750 in hybridization buffer (70% formamide, 0.25% blocking reagent [Roche], 10mM Tris pH 7.5, 4.1 mM Na2HPO4, 1.25 mM MgCl2, 0.45 mM citric acid) was added to glass slides and coverslips were flipped onto it. Slides were denatured at 85°C for 3 minutes and then placed in a humidified chamber at 37°C overnight. Coverslips were washed with 2X SSC and formamide mixed 1:1 three times for 5 minutes each at 37°C, and then three times in just 2X SSC at 37°C. One μg/mL DAPI (4′,6-diamidino-2-phenylindole) 1:1000 was added into 2X SSC for a final 10-minute wash at room temperature. Coverslips were mounted on glass slides using vectashield mounting medium.

Anaphase Bridge Staining

Cells were grown on coverglass to 80-90% confluence, agitation was avoided as much as possible throughout the protocol. Cells were washed gently with PBS. Pre-extraction buffer A (0.2% Triton in 1X PEM buffer [20mM PIPES pH 6.8, 1 mM $MgCl_2$, 10 mM EGTA]) was added to PBS on coverslips, diluting the buffer 1:1 in PBS. Cells were incubated for 60 seconds at room temperature. Pre-extraction buffer B (0.1% triton, 8% PFA in 1X PEM) was added directly into Pre-extraction A buffer/PBS mixture, diluting Pre-extraction buffer B 1:1. Cells were incubated for 15 minutes at room temperature. Pre-extraction buffers were removed from the cells and discarded, cells were washed 3 times for 5 minutes each with PBS. Coverslips were incubated at 4°C overnight in PBSAT (3% BSA, 0.5% triton in 1X PBS).

Coverslips were then incubated in primary antibody diluted in PBSAT in a humidified chamber overnight at 4°C. The following day, coverslips were washed three times for 10 minutes each in PBSAT at room temperature. Next, the cells were incubated in secondary antibody diluted in PBSAT for 2 hours at room temperature. Coverslips were washed three times for 15 minutes each with PBSAT, and then one time for 10 minutes with PBS. Antibodies were fixed with post-staining fixation buffer (4% PFA in 1X PBS) for 5 minutes at room temperature. The coverslips were washed three times for 5 minutes each with PBS. After the washes, the coverslips were incubated with DAPI diluted in PBS for 5 minutes. Cells were washed a final time with PBS and then rinsed with H_2O. The coverslips were mounted with VectaShield mounting media. (Mason-Osann et al. 2020).

Analysis

Statistical Analysis

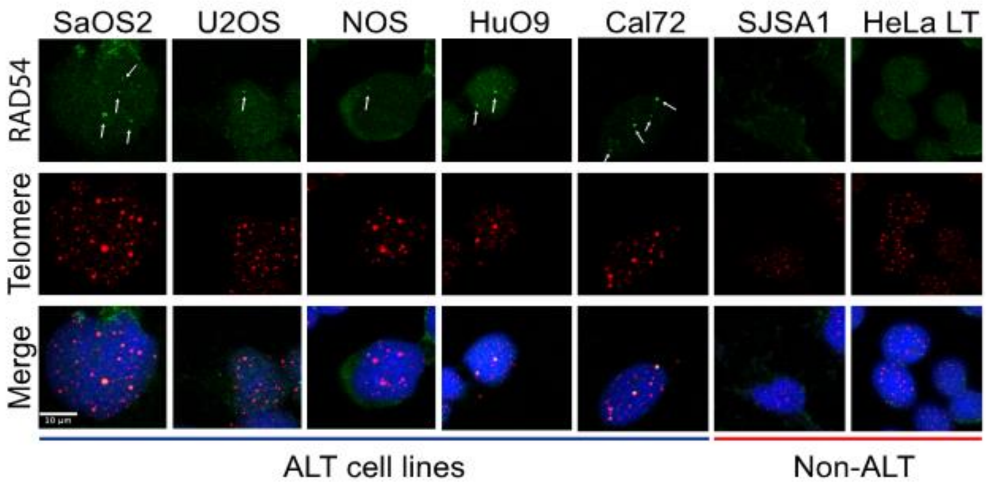
Experiments were performed at least 3 times independently and statistical analysis was done by Dr. Mason-Osann using GraphPad Prism8 software. Exact p-values are given for each test, and p-values were considered significant if they were <0.05.

RESULTS

RAD54 is Enriched at ALT Telomeres

To determine if RAD54 is involved in break induced replication (BIR) at telomeres we needed to confirm that RAD54 is enriched at ALT telomeres. To do this, we performed combined IF-FISH on a series of known ALT cell lines and non-ALT cell lines. Cells were treated with RAD54 antibody, telomere probe, and fluorescent DNA stain. Five of the cell lines were ALT cell lines and two were non-ALT cell lines. Based on evidence that RAD54 is a DNA-dependent ATPase (Tan et al., 2003), we hypothesized that RAD54 is involved in homologous recombination at ALT telomeres. We expected a greater number of colocalization events between RAD54 and telomere foci in ALT cell lines as compared to non-ALT cell lines. Results from the IF-FISH experiments showed that there was a significantly higher number of positive colocalizations between telomere foci and RAD54 foci in ALT cell lines. We counted cells as positive if they contained one or more colocalizations between RAD54 and telomere foci. Although there was variation in the percentage of RAD54 colocalizations among the ALT cell lines, all ALT cell lines had a significantly greater percentage compared to the two non-ALT cell lines.

A.



B.

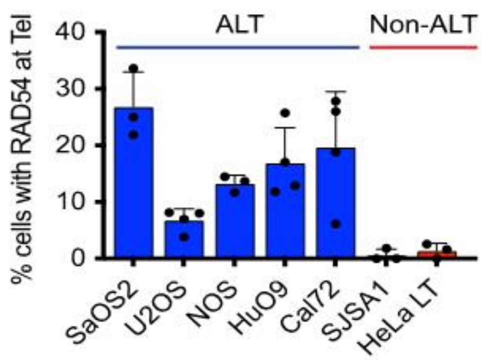
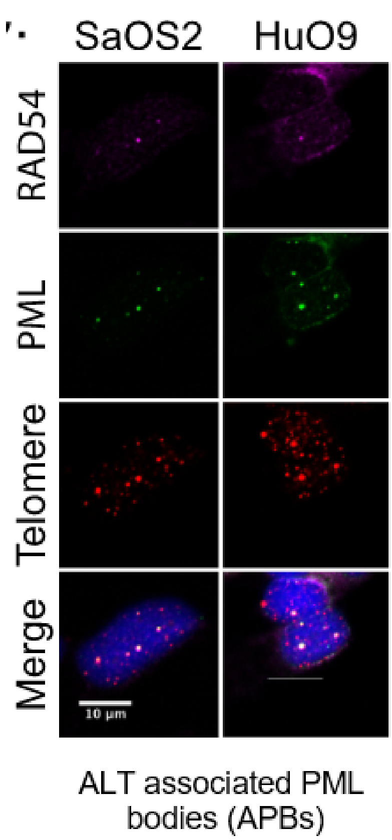


Figure 3. A. Combined IF and DNA FISH analysis of RAD54 (IF) and telomeres (FISH) in ALT and non-ALT cell lines. White arrows indicate RAD54 foci that colocalize with telomeres. Scale bars = 10 µm. **B**. Quantification of data in A. A cell was counted positive if it contained 1 or more colocalization events between RAD54 and telomere. At least 100 cells were counted per cell line per repeat. For SaOS2, NOS, SJSA1, HeLa LT n = 3. For Cal72, U2OS, HuO9 n = 4. Values shown are n±sd.

RAD54 is Found Within APBs

To further conclude that RAD54 contributes to ALT function, we performed combined IF-FISH for PML. APBs are thought to be sites of active telomere recombination (Yeagar et al., 1999). PML is a scaffold protein and therefore this is where telomere elongation is thought to occur. Cells were incubated overnight with RAD54 and then the following day incubated with PML and the rest of the IF-FISH protocol was followed as normal. Analysis of these SaOS2 and HuO9 cell lines showed that the majority of RAD54 foci localized to APBs. As can be seen in the merged image (Fig. 4), we found that PML foci, RAD54 foci, and telomere foci all colocalize greater than 75% of the time.



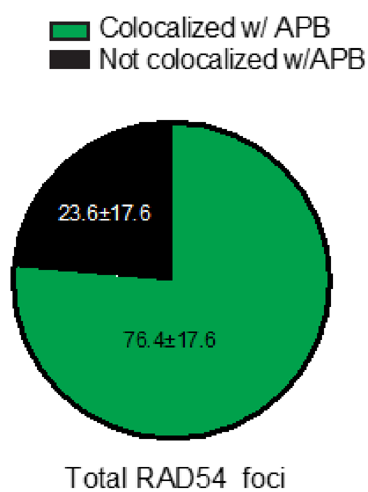


Figure 4. RAD54 localized to ALT associated PML bodies (APBs), which are thought to be sites of active telomere recombination. Combined IF and DNA FISH analysis of RAD54 (IF), PML (IF) and telomeres (FISH) in ALT cell lines SaOS2 and HuO9.

Successful Depletion via siRNA Reverse Transfection

When siRNA was used to deplete RAD54 or RAD51 we confirmed successful knockdown via western blot analysis. Tubulin was used as a loading control. Cases in which the control bands showed even loading, the presence of a band at the expected location, and lack of a band in the siRNA column confirmed knockdown. As can be seen in Figure 5, RAD51 was successfully knocked down. There are even bands for RAD54 and tubulin, but a much fainter band for RAD51 in the second column which contains an siRAD51 sample as compared to the mock sample in the first lane. Our results also showed successful knockdown of RAD54 when that was the target. It was important to ensure successful knockdowns so that the results of other experiments could be interpreted in the correct context.

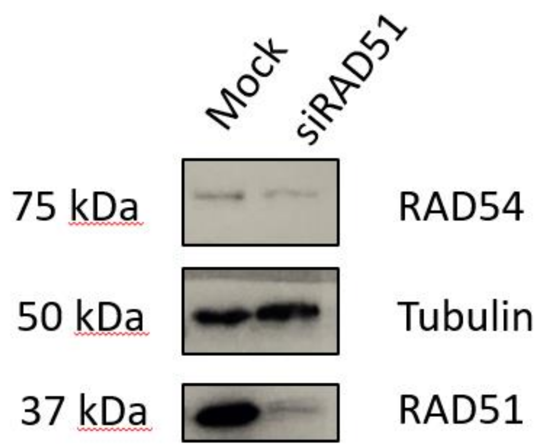


Figure 5. Western blot analysis of HuO9 mock and siRAD51. The top section was incubated with RAD54 primary antibody and mouse HRP, the middle section was the control for tubulin and rabbit HRP, the bottom section was incubated with RAD51 primary antibody and detected with rabbit HRP secondary antibody. The mock sample was loaded in the first lane next to the ladder and the HuO9 siRAD51 sample was loaded in the second lane. A significant decrease in the band for RAD51 can be seen under the knockdown conditions.

RAD54 Promotes ALT Activity

To determine whether RAD54 was contributing to ALT activity, we performed a C-circle assay and another series of IF-FISH experiments, this time to detect EdU incorporation. C-rich extrachromosomal telomeric repeats (C-circles) are one of the unique markers of ALT positive human cancer cells. A C-circle assay produces long telomeric ssDNA molecules through a process in which a Φ29 DNA polymerase is auto-primed by the partial G—strand and there is there is rolling circle amplification of partially double-stranded C-circles. We saw a significant decrease in signal in the DNA dot blot when RAD54 was knocked down in Hu09 cells in the C-circle assay. The two conditions in Figure 6B, +Φ29 and –Φ29, represent being in the presence (+Φ29) or absence (–Φ29) of the polymerase, the latter being the negative control in which, as was expected, we do not see DNA present in the dot blot. The dot blot was quantified with densitometry, normalized to the mock condition. As can be seen in Figure 6C, under RAD54 knockdown conditions there is a significant decrease in the amount of C-circles in the absence of RAD54.

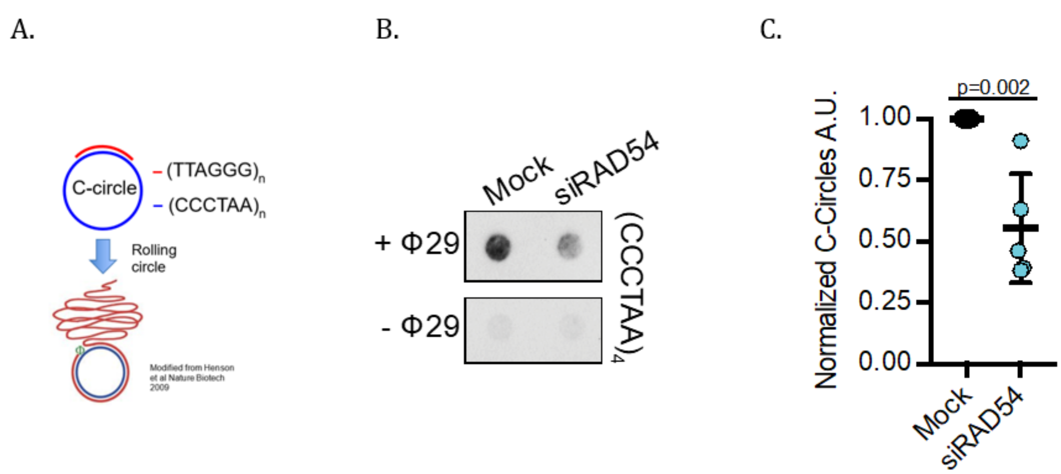


Figure 6. A. This image is a depiction of C-circle rolling amplification. Modified from (Hensen et al., 2009). **B.** Representative DNA dot blot from C-circle assay on HuO9 cells, either mock transfected or transfected with 20nM siRAD54for 48 hours. **C.** Quantification of C-circle assay in B. Signal was quantified with densitometry, background (- Φ29) was subtracted and signal was normalized to mock. N=5, values shown are mean±sd. Conditions were analyzed using unpaired two-tailed student's t-test.

EdU is a synthetic nucleotide that we pulsed into dividing cells and left to incubate for 1.5 hours. We quantified EdU incorporation at telomeres in control cells and cells transfected with siRNA for RAD54 by IF-FISH. Cells were counted as positive for EdU incorporation when they contained at least one EdU foci localizing with telomeres. Cells that stained everywhere for EdU were discounted, as they were considered to be S-phase cells, and thus undergoing complete DNA synthesis. Our data demonstrated that when RAD54 was knocked down there was a significant decrease in the percentage of cells with EdU incorporation at telomeric DNA. Both SaOS2 and HuO9 ALT cell lines showed decreased EdU incorporation when RAD54 was depleted, suggesting that RAD54 promotes DNA synthesis at ALT telomeres.

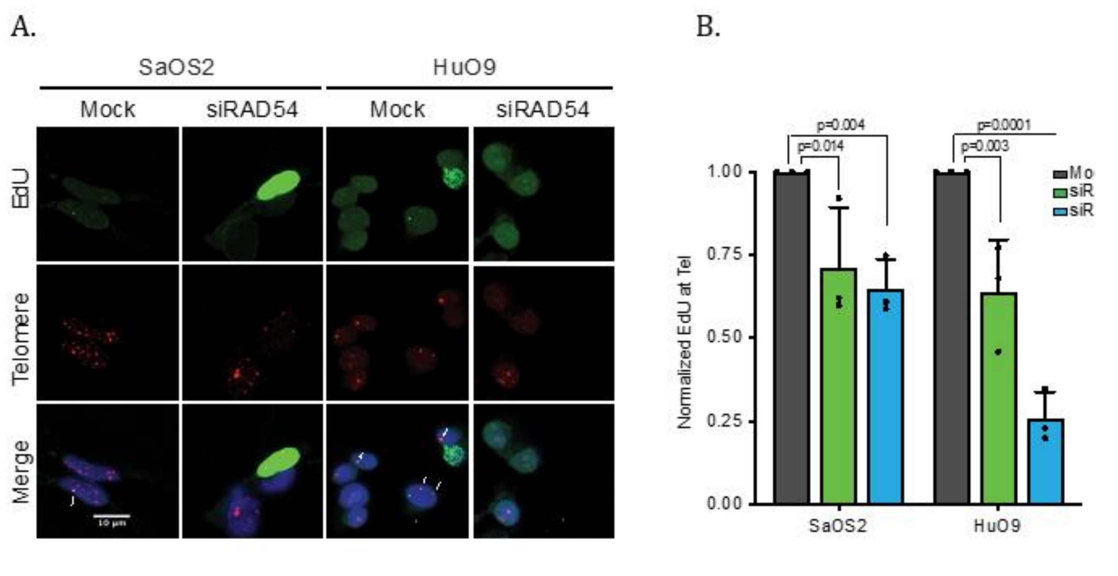


Figure 7: A. Representative images of combined EdU staining (Click-it) and DNA FISH for telomeres. HuO9 or SaOS2 cells were transfected with 20nM siRAD54#1 for 48 hours followed by a 1.5 hr pulse of EdU. Pan-nuclear EdU stain represents S-phase cells. White arrows indicate non-S-phase cells containing EdU colocalizing with telomeres. **B.** Quantification of data shown in A. Non-S-phase cells were considered positive if they contained at least one EdU foci colocalizing with telomeres.

Branch Migration Activity of RAD54

To determine what role RAD54 plays in ALT activity we performed a series of rescue experiments with both WT and mutant RAD54 vectors. We performed IF-FISH on HuO9 cells that had been forward transfected with an empty vector, RAD54-WT, RAD54-K189R, or RAD54-S49E that contained silent mutations so that they would be resistant to siRNA. After 24 hours we transfected the cells with siRAD54 to knock down endogenous RAD54 and then 48 hours later pulsed them with EdU for 1.5 hours. When RAD54 was knocked down in the empty vector cells, we observed the expected decrease in EdU incorporation. When RAD54 was knocked down in the cells with RAD54-WT vector, we saw that there was a full rescue in RAD54 function in relation to EdU incorporation, as there was no significant change between the mock and the siRAD54. This indicated that knocking down RAD54 via siRNA reverse transfection was not disturbing other functions because it could be rescued with the wild type vector.

The RAD54-K189R mutation causes impairment of the ATPase function of RAD54 (Agarwal et al., 2011). When endogenous RAD54 was knocked down in the RAD54-K189R mutant we saw a significant decrease in EdU incorporation, suggesting that the ATPase activity of RAD54 is critical for DNA synthesis at telomeres. The RAD54-S49E is a phosphomimetic mutant. Phosphorylation at this location inhibits oligomerization and subsequent branch migration (Goyal et al., 2018). In the RAD54-S49E cells we also observed a significant decrease in EdU incorporation when endogenous RAD54 was knocked down as compared to the control. These data suggest that the oligomerization activity of RAD54 is necessary for ALT activity at telomeres.

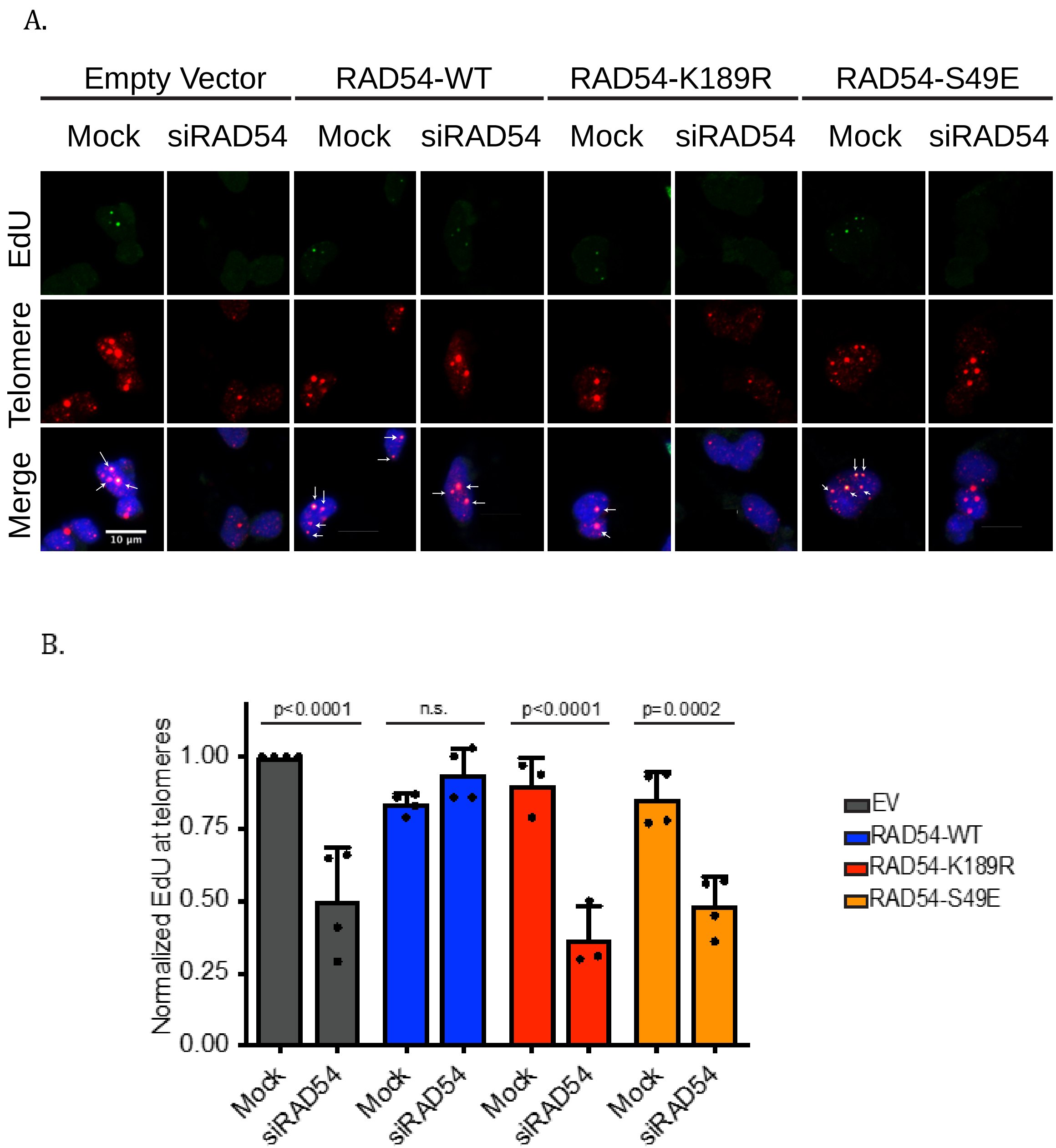
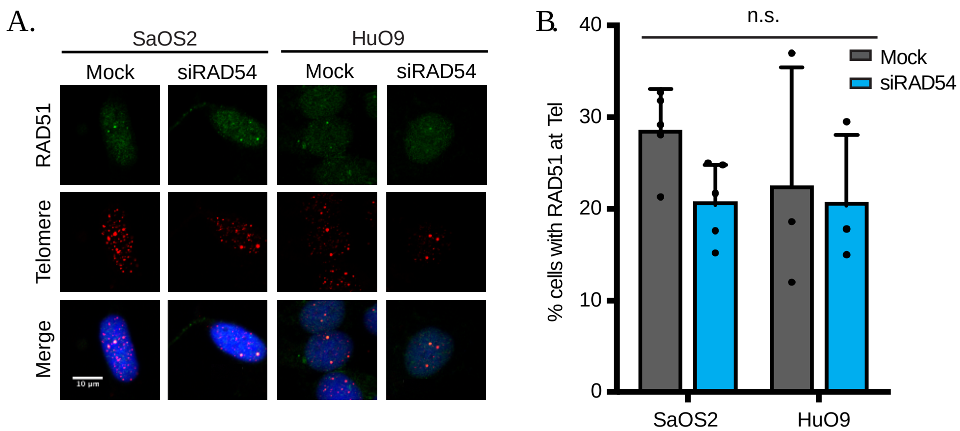


Figure 8: A. Combined EdU and telomere FISH. HuO9 cells were forward transfected with 2µg empty vector (EV), RAD54-WT, RAD54-K189R, or S49E construct. After 24 hours, cells were transfected with 20 nM siRAD54#2 for 48 hours followed by a 1.5 hr EdU pulse. White arrows show colocalization between the telomeres and EdU.

B. Quantification of data shown in A. Data were normalized to EV-Mock condition for each repeat. At least 100 non-S-phase cells were counted per condition per repeat, n=4 for EV, WT, S49E, n=3 for K189R. Values shown are mean±sd. Data were analyzed using two-way ANOVA followed by Sidak's multiple comparison test.

RAD54 is Not Playing a Role in RAD51 Mediated Synapsis

RAD51 is another gene in the RAD52 epistasis group and is involved in homologous recombination (Symington, 2002). BIR at ALT telomeres can be RAD51-dependent or RAD51-independent, as such, RAD51 is not essential for BIR at ALT telomeres (Min et al., 2017). We performed a series of experiments to determine whether or not RAD54 was playing a role in RAD51 mediated synapsis. This was done by knocking down RAD54 and doing IF-FISH for RAD51, and conversely knocking down RAD51 and doing IF-FISH for RAD54. In both of these instances, we saw no significant change in colocalization of foci with telomeres between the control cells and the transfected cells. These results support that RAD51 does not recruit RAD54 to telomeres, nor does RAD54 recruit RAD51. In HuO9 cells reverse transfected with siRAD54 and stained for RAD51, both the mock cells and the transfected cells had around 20% of total cells counted with RAD51 at telomeres. In HuO9 cells that were reverse transfected with siRAD51 and stained for RAD54 there was a slightly higher percentage (~30%) of cells with RAD54 at telomeres, but again we saw no significant difference between the control cells and the transfected cells.



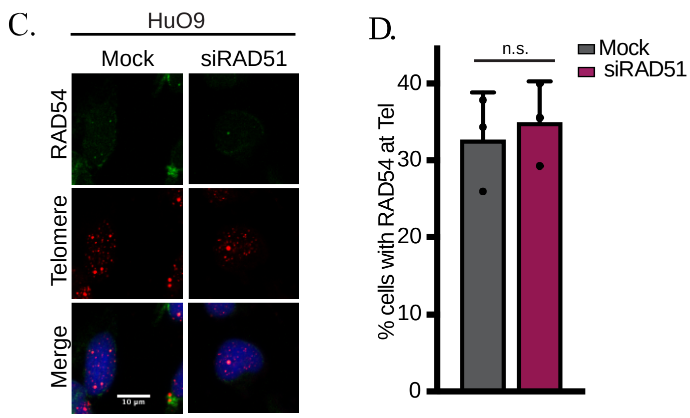


Figure 9. A. Combined IF and DNA FISH on SaOS2 and HuO9 cells stained for RAD51. Mock conditions or cells were reverse transfected with 20nM siRAD54 for 48 hours prior to staining. Scale bar = 10 µm. **B.** Quantification of data in A. At least 100 cells were counted per condition. Cells were counted as positive if they contained at least one colocalization between telomeres and RAD51. **C.** Combined IF and DNA FISH on SaOS2 and HuO9 cells stained for RAD54. Mock conditions or cells were reverse transfected with 20nM siRAD51 for 48 hours prior to staining. Scale bar = 10 µm. **D.** Quantification of data in C. At least 100 cells were counted per condition. Cells were counted as positive if they contained at least one colocalization between telomeres and RAD54.

RAD54 Regulates post-Synaptic Processing of Recombination Intermediates

RAD54 is believed to be involved in resolution of recombination intermediates via homologous recombination and the BTR (BLM, TOP3A, RMI1/2) complex. An alternative way for these recombination intermediates to be resolved is via the SMX (SLX1-4, MUS81-EME1, XPF-ERCC1) complex, which includes the nuclease MUS81.

To understand the role of RAD54 in resolution of these intermediates we examined what happened when RAD54 was depleted. HuO9 cells were transfected with siRAD54 for 48 hours and then stained for MUS81 and telomeres. Around 38% of the transfected cells had MUS81 at telomeres whereas the mock cells had MUS81 at telomeres only around 14% of the time. These results show a significant increase in recruitment of MUS81 to telomeres in the absence of RAD54, suggesting that MUS81 compensates for loss of RAD54 in resolving recombination intermediates.

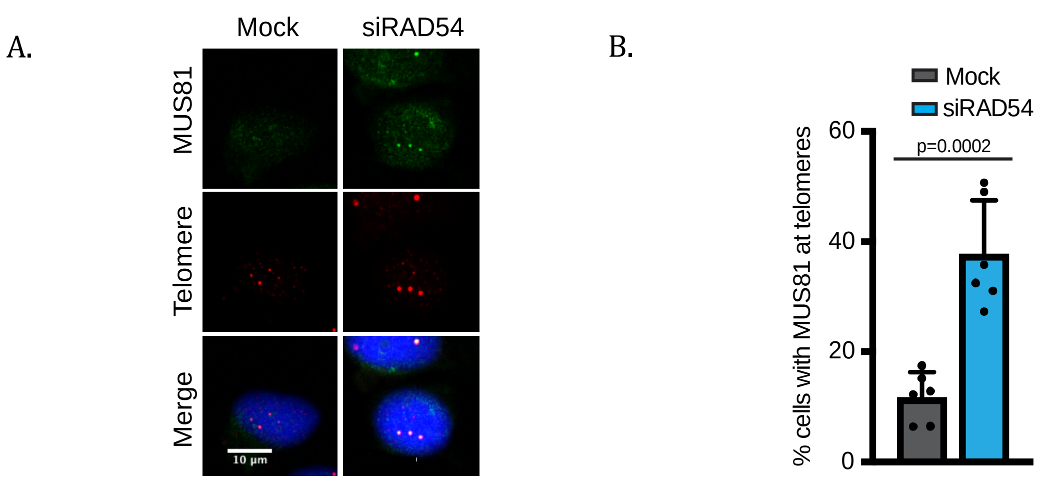


Figure 10. A. Image showing example of colocalization between MUS81 and telomeres for HuO9 cells using combined IF and DNA FISH. Cells were treated with 20 nM siRAD54 for 48 hours prior to staining. Scale bars = 10 μm. **B.** Quantification of data in A. Cells were counted as positive if they contained at least 1 colocalization event between MUS81 and telomeres.

SLX4 is another component of the SMX complex. SLX4 is associated with MUS81 and its major function is to resolve recombination intermediates via cleavage (Verma et al., 2019). We hypothesized that by inhibiting both the BTR and SMX complex there would be an increase in unresolved recombination intermediates. These unresolved recombination intermediates could be visualized as ultrafine anaphase bridges in mitosis. We performed a series of experiments to visualize anaphase bridges in HuO9 cells that were stained for shelterin protein, TRF2. Mock HuO9 cells and HuO9 cells transfected with siRAD54 for 72 hours both had around 20% of total cells with visualized anaphase bridges. When HuO9 cells were cotransfected with both siRAD54 and siSLX4 for 72 hours, 45% of cells had visualized anaphase bridges. This is a significant increase from the 20% of anaphase bridges observed in the mock cells or the cells transfected with siRNA for RAD54 alone.

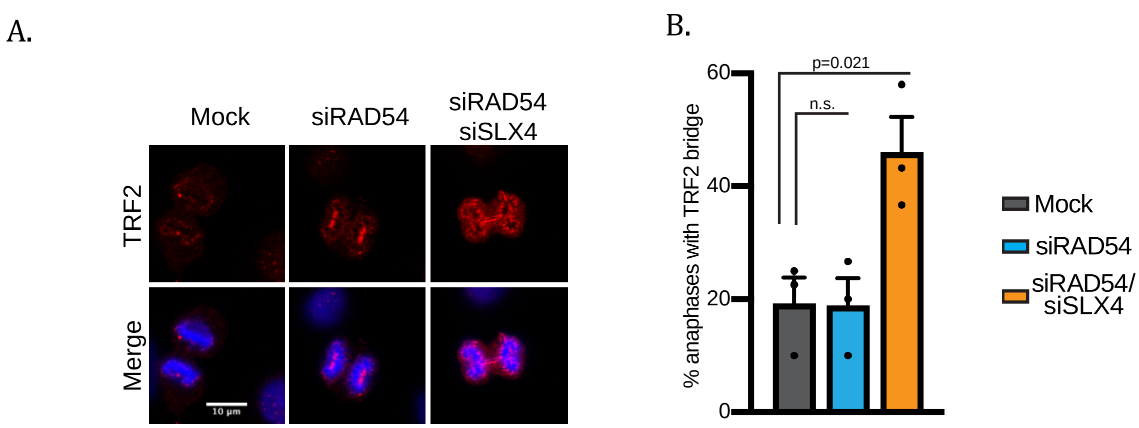


Figure 11. A. Anaphase bridge staining for TRF2 in HuO9 cells. Cells were transfected with 20nM siRAD54#2 alone or cotransfected with 20 nM siSLX4 for 72 hours. **B**. Quantification of data shown in A. At least 90 Anaphases were scored from 3 independent experiments. Values are mean±sd.

DISCUSSION

RAD54 has been shown to be relevant to the ALT pathway for telomere elongation in murine cells, however few studies have evaluated RAD54's role in human cancer cells. RAD54 is a central player in HR, and RAD54 deficiency in mice leads to significantly shorter telomeres (Jaco et al., 2003). The purpose of this analysis was to define the role that RAD54 plays in promoting the ALT mechanism in human cancer cells, and to determine if it is involved in regulating BIR.

RAD54 is Enriched at ALT Telomeres

To determine if RAD54 functions to promote BIR telomere elongation at ALT telomeres, we first determined if RAD54 is associated with telomeres. Based on the results of this study it is clear that RAD54 is located at ALT telomeres. The results from the combined IF and DNA FISH experiments on a panel of ALT cell lines and non-ALT cells demonstrate that RAD54 is enriched at telomeric DNA in ALT positive cells. We were also able to discount the possibility that RAD54 was at ALT telomeres just as a consequence of extended telomere length in these cells. This was confirmed due to RAD54 not being enriched in the HeLa-LT cell line, which has long telomeres and does not use the ALT mechanism. This panel of cells demonstrates that RAD54 is enriched at the ALT telomeres, but does not yet give any evidence that RAD54 is aiding in the ALT process.

RAD54 Localizes With APBs

Some of the well-known markers of ALT are heterogeneous telomere length, high levels of telomere-sister chromatid exchanges (T-SCE), extrachromosomal telomeric DNA repeats (ECTRs), and a specialized telomeric nuclear structure called ALT-associated PML (promyelocytic leukemia protein) bodies (APBs) (De Vitis et al., 2018). APBs contain PML protein, telomeric DNA, hTRF1, hTRF2, replication factor A (RPA), RAD51, RAD52 and appear at a similar time as activation of ALT (De Vitis et al., 2018). These factors suggest that APBs are the site of activity for the ALT mechanism. Our results show that RAD54 localizes with APBs the majority of the time, which indicates that RAD54 is located at the site of active telomere elongation, and is contributing to the ALT mechanism.

RAD54 Promotes ALT Activity

C-Circles arise as a byproduct of the recombination interaction at ALT telomeres and correlate with ALT activity (Henson et al., 2009). ALT involves recombination-dependent DNA replication that elongates telomeres via a sudden and large increase in telomere length. This type of lengthening is indicative of a method involving a long, linear telomeric template or a rolling mechanism, as in C-circles (Henson et al., 2009). C-circles are a hallmark of ALT activity, so we wanted to determine whether a decrease in RAD54 would lead to a decrease in C-circles. A C-circle is a partially single-stranded telomeric $(CCCTAA)_n$ DNA repeat, of which part of the single-stranded DNA may be circular. A C-circle assay detects extrachromasomal C-circles in the cell. In the C-circle assay performed we used HuO9 cells mock transfected and transfected with siRNA to deplete RAD54. We ran the assay in the presence of the Φ29 polymerase and in its absence for a negative control. The dot blot was quantified with densitometry and showed a significant decrease in signal in cells depleted for RAD54. These results indicate that without RAD54 there is a decrease in C-circles, and thus a decrease in ALT activity.

RAD54 Promotes DNA Synthesis

The synthetic nucleotide EdU (5-ethynyl-2'-deoxyuridine) is a thymidine analog we can detect with click-it chemistry when incorporated into the cell. This allows us to monitor newly synthesized DNA during mitosis, or in S and G2/M phase, whenever a cell is undergoing replication-dependent recombination (Min et al., 2019). By quantifying the number of EdU-positive telomeres in cells under normal conditions and cells that had been transfected with siRNA to deplete RAD54, we were able to determine if RAD54 was playing a role in promoting DNA synthesis. We discounted cells that had pan-nuclear EdU staining as these cells were believed to be S-phase cells and we only wanted to see if RAD54 was promoting DNA synthesis at telomere foci. The reduced number of cells with positive EdU incorporation when RAD54 was depleted indicates that RAD54 is likely involved in DNA synthesis at ALT telomeres, and is thus promoting ALT activity.

RAD54 Branch Migration and ATPase Activity are Necessary for ALT

Break induced replication (BIR) is initiated by RAD51-mediated strand invasion to form a displacement loop (D-loop), followed by replication fork assembly and DNA synthesis (Malkova and Ira, 2013). Branch migration of the mature D-loop carries out BIR by extending the lagging strand and then the telomeric C-strand is filled in later. RAD54-S49E is a phosphomimetic mutant which is phosphorylated at the Serine-49 CDK2 consensus site (Goyal et al., 2018). This site regulates oligmerization and is inhibited when phosphorylated. Inhibition of oligmerization thus inhibits branch migration. We saw a reduction in EdU incorporation in cells with this mutation introduced and that had been transfected to deplete RAD54. These results suggest that the branch migration activity of RAD54 is critical for DNA synthesis at ALT telomeres. This is because the RAD54-S49E mutant was unable to rescue the EdU incorporation defect caused by RAD54 depletion.

RAD54 is an ATP-dependent DNA translocase. Branch migration is dependent on ATP hydrolysis. The RAD54-K189R mutant is a mutant construct in which the lysine at position 189 was replaced with arginine. This mutation depletes the ATPase activity of RAD54 greater than 100 fold as compared to wild-type (Argawal et al., 2011). We depleted RAD54 in HuO9 cells to cause the known defect in EdU incorporation in the absence of RAD54, and then sought to conclude whether replacing this depletion with RAD54-K189R mutant could rescue the DNA synthesis. There was a significant reduction in EdU incorporation under these conditions, indicating that without the ATPase activity, ALT activity is hindered.

RAD54 is Not Playing a Role in RAD51 Mediated Synapsis

RAD51 is a recombinase protein that forms a RAD51-ssDNA nucleofilament, is involved in the strand-invasion step of homologous recombination, and is known to interact with RAD54 (Claussin and Chang, 2015). In human cancers ALT has been shown to proceed via two different mechanisms, one of which is RAD51-dependent and one which is RAD51-independent (Min et al., 2017). Due to RAD54 interacting with RAD51 during the pre-synapsis phase of homologous recombination, we examined if RAD54 was interacting with RAD51 in the next phases. To determine whether or not RAD54 was playing a role in RAD51 mediated synapsis, we performed a series of experiments in which we depleted either RAD54 or RAD51 via siRNA reverse transfection. After siRNA knockdown we performed IF-FISH on the cells staining for the opposite protein from the one that was knocked down. We hypothesized that if one was playing a role in recruiting the other then there would be a reduction in colocalization of foci at telomeres in the knockdown cases. Because we saw no change in the percentage of cells with colocalization events between the mock and knockdown instances, we concluded that RAD54 is not required for RAD51 nucleofilament formation at ALT telomeres, and perhaps there are factors that compensate, such as RAD52.

Proposed Model of RAD54 Activity at ALT Telomeres

The ALT mechanism is thought to work via homologous recombination (Nabetani and Ishikawa, 2011). Homologous recombination corrects errors during DNA replication and restores damaged DNA using intact and homologous DNA as a template (Tan et al., 2003). RAD54 is part of the homologous recombination machinery and thought to be necessary for telomere length maintenance (Jaco et al., 2003). We hypothesize that the role of RAD54 in homologous recombination is to promote telomere synthesis via branch migration during BIR.

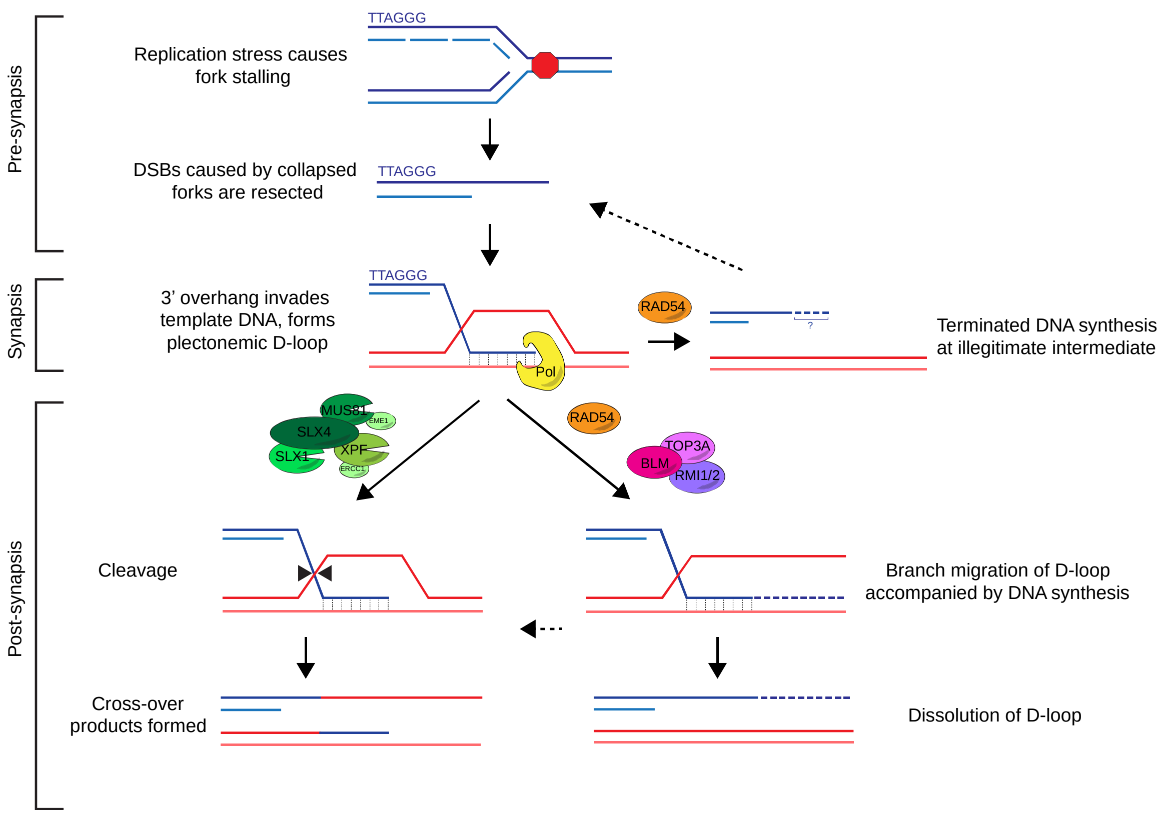


Figure 12. Model of Homologous Recombination. In this model three different stages of homologous recombination are shown: pre-synapsis, synapsis, and post-synapsis. The proposed function of RAD54 in homologous recombination is within the synapsis stage in regulating the processing of D-loop formation and promoting DNA synthesis at ALT telomeres via BIR. RAD54 is also proposed to have a role in resolution of recombination intermediates

Included in our proposed model of homologous recombination are three steps: 1) pre-synapsis during which damage is incurred and a homology search is initiated, 2) synapsis which includes strand-invasion and formation of the mature D-loop, and 3) post-synapsis where there is resolution of the joint recombination intermediates. We suggest that RAD54 has a more significant role in post-synapsis. Specifically, we hypothesize that RAD54 promotes DNA synthesis at ALT telomeres via BIR to repair the single-ended double strand break.

The model of homologous recombination (Fig. 12) depicts two options for post-synaptic resolution of recombination intermediates. The option on the left shows cleavage via the SMX complex (SLX1-4, MUS81-EME1, XPF-ERCC1) and the formation of cross-over products. Cleavage does not lead to telomere extension. The pathway on the right shows resolution through branch migration via the dissolvasome complex BTR (BLM, TOP3α, RMI1, RMI2). DNA synthesis followed by dissolution generates true telomere extension events. RAD54 is hypothesized to work in conjunction with the BTR complex and promote BIR extension and dissolution via branch migration.

RAD54 Regulation of Post-Synaptic Events

We hypothesized that when RAD54 was depleted, the resolution of recombination intermediates would become more dependent on the SMX complex. To test this hypothesis, we performed IF-FISH on HuO9 cells in which RAD54 had been depleted. We observed that when RAD54 was depleted there was a greater percentage of cells with MUS81 localization at ALT telomeres. The increase in recruitment of MUS81 to ALT telomeres suggests that MUS81 is recruited to sites of unresolved recombination intermediates to resolve these structures in the absence of RAD54. Under these conditions, recombination intermediates seem to be resolved via cleavage rather than branch migration.

After observing that when one of the recombination intermediate resolution pathways is inhibited, the other compensates, we looked at the result when both proposed pathways are inhibited. We anticipated that by inhibiting both the cleavage pathway and the branch migration pathway there would be an increase in unresolved recombination intermediates. These unresolved intermediates can be visualized as ultra-fine anaphase bridges. The combined depletion of RAD54 and the SMX complex enzyme SLX4 led to a big increase in the quantity of visualized anaphase bridges. This result suggests that with the inhibition of the SMX complex via SLX4 depletion, and inhibition of branch migration by RAD54 depletion, it is more difficult for recombination intermediates to be resolved. Thus, we concluded that together with the SMX and BTR complexes, RAD54 contributes to the regulation of post-synaptic processing of recombination intermediates.

RAD54 is clearly involved in ALT telomere maintenance. While initially RAD54 seemed like a good target for inhibiting the ALT mechanism due to its involvement in ALT and non-essentiality, knocking down RAD54 alone ultimately did not lead to ALT cell death. ALT cells likely have other mechanisms to compensate for loss of RAD54 and maintain the ALT pathway, making RAD54 alone a poor target for inhibiting ALT telomere elongation, but perhaps combined inhibition of RAD54 and something else could be successful.

LIST OF JOURNAL ABBREVIATIONS

Am J Pathol	The American Journal of Pathology
AJPATH	The American Journal of Pathology
BBRC	Biochemical and Biophysical Research Communications
Cancer Res	Cancer Research
Curr Opin Genet Dev	Current Opinion in Genetics and Development
Genes Dev	Genes & Development
Genome Med	Genome Medicine
Int J Mol Sci	International Journal of Molecular Science
J Biochem	The Journal of Biochemistry
J Cell Biol	Journal of Cell Biology
Microb Cell	Microbial Cell
Mol Cell Biol	Molecular and Cellular Biology
MMBR	Microbiology and Molecular Biology Reviews
Nat Cell Biol	Nature Cell Biology
Nat Commun	Nature Communications
NRG	National Reviews Genetics
Sci Rep	Scientific Reports
Trends Biochem Sci	Trends in Biochemical Sciences

REFERENCES

Agarwal S, van Cappellen WA, Guénolé A, et al. ATP-dependent and independent functions of Rad54 in genome maintenance. J Cell Biol. 2011;192(5):735-750. doi:10.1083/jcb.201011025

Bass TE, Luzwick JW, Kavanaugh G, et al. ETAA1 acts at stalled replication forks to maintain genome integrity. Nat Cell Biol. 2016;18(11):1185-1195. doi:10.1038/ncb3415

Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nature Reviews Genetics. 2010;11(5):319-330. doi:10.1038/nrg2763

Cesare AJ, Kaul Z, Cohen SB, et al. Spontaneous occurrence of telomeric DNA damage response in the absence of chromosome fusions. Nature Structural & Molecular Biology. Published November 22, 2009. Accessed March 13, 2020.

Claussin C, Chang M. The many facets of homologous recombination at telomeres. Microb Cell. 2(9):308-321. doi:10.15698/mic2015.09.224

Clynes D, Jelinska C, Xella B, et al. Suppression of the alternative lengthening of telomere pathway by the chromatin remodelling factor ATRX. Nature Communications. 2015;6(1):1-11. doi:10.1038/ncomms8538

Dagg RA, Pickett HA, Neumann AA, et al. Extensive Proliferation of Human Cancer Cells with Ever-Shorter Telomeres. Cell Reports. 2017;19(12):2544-2556. doi:10.1016/j.celrep.2017.05.087

De Vitis M, Berardinelli F, Sgura A. Telomere Length Maintenance in Cancer: At the Crossroad between Telomerase and Alternative Lengthening of Telomeres (ALT). Int J Mol Sci. 2018;19(2). doi:10.3390/ijms19020606

Dilley RL, Verma P, Cho NW, Winters HD, Wondisford AR, Greenberg RA. Break-induced telomere synthesis underlies alternative telomere maintenance. Nature. 2016;539(7627):54-58. doi:10.1038/nature20099

Episkopou H, Diman A, Claude E, Viceconte N, Decottignies A. TSPYL5 Depletion Induces Specific Death of ALT Cells through USP7-Dependent Proteasomal Degradation of POT1. Molecular Cell. July 2019. doi:10.1016/j.molcel.2019.05.027

Fasching CL, Neumann AA, Muntoni A, Yeager TR, Reddel RR. DNA Damage Induces Alternative Lengthening of Telomeres (ALT)–Associated Promyelocytic

Leukemia Bodies that Preferentially Associate with Linear Telomeric DNA. Cancer Res. 2007;67(15):7072-7077. doi:10.1158/0008-5472.CAN-07-1556

Flynn RL, Cox KE, Jeitany M, et al. Alternative lengthening of telomeres renders cancer cells hypersensitive to ATR inhibitors. Science. 2015;347(6219):273-277. doi:10.1126/science.1257216

Gocha ARS, Nuovo G, Iwenofu OH, Groden J. Human sarcomas are mosaic for telomerase-dependent and telomerase-independent telomere maintenance mechanisms: implications for telomere-based therapies. Am J Pathol. 2013;182(1):41-48. doi:10.1016/j.ajpath.2012.10.001

Goyal N, Rossi MJ, Mazina OM, et al. RAD54 N-terminal domain is a DNA sensor that couples ATP hydrolysis with branch migration of Holliday junctions. Nature Communications. 2018;9(1):1-10. doi:10.1038/s41467-017-02497-x

Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013

Heaphy CM, de Wilde RF, Jiao Y, et al. Altered Telomeres in Tumors with ATRX and DAXX Mutations. Science. 2011;333(6041):425. doi:10.1126/science.1207313

Heaphy CM, Subhawong AP, Hong S-M, et al. Prevalence of the alternative lengthening of telomeres telomere maintenance mechanism in human cancer subtypes. Am J Pathol. 2011;179(4):1608-1615. doi:10.1016/j.ajpath.2011.06.018

Henson JD, Cao Y, Huschtscha LI, et al. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. Nature Biotechnology. 2009;27(12):1181-1185. doi:10.1038/nbt.1587

Ivanov EL, Haber JE. DNA repair: RAD alert. Current Biology. 1997;7(8):R492-R495. doi:10.1016/S0960-9822(06)00246-6

Jaco I, Muñoz P, Goytisolo F, et al. Role of Mammalian Rad54 in Telomere Length Maintenance. Mol Cell Biol. 2003;23(16):5572-5580. doi:10.1128/MCB.23.16.5572-5580.2003

Jafri MA, Ansari SA, Alqahtani MH, Shay JW. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. Genome Med. 2016;8. doi:10.1186/s13073-016-0324-x

Lawlor RT, Veronese N, Pea A, et al. Alternative lengthening of telomeres (ALT) influences survival in soft tissue sarcomas: a systematic review with meta-analysis. BMC Cancer. 2019;19(1):232. doi:10.1186/s12885-019-5424-8

Liu H, Xie Y, Zhang Z, et al. Telomeric Recombination Induced by DNA Damage Results in Telomere Extension and Length Heterogeneity. Neoplasia. 2018;20(9):905-916. doi:10.1016/j.neo.2018.07.004

Lovejoy CA, Li W, Reisenweber S, et al. Loss of ATRX, Genome Instability, and an Altered DNA Damage Response Are Hallmarks of the Alternative Lengthening of Telomeres Pathway. PLOS Genetics. 2012;8(7):e1002772. doi:10.1371/journal.pgen.1002772

Malkova A, Ira G. Break-induced replication: functions and molecular mechanism. Curr Opin Genet Dev. 2013;23(3):271-279. doi:10.1016/j.gde.2013.05.007

Min J, Wright WE, Shay JW. Alternative lengthening of telomeres can be maintained by preferential elongation of lagging strands. Nucleic Acids Res. 2017;45(5):2615-2628. doi:10.1093/nar/gkw1295

Min J, Wright WE, Shay JW. Alternative Lengthening of Telomeres Mediated by Mitotic DNA Synthesis Engages Break-Induced Replication Processes. Molecular and Cellular Biology. 2017;37(20). doi:10.1128/MCB.00226-17

Mason-Osann E, Terranova K, Lupo N, Lock YJ, Carson LM, Flynn RL. RAD54 promotes Alternative Lengthening of Telomeres by mediating branch migration. Embo Reports. 2020

Min J, Wright WE, Shay JW. Clustered telomeres in phase-separated nuclear condensates engage mitotic DNA synthesis through BLM and RAD52. Genes Dev. 2019;33(13-14):814-827. doi:10.1101/gad.324905.119

Nabetani A, Ishikawa F. Alternative lengthening of telomeres pathway: recombination-mediated telomere maintenance mechanism in human cells. J Biochem. 2011;149(1):5-14. doi:10.1093/jb/mvq119

Raoul Tan TL, Kanaar R, Wyman C. Rad54, a Jack of all trades in homologous recombination. DNA Repair. 2003;2(7):787-794. doi:10.1016/S1568-7864(03)00070-3

Sykes DB, Kfoury YS, Mercier FE, et al. Inhibition of Dihydroorotate Dehydrogenase Overcomes Differentiation Blockade in Acute Myeloid Leukemia. Cell. 2016;167(1):171-186.e15. doi:10.1016/j.cell.2016.08.057

Symington LS. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microbiol Mol Biol Rev. 2002;66(4):630-670, table of contents. doi:10.1128/mmbr.66.4.630-670.2002

Tavares EM, Wright WD, Heyer W-D, Cam EL, Dupaigne P. In vitro role of Rad54 in Rad51-ssDNA filament-dependent homology search and synaptic complexes formation. Nat Commun. 2019;10(1):1-12. doi:10.1038/s41467-019-12082-z

Verma P, Dilley RL, Zhang T, Gyparaki MT, Li Y, Greenberg RA. RAD52 and SLX4 act nonepistatically to ensure telomere stability during alternative telomere lengthening. Genes Dev. 2019;33(3-4):221-235. doi:10.1101/gad.319723.118

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR. Telomerase-negative Immortalized Human Cells Contain a Novel Type of Promyelocytic Leukemia (PML) Body. Cancer Res. 1999;59(17):4175-4179.

Yost KE, Clatterbuck Soper SF, Walker RL, et al. Rapid and reversible suppression of ALT by DAXX in osteosarcoma cells. Sci Rep. 2019;9(1):4544. doi:10.1038/s41598-019-41058-8

Zeman MK, Cimprich KA. Causes and Consequences of Replication Stress. Nat Cell Biol. 2014;16(1):2-9. doi:10.1038/ncb2897

Zhao S, Wang F, Liu L. Alternative Lengthening of Telomeres (ALT) in Tumors and Pluripotent Stem Cells. Genes (Basel). 2019;10(12). doi:10.3390/genes10121030

Zhao S, Wang F, Liu L. Alternative Lengthening of Telomeres (ALT) in Tumors and Pluripotent Stem Cells. Genes. 2019;10(12):1030. doi:10.3390/genes10121030

Martínez P, Blasco MA. Replicating through telomeres: a means to an end. Trends Biochem Sci. 2015;40(9):504-515. doi:10.1016/j.tibs.2015.06.003

Qiang W, Wu Q, Zhou F, Xie C, Wu C, Zhou Y. Suppression of telomere-binding protein TPP1 resulted in telomere dysfunction and enhanced radiation sensitivity in telomerase-negative osteosarcoma cell line. Biochemical and Biophysical Research Communications. 2014;445(2):363-368. doi:10.1016/j.bbrc.2014.02.001

Wright WD, Heyer W-D. Rad54 functions as a Heteroduplex DNA Pump Modulated by Its DNA Substrates and Rad51 during D-loop Formation in Homologous Recombination. Mol Cell. 2014;53(3):420-432. doi:10.1016/j.molcel.2013.12.027

CURRICULUM VITAE

[redacted]

[redacted]