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THE ROLE OF RAD51 IN TRICHOMONAS VAGINALIS

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THE ROLE OF RAD51 IN TRICHOMONAS VAGINALIS

By

Dominique Hall

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In Partial Fulfillment of the
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THE ROLE OF RAD51 IN TRICHOMONAS VAGINALIS

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THE ROLE OF RAD51 IN TRICHOMONAS VAGINALIS

Abstract

By Dominique Hall

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Drug resistance to the current treatments on the market is on the rise, therefore there is strong interest in understanding what could be causing the resistance, how resistance could be spreading through the population, and finding some possible new drug targets. One protein of interest is Radiation Sensitive Protein 51 (Rad51). It is a protein that is involved in homologous recombination as well as other process such as DNA damage repair. While *Trichomonas vaginalis* traditionally has been known to replicate via binary fission, a modified form of closed mitosis, there is some evidence that meiosis, or at least some form of genetic recombination, could occur in the organisms, possibly contributing to the resistance. The focus of my project was gaining an understanding of how the *Trichomonas vaginalis* Rad51 protein (TvRad51) could, either directly or indirectly, play a role in generating diversity that could lead to resistance. Since functional assays of purified Rad51 protein were previously unsuccessful, this study attempted to investigate TvRad51's ability to interact with known binding partners via protein-protein interactions, while exploring new targets using yeast two hybrid. Localization of TvRad51 showed that it behaves differently than the human version giving more information to understand how TvRad51 functions. Lastly, preliminary studies were conducted to evaluate the potential role of TvRad51 in telomere maintenance.

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CHAPTER 1: INTRODUCTION

Disease, Treatment, and Drug Resistance

Trichomonas vaginalis is a eukaryotic parasite that is responsible for causing a Sexually Transmitted Disease (STD) called Trichomoniasis. Trichomoniasis is the most common curable STD, which according to the CDC, caused over 2 million infections in 2018 [CDC]. 70% of people that are infected with the STD are asymptomatic and there seems to be more adverse effects in women than in men [CDC]. Unless a person is showing symptoms, trichomoniasis is not part of the panel of STDs screened for during a routine gynecology appointment. Women suffering from the STD normally exhibit symptoms such as itching, burning, redness or soreness of the genitals, and smelly discharge while males show itching or burning of the penis, burning after urination or ejaculation, or discharge [CDC]. Women usually have a predisposition to HIV, cervical cancer, and even preterm pregnancy while men are more susceptible to urethritis and prostatitis [CDC]. The current treatments are metronidazole and tinidazole, however resistance to metronidazole is increasing [CDC].

The decision of which treatment, metronidazole or tinidazole, prescribed is determined based on meta-analysis and data from a randomized trial of mostly women without HIV [CDC]. The dosage for tinidazole is the same for both men and women (2g orally in a single dose) whereas for metronidazole it differs based on gender; for men it's 2g orally in a single dose while for women it's 500mg 2 times/day for 7 days. Most people are cured after that treatment regimen; however, reoccurrence of infection can occur. Normally when this happens doctors attribute it to one of three possibilities: antimicrobial resistance, lack of adherence, or reinfection from an infected sexual partner [CDC]. Occurrence of reinfection differs slightly depending on

the treatment; metronidazole being 4%-10% and ~1% for tinidazole (although less studied) [CDC]. Antibiotic resistance is of concern as the nitroimidazoles are the only class of approved antimicrobials that are effective against trichomonas infection [CDC]. If a person is reinfected the guidance is to start a new round of treatment increasing the dosage of metronidazole or tinidazole. If the issue still persists, a kit from the CDC is recommended to confirm antibiotic resistance and a consultation with an infectious disease specialist is needed to determine and alternative treatment [CDC].

Morphology and Life Cycle

T. vaginalis belongs to the Trichomonad order, among a genus that consists of eight described members. Out of these species *T.vaginalis* and *T.foetus* are the most similar, varying slightly in morphology and infected host range (*T.foetus* causes Trichomoniasis in cattle) [Schwebke et al., 2004]. *T. vaginalis* is an anaerobic protozoan parasite which is normally in a pyriform state in culture [Schwebke et al., 2004]. However, when it adheres to the epithelium of the vaginal wall or urethra it changes into the amoeboid morphology [Schwebke et al., 2004]. It is 9 by 7 μm and has four flagella [Schwebke et al., 2004].

Figure 1

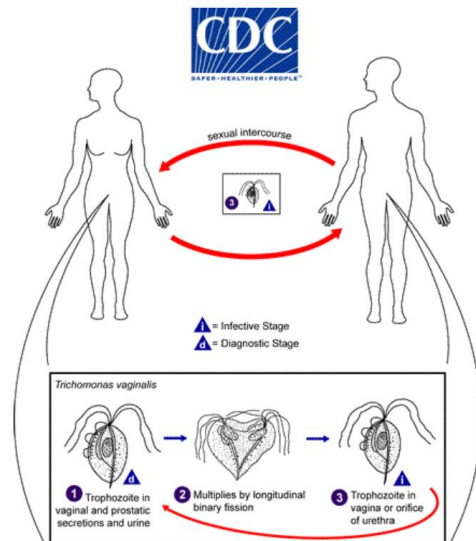
A Magnified Microscope Image of Two Trichomonas Vaginalis Parasites



Its most obvious organelles consist of a nucleus, an axostyle that runs from the anterior to the posterior of the parasite, and, instead of having mitochondria, it has hydrogenosome to perform its metabolic processes [Schwebke et al., 2004]. The life cycle of *T. vaginalis* is simple in that it is transmitted in a trophozoite form and does not have a cyst form [Schwebke et al., 2004].

Figure 2

The Life Cycle of Trichomonas Vaginalis.



Trichomonas vaginalis resides in female lower genital tract and the male urethra and prostate (1) where it replicates via binary fission (2). The parasite does not appear to have a cyst form, and does not survive well in the external environment. *Trichomonas vaginalis* is transmitted among humans, its only known host, primarily through sexual intercourse (3). From CDC website

<https://www.cdc.gov/dpdx/trichomoniasis/index.html>

T.vaginalis has been shown to reproduce asexually via a process called binary fission, in which the nucleus does not break down during division and there is a reduced mitotic spindle [Benchimol et al., 2000 and Arroyo et al., 2000]. While meiosis has not been observed, genes that are involved in meiosis have been found in its genome, suggesting that sexual processes could be occurring [Malik et al., 2008]. Some of the genes are recombinases that are involved in meiosis and homologous recombination, which could allow for the parasite to have daughter cells that are not genetically identical and increases variation via sexual reproduction, potentially aiding in the increase of antibiotic resistance. If these genes are shown to not be involved in meiosis then they could serve another purpose in the organism, such as DNA damage repair.

DNA Repair and Homologous Recombination

Results from a phylogenetic analysis of 730 polymorphic molecular markers in 20 strains of *T.vaginalis* provides evidence of genetic variation that suggests meiotic recombination could be occurring, although it has never been seen [Malik et al., 2008]. Recombination genes such as Spo11, Hop1, Hop2, Rad51 and DMC1, have been found to function in meiosis in animals, fungi, and protists (AFP) and have homologs in *T.vaginalis* [Malik et al., 2008]. Many of these genes are also involved in the repair of DNA double strand breaks in addition to being important for meiosis. Likewise, genes involved in DNA repair, like Rad50, Rad21, and Mre11, have also been found in *T.vaginalis*, however the function of these genes in the parasite is still being determined [Malik et al., 2008].

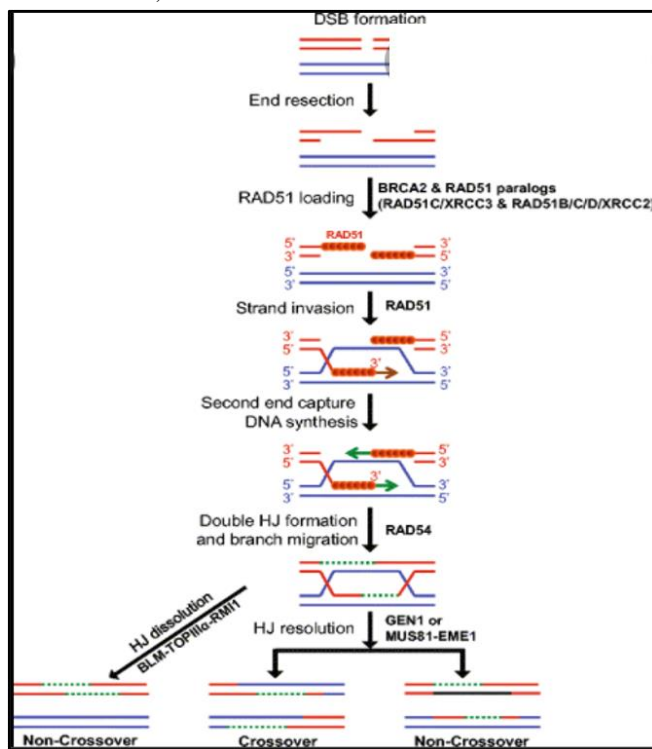
In humans, there are two major pathways that are used for the repair of double strand breaks: non-homologous end joining and homologous recombination. The focus of my research is on genes that might play a role in homologous recombination, because many of these genes function in a similar role during meiosis as well. Double strand breaks can occur from ionizing

radiation, chemical damage, and during DNA replication as a result of the stalling or collapse of replication forks [Masson et al., 2001]. If gone unrepaired this can result in chromosomal deletions, translocations, tumorigenesis or apoptosis as a result of chromosomal instabilities [Masson et al., 2001]. These mechanisms are crucial in maintaining genomic integrity.

Homologous recombination (HR) is a process used to repair double strand breaks that occur during meiotic recombination between homologous chromosomes, resulting in haploid cells with unique characteristics [Masson et al., 2001]. This process shares a lot of the same enzymes and genes used in double strand break repair in dividing somatic cells, except recombination occurs between sister chromatids after DNA replication [Masson et al., 2001]. The process starts with either of two sister chromatids where a double strand break is induced by Spo11 on one of the chromosomes; then the nuclease, Mre11, digests away the 3' end of the DNA making it more accessible for recombinases, Rad51 alone or Rad51 and DMC1, to perform the next step [Masson et al., 2001]. Subsequently, the strand invasion portion occurs where Rad51 (with DMC1 during meiosis) finds homology with the opposing strand from a homologous chromosome and forms a displacement loop. DNA synthesis then occurs from the invading strands, where are then ligated back to the original cut chromosome. Once ligated a Holliday junction is formed. Lastly, the DNA is cut to resolve the Holliday junction and a crossing over or non-crossing event results.

Figure 3

*Schematic of Homologous Recombination and Some of the Proteins Involved in the Mechanism
Adapted from Tarsounas et al., 2015.*



Although there are several proteins that are involved in this mechanism my focus is on Rad51 and its role in these processes.

Radiation Sensitive Protein 51 (Rad51)

Rad51 is a highly conserved recombinase that is one of the eukaryotic homologues of the *Escherichia coli* gene, RecA. Human Rad51 is 37 kDa and contains a core domain that has Walker A and B domains that are used for ATP binding and hydrolysis [Walker et al., 1982]. The sequence identity of the *T.vaginalis* version compared to the human Rad51 is ~71% sequence identity.

Figure 4

BLAST Protein Alignment of TvRad51 (Query) and Human Rad51 (Subject).

Score	Expect	Method	Identities	Positives	Gaps
496 bits(1278)	0.0	Compositional matrix adjust.	233/328(71%)	282/328(85%)	1/328(0%)
Query 2	DTEVAQEGAAGPLLIQTLEKQGIAGDIKKLQEAGYNTVESVAFSTKKQLITVKGISEAK	61			
Sbjct 12	DT V +E + GP I LE+ GI A D+KKL+EAG++TVE+VA+ KK+LI +KGISEAK	70			
Query 62	ADKIMAAAAQLVPMGFASADYNQIRKEMVYLTGSTEELDKLLGGGIETGSVTEIFGEFR	121			
Sbjct 71	ADKI+A AA+LVPMGF +A++++Q R E++ +TTGS ELDKLL GGIETGS+TE+FGGEFR	130			
Query 122	TGKTQLCHTLAVTCQLPIENGSGGKCLWIDTEGTFRPERIPIAQRFGLDPPDDVMDNIA	181			
Sbjct 131	TGKTQ+CHTLAVTCQLPI+ GGG+GK ++IDTEGTFRPER++ +A+R+GL DV+DN+A	190			
Query 182	YARAYNSDHQSLLVTASAMMAEAYALLIVDSSTNLRYTDYSGRGELSARQMHLGQFLR	241			
Sbjct 191	YARA+N+DHQ+QLL ASAMM E+ YALLIVDS+T LYRTDYSGRGELSARQMHL +FLR	250			
Query 242	NIQRLADEYGIQVAVITNQVVAQVDGAAMFVSDPKKPIGGNIMAHASQTRLRLKGRGETR	301			
Sbjct 251	+ RLADE+G+AVVITNQVVAQVDGAAMF +DPKKPIGGNI+AHAS TRL LRKGRGETR	310			
Query 302	VCKIYDSPSLPENEAFTQITNGGITDAE	329			
Sbjct 311	+CKIYDSP LPE EA F I G+ DA+ ICKIYDSPCLPEAEAMFAINADGVGDAK	338			

Interestingly, while the human version of Rad51 cycles between the nucleus and cytoplasm, the *T.vaginalis* version stays in the nucleus. When in the cytoplasm Rad51 is in an inactivated

heptameric ring structure form; however, in the presence of DNA it binds to DNA and forms a multisubunit nucleoprotein filament. Once this filament has formed the recombinase then extends and unwinds the DNA [Masson et al., 2001]. Studies have shown that one molecule of Rad51 binds three nucleotides of single stranded DNA (ssDNA) however Rad51 also has a high binding affinity for double stranded DNA (dsDNA) as well [Bhattacharyya et al., 2008]. Studies of Rad51 function in different ammonium sulfate salt concentrations showed that Rad51 is able to differentiate between ssDNA and dsDNA and undergoes a conformational change leading to nucleoprotein filament elongation on ssDNA [Kagawa et al., 2010]. Replication protein A (RPA) stimulates the Rad51 nucleoprotein filaments to perform DNA pairing, strand invasion, and strand exchange through the removal of the secondary structure on the ssDNA tail [McIlwraith et al., 2000] and calcium levels can decrease the ATPase activity, causing the filament to be in an actively, stable state [Bugreev and Mazin, 2004; Chi et al., 2006]. In order for the nucleoprotein filament of Rad51 to assemble, two to five monomers have to assemble to have a stable nucleation event and once that has occurred it grows. When double strand breaks are induced Rad51, along with the other proteins involved in HR, are localized to the nucleus where they make nuclear foci, which are subcomplexes the proteins form on the DNA [Haaf et al., 1995; Scully et al., 1997].

Among the protozoan parasites such as *Entamoeba histolytica*, *Giardia lamblia*, and *Plasmodium falciparum*, the process of homologous recombination as a double strand break repair mechanism has been conserved between these organisms [Marchat et al.]. In these same parasites when the *E. histolytica* protein sequence was compared to the orthologues of *G. lamblia*, *P. falciparum*, and *T. vaginalis*, the results showed that Rad51 was the most conserved recombinase with the percentages being 51%, 58%, and 64% [Marchat et al.]. This gives more

evidence for the importance that homologous recombination plays in these organisms as well as further shows how crucial Rad51 is to *T.vaginalis*. In other eukaryotes there have been studies showing adverse effects during development as well as maintaining chromosomal integrity when Rad51 is depleted. For example, mice exhibiting a homozygous Rad51^{-/-} phenotype died as an embryo during development due to Rad51 being knocked out [Masson et al., 2001].

Furthermore, when an inducible Rad51 transgene was made in chicken DT40 cells, when Rad51 was not being expressed more spontaneous chromosomal breaks occurred showing the importance of Rad51 in the maintenance of replication forks and chromosomal integrity [Masson et al., 2001].

Breast Cancer Gene 2 (BRCA2) and Its Supporting Role

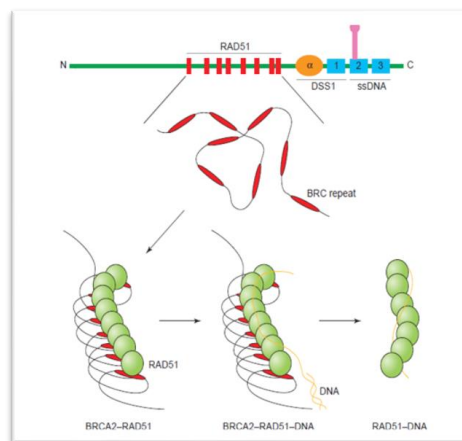
BRCA2 is most notable for being mutated in most breast cancer cases. The human BRCA2 protein was discovered in 1995 and orthologs have been shown in mouse, rat, *Xenopus*, *Caenorhabditis elegans*, *Trypanosoma* and *Leishmania*, however it is not present in yeast, archaea, and bacteria [Genois et al, 2012]. Emerging evidence has proven that BRCA2 is involved in DNA damage response processes. It has been seen that when BRCA2 is disrupted DSBs cannot be properly be repaired due to its interaction with Rad51 [Pellegrini et al., 2004]. Additionally, BRCA2-deficient hamster cells treated with hydroxyurea in order to stall and collapse the replication fork, showed defects in the maintenance of nascent DNA strand length, potentially showing that BRCA2 protects nascent strands for degradation [Roy et al., 2012].

Additionally, BRCA2 is a large protein with a size of 384 kDa [Genois et al, 2012] and contains eight BRC repeat motifs which are encoded by exon 11 [Pellegrini et al., 2004]. These BRC sequences are conserved between all mammalian BRCA2 proteins [Lo et al., 2003]. The motifs are about 30 amino acids long and are separated by linker regions that span 60 to 300

amino acids long, which have not been found to be conserved [Bork, Blomberg, and Nilges, 1996; Bignell et al., 1997]. The BRC motifs are how Rad51 binds to BRCA2. In HR BRCA2 is

Figure 5

Schematic Showing Interaction of Rad51 with the BRC Repeat Domain of BRCA2 From Venkitaraman et al., 2004



responsible for the localization of Rad51 to the nucleus as well as facilitating the interaction between Rad51 and ssDNA [Pellegrini et al., 2004]. The exact mechanism of how of BRCA2-dependent Rad51 transfer to DNA is not known, however there is a proposed idea: upon the induction of DSB breaks in the DNA and the formation of ssDNA tails by Mre11, the BRC repeats 1-4 bind monomeric Rad51 and improve binding to ssDNA not dsDNA [Carreira and Kowalczykowski, 2011]. Rad51 then nucleates onto the ssDNA where then BRC repeat 5-8 stabilizes the developing nucleoprotein filament by limiting ATP hydrolysis by Rad51 [Carreira and Kowalczykowski, 2011]. Subsequently, the nucleoprotein filament starts to grow displacing RPA from the ssDNA and the BRCA2 is released, where it then helps nucleation of Rad51 at

another site [Carreira and Kowalczykowski, 2011]. An ATP bound nucleoprotein filament capable of homologous pairing is then synthesized [Carreira and Kowalczykowski, 2011].

The Rad51-BRCA2 interaction in HR and DSB aids in the survival and transmission of the protozoan parasite *Trypanosoma brucei* in its hosts. This parasite uses these mechanisms as a means of generating antigenic variation by performing variant surface glycoprotein switching which allows it to evade a host immune response [Genois et al, 2012]. Likewise, *Leishmania* uses HR and DSB to elicit drug resistance [Genois et al, 2012].

Disrupted Meiotic cDNA 1 (DMC1) and Its Relationship to Rad51

Disrupted Meiotic cDNA 1 is a 37 kDa recombinase that is involved in HR and like Rad51 is a homolog of the *Escherichia coli*, RecA, however as of yet it has only been shown to be used in meiotic recombination [Masson et al., 2001]. Deficiencies in DMC1 can result in infertility like in *Arabidopsis* or in mice with a *dmc1*^{-/-} knockouts, which had smaller reproductive organs than usual and were sterile [Masson et al., 2001]. DMC1 mutations in *S. cerevisiae* SK1 strain had abnormal formation of synaptonemal complexes, a decrease in chromosomal synapsis, as well as defects in reciprocal recombination [Masson et al., 2001]; thus proving the importance of DMC1 in meiotic recombination.

DMC1 homologues have been found in protozoan parasites such as *Plasmodium* and *Leishmania* [Bhattacharyya et al., 2004]. The role of DMC1 is to help in the search for homology between homologous chromosomes; however, when absent this is eradicated leading to interactions between non-homologues [Masson et al., 2001]. In order for DMC1 to perform its job it needs the help of Rad51. Rad51 promotes the recombination of sister chromatids during mitosis, but works with homologs in meiosis and it has been shown that DMC1 assists Rad51 in doing that [Bhattacharyya et al., 2004]. In the absence of DNA DMC1 forms

octameric rings whereas in the presence is creates stacked rings [Kagawa et al., 2010]. Electron microscope and X-ray crystallography studies have suggested that the DNA passes through the center of the octameric rings through a tail-to-tail association between the octameric ring and the DNA [Kagawa et al., 2010]. However, there has not yet been a correlation between the ring structures of DMC1 and its function. During the strand invasion portion of HR DMC1 forms helical filaments with ssDNA which are similar to those made with Rad51 [Kagawa et al., 2010]. DMC1 is able to differentiate between ssDNA and dsDNA by Ca^{2+} binding to a site that is different than the ATP binding site, causing a conformational change [Kagawa et al., 2010].

Telomere Maintenance by Telomerase and Alternative Lengthening of Telomeres (ALT)

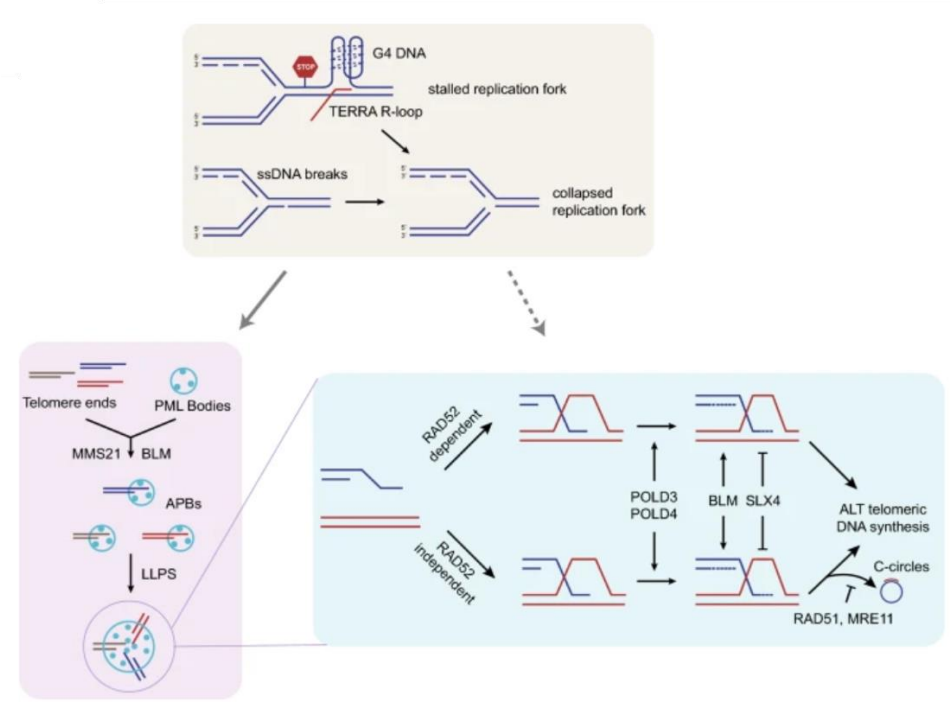
Telomeres are tandem repeats of DNA located at the end of chromosomes and function as a cap to prevent deterioration of the end DNA [Blackburn, 2004]. Without the telomeres, incorrect recombination events, telomere end fusion, and degradation of terminal regions within the chromosome would occur resulting catastrophic events such as loss of genetic information [Blackburn, 2004]. The telomeric DNA sequence is conserved between higher eukaryotes with nucleotide sequence differences in the ciliate protozoa *Tetrahymena*, *Euplotes*, and *Plasmodium* [Blackburn, 1991]. Telomeres are maintained by a ribonucleoprotein called telomerase which consists of two components, telomerase reverse transcriptase (TERT) and telomerase RNA template (TR) [Blackburn, 2004]. In order for the telomeres to be extended, the telomerase is going to recognize the 3' flanking end of the telomere that has a complementary sequence to the RNA template [Blackburn, 2004]. Subsequently using the RNA template the parental strand is elongated in the 5' to 3' direction, having additional repeats added as it moves down the strand [Blackburn, 2004]. The telomerase then relocates to the 3' end of the parental strand and then starts adding bases to the 3' end of the leading strand in the 5'→3' direction [Blackburn, 2004].

The lagging strand is then extended using DNA polymerase that contains DNA primase, however a small 3' overhang of the leading strand always remains on the chromosomes [Blackburn, 1991].

Telomerase activity has been seen many organisms such as ciliates, vertebrates, yeasts, plants, nematodes, and insects [Munoz et al., 2004]. Deficiencies in telomerase results in telomere length not being maintained. Strains of yeast and cultured mammalian cells deficient in telomerase can still maintain their telomeres by a method called the alternative lengthening of telomeres (ALT) mechanism. Although the telomeres are maintained in mice this mechanism is not enough on its own to keep the mice healthy and reproduce further than a few generations [Munoz et al., 2004].

The Alternative Lengthening of Telomeres (ALT) is a method used by 5% to 10% of cancer cells (more specifically usually sarcomas), and is a telomerase-independent method for telomere maintenance [Marciniak et al., 2005]. While the mechanism of how ALT works is not highly understood, what is known is that it is based on recombination events relying on proteins involved in that process (like Rad51) to maintain the telomeres [Marciniak et al., 2005]. It has been shown that in *S. cerevisiae* when telomerase is inactive most of the yeast die; however, a small percentage lives and that surviving population was shown to have higher levels of Rad51 [Marciniak et al., 2005]. It was even proposed that *T. brucei* could be potentially be using this mechanism to maintain its telomere in a telomerase-independent environment [Dreesen et al., 2006]. Initially it was unclear how *T. vaginalis* maintained its telomeres because homologs for the telomerase genes were found in its published genome (cite 2007 genome science paper). Since the ALT method relies on recombination it was a possibility that ALT could be the dominant process in maintaining telomeres in *Trichomonas*.

Figure 6
Framework of the ALT pathways



(Figure 6 continued)

(Upper section) The replication stress at telomeres may be a trigger for ALT activation. The accumulation of R-loops, G-quadruplexes, and DNA single-strand breaks at telomeres may interfere with DNA replication, leading to collapse of replication forks and formation of one-ended DSBs. (Lower left section) the replication stress or DNA damage at telomeres may induce SUMOylation of telomere proteins, which recruit PML and trigger APB formation through SUMO/SIM-mediated LLPS. The clustering of telomeres and enrichment of DNA repair, recombination, and replication proteins in APBs may drive ALT efficiently. (Lower right section) In APBs, BIR is triggered by the one-ended DSBs at telomeres. ALT can take place through RAD52-dependent and -independent BIR pathways. The conservative DNA replication during BIR is dependent on POLD3/POLD4, promoted by BLM, and inhibited by SLX4. C-circles are generated by the RAD52-independent BIR pathway, which is suppressed by RAD51 and MRE11. From Zou et al., 2020

CHAPTER 2: MATERIALS AND METHODS

PCR Conditions

In order to amplify the DMC1 insert was amplified in a reaction containing 10 µl of 5X Reaction Buffer, 0.4 µl of 25 mM dNTPs, 1 µl each of 20 µM Forward and Reverse primers for DMC1, 1 µl of Genomic DNA G3, 0.5 µl of Q5 High-Fidelity DNA polymerase, and 36.1 of water to get a final volume of 50 µl. The sample was then placed into the Thermocycler and run for 35 cycles using the program 94° C for 3 minutes, 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute, and ending with 10°C for forever. To ensure that the PCR worked the samples were run on a 0.8% agarose gel to check for a 1kb band.

Gel Purification

DNA samples were purified from agarose gels using the QIAquick Purification Kit from Qiagen. The protocol was followed exactly according to the manufacturer. The purified samples were later examined on a 0.8% agarose gel containing Ethidium Bromide (10mg/mL) to ensure that the purification worked properly.

Ligation

Gel purified samples were ligated into the appropriate vector using 6 µl of gel purified sample and 2 µl of vector. Additionally, 1µl of 10X Ligase Buffer, 0.5µl of T4 DNA Ligase, and 0.5µl of water was added to the reaction for a total volume of 10µl. The reaction was run at 14°C overnight using the Thermocycler.

Transformation into *E.coli* Cells

Ligated products were transformed into either JM109 or Top10 competent cells using 25µl of competent cells and 2µl of the vector. The mixture was incubated on ice for 20-30

minutes where subsequently it was heat shocked in a 42°C water bath for 3 minutes. After the heat shock it was then placed in ice for 30 seconds where then 500µl of Luria-Bertani (LB) was added to the mixture and then place in a 37°C shaker at 220 rpm for 45 minutes. Afterwards the mixture is spread on a LB plate with the appropriate antibiotic and placed in a 37°C incubator overnight.

10 Minute Miniprep

1.5ml of an overnight liquid culture containing the transformed vector of insert is centrifuged at 16,000 x g for 1 minute. The supernatant is then removed and 300 µl of TENS (1X TE (10mM Tris and 1mM EDTA), 0.1M NaOH, and 0.5% SDS) was added to the pellet and resuspended. 150µl of 3M NaOAc at pH 5.2 was added to the mixture where then it was inverted three times and vortexed for 30 seconds in order to mix the sample. The sample was then centrifuged for 2 minutes at 16,000 x g. The supernatant was the removed and added to a new micocentrifuge tube where 1 ml of 95% ethanol was added and then the sample was shaken vigorously for 1 minute. The sample again was centrifuged for 2 minutes at 16,000 x g and the supernatant decanted leaving a small white pellet in the tube. The pellet was washed with 70% ethanol and centrifuged at 16,000 x g for 2 minutes. The supernatant was removed and the pellet is place in the 37°C incubator to air dry for 3 to 4 hours. Once the pellet is dry 30µl of TE Buffer (10mM Tris and 1mM EDTA) with RNase (10µg/ml) is added and the sample.

Restriction Digestion

PCR products were digested from the pCR2.1 vector with the appropriate restriction enzymes in preparation to later be ligated into either the pEGFP C2 or the pGAD vector. The pEGFPC2 vector was cut with Nde1 and Pst1 while Nde1 and BamH1 were used for the pGAD vector. For the reaction 4 µl of vector, 3 µl of 10X of NEB Buffer 3 or 4, 3 µl of 10X BSA, 19

μl of water, and 0.5 μl of each restriction enzyme was used to make a 30 μl reaction. The sample was then placed in a 37°C incubator overnight.

HeLa and HEK293T Cell Culture

HeLa and HEK293T cells were maintained in Dulbecco's Modified Eagle Medium that contained 1% penicillin/streptomycin and 10% fetal bovine serum in a humidified 5% CO₂ atmosphere at 37°C.

Transfection into HeLa and HEK293T Cells

HeLa and HEK293T cells were passed in at a 1:4 dilution in Dulbecco's Modified Eagle Medium (DMEM) that contained 1% penicillin/streptomycin and 10% fetal bovine serum the night before and placed in a humidified 5% CO₂ atmosphere at 37°C. The next day 250 μl DMEM with penstrep and FBS was added to a microcentrifuge tube along with either 0.5 μg or 0.75 μg of Plus reagent, either 0.5 μg or 0.75 μg of DNA, or 1.25 μl or 2.25 μl of Lipofectamine LTX. This mixture incubates at room temperature for 30 minutes where then it is added to the corresponding plate of cells in a dropwise fashion. The plates are incubator overnight at 37°C in a humidified 5% CO₂ atmosphere. The next day they are harvested for SDS-PAGE/ Western Blot analysis.

Immunofluorescence

Hela and Heks mammalian cells were grown to approximately 60% confluency. To induce double strand breaks cells were exposed to 10μM Bleomycin at different timepoints 1 hour, 3 hours, and 6 hours to see translocation into the nucleus. The cells were washed using 1X PBS. Cells were fixed for 5 minutes using cold methanol and subsequently washed twice using 1X PBS. Fluorescence was observed using GFP under a Leica DMIRE2 inverted fluorescence microscope using a Yokogawa CSU-X1 confocal scanner unit.

***Trichomonas Vaginalis* Cell Culture**

Transfected Rad51-inducible clones were maintained in TYM media that contained 16µl of G418 (100mg/ml) and grown overnight at 37°C.

SDS-Page, Western Blot for Protein Analysis

Transfected or induced cells were loaded and run on a SDS-PAGE gel at 100V for 1 hour. Once completed the gel is placed in a sandwich between fiber pads, fiber paper, and placed next to a nitrocellulose membrane so that the contents for the gel can be transferred onto the membrane. This portion runs for 1 hour at 100V. Subsequently, the nitrocellulose membrane is placed in 5% blocking buffer (2.5g Block, 50ml TBS) and placed on a rotator for 5 minutes. The appropriate primary antibody is then added to the nitrocellulose membrane container that has 2 ml of the 5% blocking solution and 8 ml of 1X TBS- T (1X TBS and 1:1000 Tween-20) and placed on a rocker at 4°C overnight. The next day the membrane is washed 4 times for 5 minutes with 1X TBS. Once the washes are complete then 10ml of 1X TBST is added to the membrane and the peroxidase labelled secondary antibody is added at a 1:5000 dilution. This is then placed on a rotator for 1 hour. The membrane is then washed 4 times for 5 minutes with TBST. 2ml of Luminata Crescendo Western HRP substrate is added to the membrane and sits for 2 minutes. Once, the 2 minutes has passed the membrane is imaged using the Bio-Rad Molecular Imager Chemidoc XRS+ Imaging System.

TRAP Assay for Detection of Telomerase Activity

The TRAP assay was done exactly according to a protocol by Dr. Xue in the Chemistry department. The only variation was that T1, G3, and B7RC2 *Trichomonas vaginalis* samples were used and they were grown as written above however the TYM media did not contain any antibiotics.

Yeast Two Hybrid

Yeast two hybrid studies were done in accordance with Matchmaker Gold Yeast Two-Hybrid System User Manual by Clontech. The cDNA HeLa normalized library was purchased from Clontech [Cat #, 630479] already transformed into the Y187 yeast cells. The vectors used were constructed in the Wrischnik lab by myself or other graduate students. The mated colonies were then PCR amplified and subsequently sent to Sequetech for analysis. BLAST was then used with the sequencing results to identify what the protein was. Subsequently, the proteins were checked against Genbank, InterAct databases to known binding partners. The remaining list was screened using the criteria detailed in the Golemis et al. 2001 paper (detailed in References). All proteins that were eliminated based on that criterion were then chosen as potential new binding partners.

ONPG Assay

The ONPG assay was done using the manufacturers protocol.

CHAPTER 3: RESULTS

Telomerase Activity in *Trichomonas Vaginalis* Strains

It has been shown that recombinase proteins, which may include Rad51, are involved in the alternative lengthening of telomeres mechanism, which is a recombination-based event [Zou et al, 2020]. While, that method is used 10% of the time to maintain or elongate telomeres, 90% of the time telomerase is used. At this point, the genome of *T.vaginalis* had been sequenced and did not show evidence of having the TERT or TR homologs [Carlton et al., 2007]. In order to test if *T.vaginalis* has telomerase a TRAP assay was run using three different lysates varying in virulence. As shown in Figure 7, all three lysates are positive for telomerase which is a novel find.

Figure 7
TRAP Assay Showing Telomerase Activity in T.vaginalis Strains T1, G3, and B7RC2

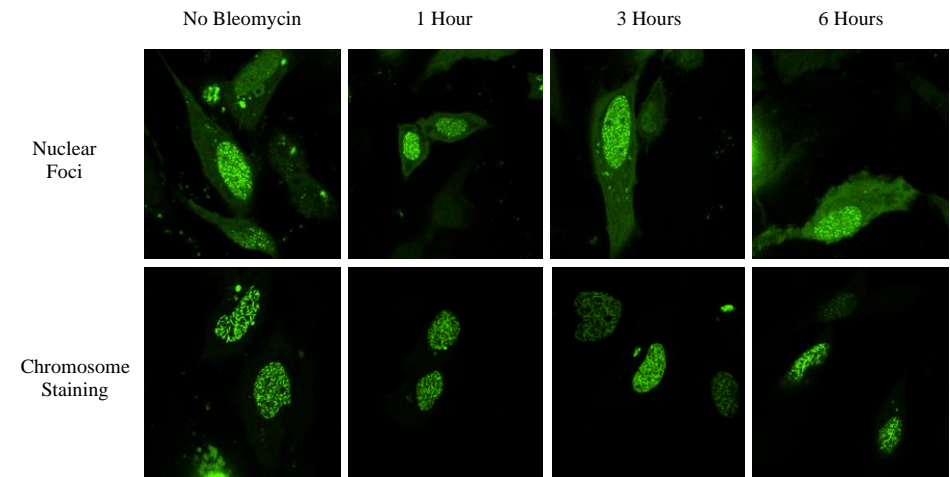


This result suggested that use of the ALT method was unlikely to be the main mechanism of telomeres maintenance in *T. vaginalis*. In addition, a more recent re-sequencing of the genome did show the presence of the predicted telomerase homologs. Our collaborators in Dr. Xue's lab also showed that a known human telomerase inhibitor, PyMp could disrupt the function of *T. vaginalis* telomerase. Dr. Wrischnik subsequently tested this telomerase inhibitor to see how *T. vaginalis* cell growth was affected. Unfortunately, the inhibitor didn't function as expected and did not delay growth after 2 months of treatment (data not shown).

Localization of PEGFP-Rad51 in HeLa Cells

Rad51 is a protein that is normally localized outside the nucleus unless a double strand break occurs. Data with an inducible version of TvRad51 showed that it appeared to be primarily nuclear when expressed in *T. vaginalis* (Wrischnik lab, data not shown). In order to examine the localization pattern of TvRad51 in human cells, TvRad51 was ligated into a peGFPC2 (vector built by Dr. Wrischnik and Ebony Tinsley) and transfected into HeLa cells. The cells were transfected using Lipofectamine-LTX and XtremeGENE HP at a ratio of 3:1 (DNA: Transfection reagent) and exposed to 10 μ M of Bleomycin at different time points (1 hour, 3 hours, and 6 hours). Bleomycin is an antibiotic that induces double strand breaks and by exposing the HeLa cells to the agent we wanted to see if there was a shift from cytoplasmic to nuclear, and if nuclear foci form. Figure 8 is showing where the TvRad51 was localized to in the HeLa cells at different time points. The Rad51 seems to be localized primarily to the nucleus regardless of the amount of exposure time to the antibiotic or not.

Figure 8
Localization of TvRad51 using Bleomycin to Induce Double Strand Breaks.



The peGFPC2-Rad51 vector was transfected in HeLa cells and exposed to 10 μ M of Bleomycin at different time points. The top panel shows nuclear foci that were formed during all the exposed time points. The bottom panel is showing Rad51 coating the chromosomes of the HeLa cells.

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This finding could lead to further experiments using the TvRad51 construct to help elucidate why the protein behaves differently from the mammalian version. Comparison of the sequence of the two proteins (shown in Figure 9) shows differences in the nuclear import and export regions, which might help us define the control points for nuclear import and export in *Trichomonas*.

Figure 9

BLAST Protein Alignment of TvRad51 (Query) and TvDMC1 (Subject).

Score	Expect	Method	Identities	Positives	Gaps
366 bits(940)	1e-131	Compositional matrix adjust	184/314(59%)	234/314(74%)	4/314(1%)
Query 16	IQTLEKQGIAGDIKKLQEAGYNTVESVAF	TKKQLITVKGISEAKADKIMAAAAQLVP-	74		
Sbjct 24	I+ L++ GI DIKKL+EAG TV +V	TKK L VKGIS+AK DK++AAA L	83		
Query 75	IEKLQQAGINIADIKKLEAGICTVGAVLME	TKKHLANVKGISDAKVVDKLIAAAQSLSE			
Sbjct 84	-MGFASASDYNQIRKEMVYLTGSTE	LDKLLGGGIETGSVTEIFGEFRTGKTQLCHTLAV	133		
Query 134	F S + + R +++ +T+GSTE	LDKLLGGG+E+ S+TE+FGFRTGKTQLCHTL V	143		
Sbjct 144	SETFISGATCLKNRSKVIRITSGSTE	LDKLLGGGVESMSITEVFGEFRTGKTQLCHTLCV	193		
Query 194	TCQLPIENGGGQKCLWIDTEGTFRPERI	IPVIAQRFGLPDDVMDNIAYARAYNSDHQSQ	203		
Sbjct 204	T QLP+ GGQ GK +IDTEGTFRPERI	IAQRF G+D D+ ++NI YARA+ + Q Q	253		
Query 254	TAQLPLSQSGGQKVC	FIDTEGTFRPERIPVIAQRF GVDGDEALEN	263		
Sbjct 264	LLVTASAMMAE	AHYALLIVDSSTNLYRTDYSGRGELSARQMHLGQFLRN	313		
Query 314	L+ A+A MAE Y LLI+DS T L+R D+SGRGEL+ RQ LGQ + + +LA E+ IA	LIQAAAAQMAEDQYRLLIIDSITALFRVDFSGRGELAERQOTLGQMMAALT	321		
Sbjct 322	VVITNQVVAQVDGAAMFVSDPKKPIGGNIMAHASQTRLSLRKGRGETRVCKIYDPSLPE	+ ITNQV+A D +A+FV P KPIGG+I+AHAS TRL LRKG+G RV KIYDPSLPE	321		
Query 327	NEATFQITNGGITD	EA+++++ GITD	335		
Sbjct 335	AEASYELSDAGITD				

Highlighted are the domains known in human Rad51 but could potentially be the same in

TvRad51 and TvDMC1. Helix-hairpin-helix domain (48-77aa in purple). Nuclear export signal

(between 245-260aa in green). The nuclear localization domain may reside in the C-terminus

(between 259 and 339AA in red).

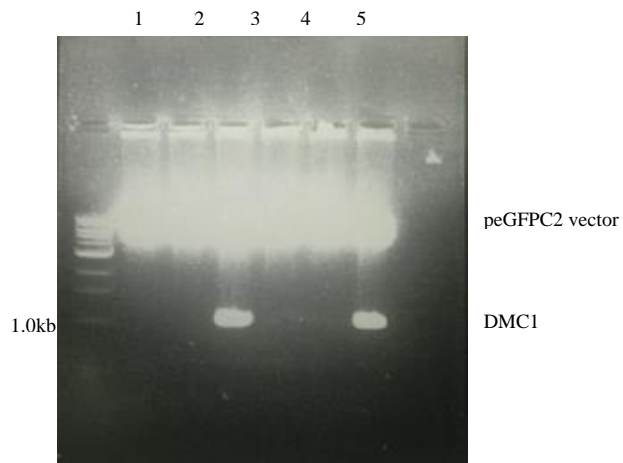
Construction of PEGFP-DMC1 Vector for Co-Immunoprecipitation Experiments

The Wrischnik Lab has been unable to express a functional version of TvRad51 in bacterial cells; experiments using purified protein in strand-exchange assays has not been successful (data not shown). To try other approaches to test if TvRad51 is a functional protein, plasmids were built to perform pull down studies to test for the interaction of TvRad51 with DMC1 and BRCA2. It has been shown in other organisms that Rad51 interacts with DMC1 during homologous recombination and that both Rad51 and DMC1 interact with BRAC2.

To show that this BRCA2 interaction occurs with the recombinases in *T.vaginalis* I created a pEGFPC2-TvDMC1 clone that can be used in doing co-IP studies along with pEGFP2-TvRad51 and a pCMVflag tag-TvBRCA2 vector containing the BRC domains. A PCR was run resulting in a 1kb DMC1 PCR product which was subsequently ligated into the pCR2.1 vector and transformed into competent *E.coli* cells. Liquid cultures were made from the colonies that grew and the pCR2.1 vector was isolated from the *E.coli* and a restriction digestion was performed to get the DMC1 insert. The insert was then ligated into the peGFPC2 vector and a restriction digestion confirmed that two clones had the DMC1 insert cloned successfully (which is shown in Figure 10).

Figure 10

Restriction Digestion of the DMC1 Insert.



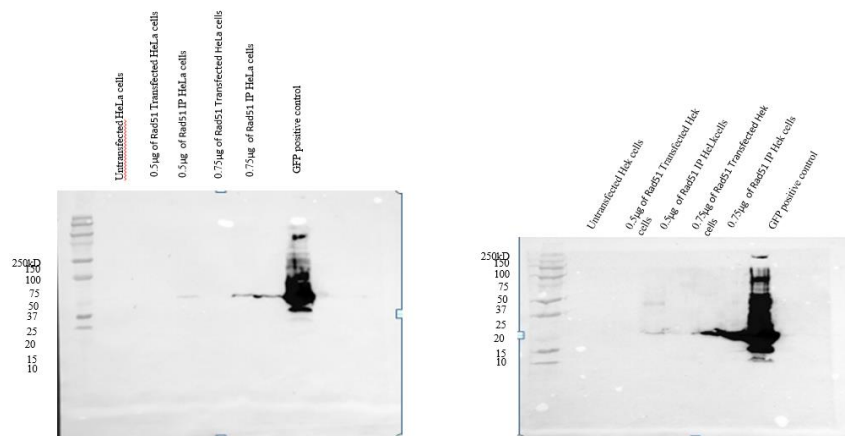
The insert was cut from the peGFPC2 vector using BamH1 and Pst1 restriction enzymes.

Immunoprecipitation Antibody Test for FLAG-BRC or GFP-Rad51 in HEK293T or HeLa Cells

In order to ensure that the antibodies for Rad51 and BRCA2 worked properly for co-IP experiments, HeLa and HEK293T cells were transfected with either the peGFPC2-TvRad51 vector or the pCMVflag tag-TvBRCA2 vector. Both HeLa and HEK293T cells were used to see which cell type expression levels were better in. The transfected cells were checked under the microscope for fluorescence of GFP to ensure that the transfection worked properly. Once the GFP expression was seen half of the sample was then used to perform an immunoprecipitation using Dr. Wrischink's protocol. Figure 11 shows the results from the IP using different concentrations of the TvRad51-GFP clone which showed faint bands in both the HeLa and HEK293T cells for the IP samples; however the band sizes were not correct. Further studies to optimize this technique are currently being done. The data for the BRCA2 transfection and IP is not shown, however it yielded similar results.

Figure 11

Western Blot Showing Transfected HeLa and Hek293T Cells with PEGFPC2-TvRad51.

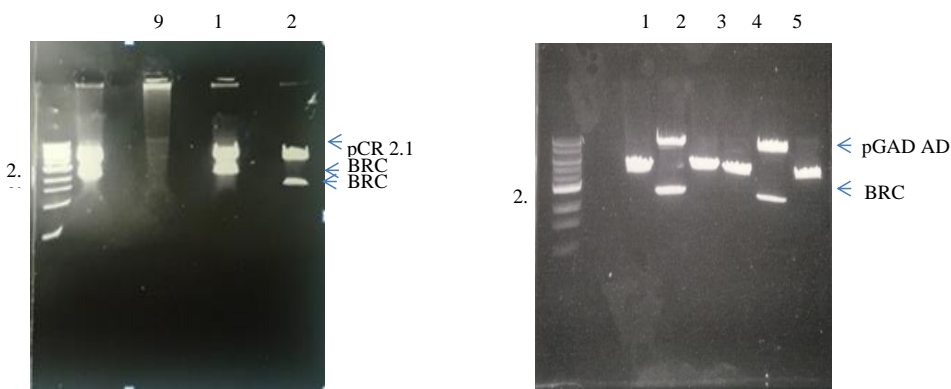


Western Blot showing transfected and immunoprecipitations using 0.5µg and 0.75µg of pEGFPC2-TvRad51 vector in HeLa (left) and Hek (right) cells. IP was done using rabbit α -TvRad51 and the IB was using mouse α -GFP (1:1000).

Construction of PGAD AD-BRC Vector for Yeast Two Hybrid Studies

Another technique that can be used to analyze protein-protein interactions is a yeast 2-hybrid screen. Along with building a clone for DMC1 for protein-protein interaction studies, a clone with the BRC repeats from BRCA2 was used to create a plasmid that could be used for the yeast two-hybrid studies. Rad51 interacts with BRCA2 via the BRC repeats and this interaction helps localize Rad51 to the nucleus in the event of a double strand break. In order to do this a previous student performed a PCR screen to assay for colonies that grew containing the BRC repeats in the pCR 2.1 vector. Those 26 samples were run on a gel and the four samples that had a band around 2 kb were grown, the plasmid was isolated from the *E.coli*, and a restriction digestion was performed. The left panel of Figure 12 is showing three of the four samples that contained the BRC insert although sample #22 had a band size of around 1.5kb. Those three samples were then ligated into the pGAD AD vector, transformed into *E.coli*, minipreped, and cut with restriction enzymes to ensure for the correct insert size. As the right panel of Figure 9 shows two out of the six minipreped samples contained the correct 2kb band indicating the BRC domain was successfully cloned into the expression vector.

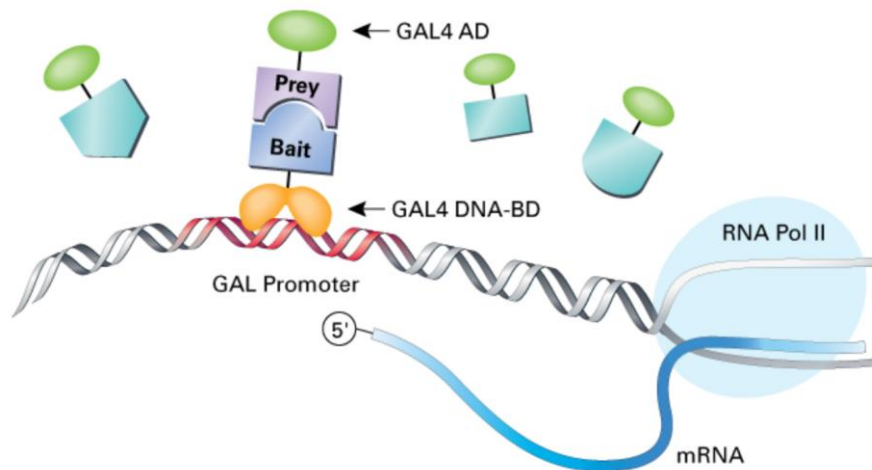
Figure 12
Restriction Digestion with BRCT Insert .



Restriction Digestion with BRCT insert from the pCR 2.1 (left) and pGAD AD (right) vectors using BamH1 and Nde1 restriction enzymes.

Yeast Two Hybrid with TvRad51, TvDMC1, and BRC Domain

It is known that Rad51 and DMC1 interact with BRCA2, and so we wanted to test the interaction of the *T. vaginalis* homologs using yeast two hybrid. To begin a control was run to ensure that the method was robust using pGBKT7-53 and pGADT7-T. When proteins are cloned into the pGBK vector, a “bait” fusion protein is created that tags your protein of interest with the GAL4 transcription factor binding domain. Clones placed into the pGAD vector will be expressed as “prey” fusion proteins containing the GAL4 transcriptional activation domain. If the two tagged proteins of interest are co-expressed in yeast cells and interact, they can produce a functional RNA polymerase 2 transcriptional activator that will induce the expression of target genes that act as reporters of this protein-protein interaction.

Figure 13*The Two-Hybrid Principle.*

Two proteins are expressed separately, one (a bait protein) fused to the Gal4 DNA-binding domain (BD) and the other (a prey protein) fused to the Gal4 transcriptional activation domain (AD). In yeast strain Y2HGold, activation of the reporters only occurs in a cell that contains proteins which interact and bind to the Gal4-responsive promoter.

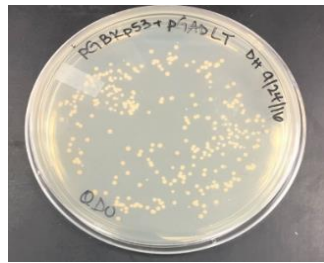
[Matchmaker Gold Yeast Two-Hybrid System Manual, Clontech Cat# 63048]

The p53 protein and the SV40 Large T antigen are known to interact, so when pGBKT7-53 and pGADT7-T plasmids are co-expressed in yeast, they should activate the target reporter genes, which can include the following: an enzyme that produces aureobacidin A resistance (AUR1-C), the ADE2 gene needed for adenine biosynthesis, the HIS3 gene needed for histidine biosynthesis, and finally the MEL1 (α -galactosidase) and LacZ (β -galactosidase) genes, which can be detected using substrates that produce color.

The positive interaction is shown in Figure 14.

Figure 14

Positive Result Showing Interaction Between PGBKT7-53 and PGADT7-T in Yeast using Quadruple Dropout Plates (-Leu, -Trp, -His, -Ade).

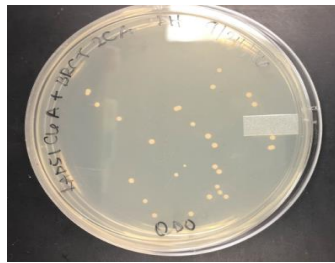


Leu and trp are used to select for the presence of the pGBKT7-53 and pGADT7-T vectors, and his and ade are used to examine protein-protein interactions.

Since the method was validated, we examined protein-protein interactions between TvRad51 and the TvBRCA2, and also TvDMC1 with the TvBRCA2. This was done using the same parameters as mentioned before but with pGBK-TvRad51 or pGBK-TvDMC1 and pGADT7-TvBRC plasmids. The positive result is shown in Figure 15.

Figure 15

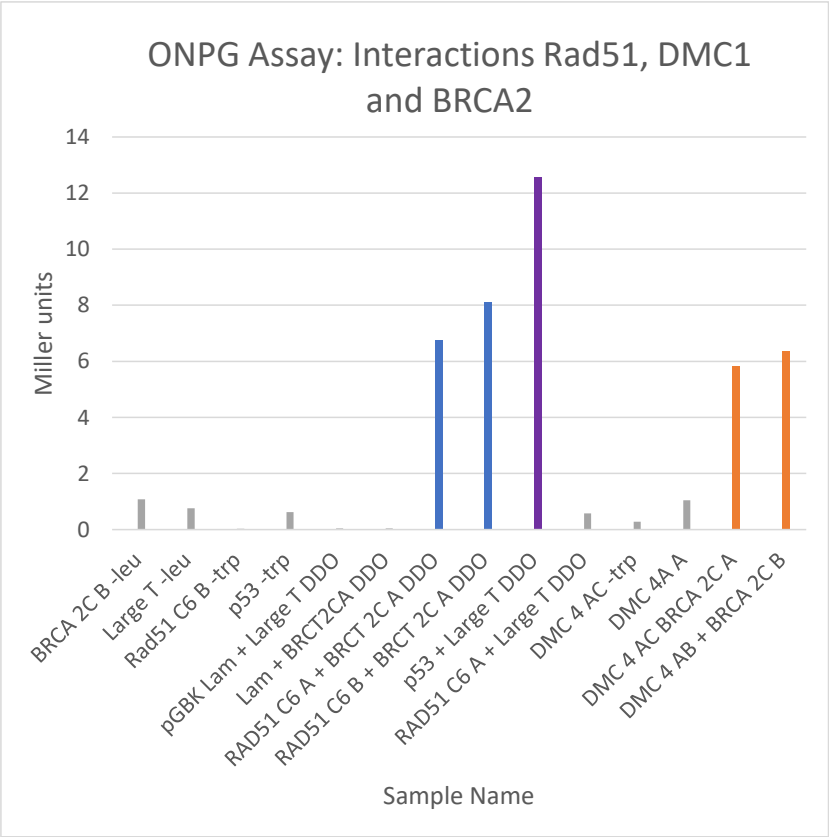
Positive Result Showing Colonies Proving Interaction Between PGBKT7-Rad51 and pGADT7-BRCT in Yeast using Quadruple Dropout Plates (-Leu, -Trp, -His, -Ade).



Leu and trp are used to select for the presence of the pGBKT7-Rad51 and pGADT7-BRC vectors, and his and ade are used to examine protein-protein interactions.

Additionally, Dr. Wrischnik's undergrad research students tested the interaction of pGBK-DMC1 and pGADT7-BRC plasmids (data not shown). The Rad51 and BRC clones and Rad51 and DMC1 clones were then used in an ONPG assay, which is a colorimetric assay that measures that amount of β -galactosidase activity produced as a reporter of the degree of protein-protein interaction between a bait and prey protein. This by proxy gives an arbitrary value of how well the proteins of interest interact compared to the control. The results of the ONPG assay are shown in Figure 16.

Figure 16
ONPG Result to Showing the Level of Interaction Between Rad51, DMC1 and BRCA.



The RAD51 and BRC sample had good interactions (blue) as did the DMC and BRC samples (orange) when compared to p53 and Large T (purple). The rest of the samples listed are negative controls which behaved as expected.

These results confirmed that these *T. vaginalis* proteins were behaving in the same way as Rad51 and DMC1 interact with BRCA2 as reported in the literature. Now, understanding what new binding partners TvRad51 has was of interest.

Yeast Two Hybrid Screen with TvRad51 and HeLa CDNA Library

The ONPG assay showed that TvRad51 and TvDMC1 could interact with a predicted binding partner (TvBRCA2) via a directed yeast 2-hybrid experiment. However, to build a better understanding of TvRad51 function, a yeast two hybrid screen was done to explore interactions with other known, and potentially new, binding partners. The yeast two hybrid analysis was utilized again; however, in this experiment a yeast strain expressing the “bait” from pGBKT7-TvRad51 (the TvRad51 fused to the Gal4 DNA binding domain) was crossed to yeast that had been transformed with a HeLa cell pGADT7-cDNA normalized library expressing “prey” proteins fused to the Gal4 activation domain. Mated diploids were selected due to their ability to grow on minimal media plates lacking leucine and tryptophan (-leu and -trp). In addition, the plates also lacked adenine and histidine, as well as containing the selective drug aureobacidin A and X- α -gal substrate, so the only yeast that could survive were the diploids with interactions between TvRad51 “bait” protein and the unknown cDNA-encoded “prey” protein that could express the reporter genes.

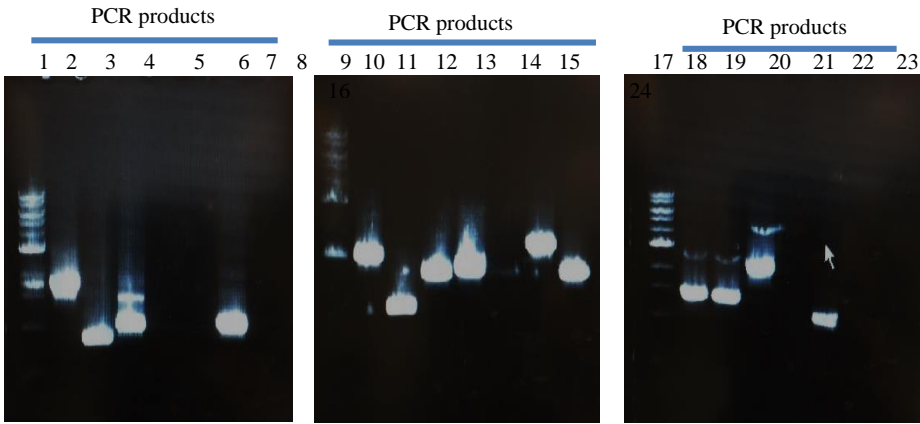
Colonies that survived on -leu, -trp, -ade and -his (also called “quad dropout plates”, or QDO plates, because they lacked 4 amino acids), in the presence of aureobacidin A, and which turned blue due to α -galactosidase expression, were picked and re-spread onto fresh QDO plates containing aureobacidin and X- α -gal. Colonies that survived the re-screen were grown up in liquid culture and the Zymoprep Yeast Plasmid Miniprep Kit (Zymo research) was used to obtain plasmid DNA from the cells. Since both pGBK and pGAD plasmids would be present in the

prep, the cDNA inserts were amplified using primers that lie outside of the pGAD vector MCS.

19 positive PCR amplifications are shown in Figure 17.

Figure 17

Agarose Gel Showing Amplified pGAD-cDNA Clones.



Agarose gel showing 19 pGAD-cDNA clones that were amplified using primers outside of the MCS; 14 clones had bands. These samples were gel-purified and sent for Sanger sequencing.

Once the sequences were made available from Sequetech Corporation we began a bioinformatics analysis to understand what the proteins were and if they could be new binding partners. An additional dataset from a less-selective double-dropout (DDO) yeast two-hybrid study, in which mated yeast were screened on –leu and –trp plates in the presence of aureobacidin A and X- α -gal, was added to the analysis. BLAST was used to identify the proteins and Genebank was used to determine known binding partners. The remaining proteins were then checked to see if they fit the criteria of typical false positives based on a paper by Golemis et al, 2001 (in References). The criteria was to remove known proteins that directly bind and activate reporter promoters, "sticky" proteins. e.g. ribosomal subunits, heat shock proteins, proteasome subunits, and cytoskeletal components, and false positives that can induce indirect effects on reporter systems. Figure 18 shows a pie chart detailing the differences between the DDO and QDO findings.

Figure 18
Breakdown of Possible New Binding Partners, Known Binding Partners, and Eliminated Proteins for Double Dropout Sequencing Results.

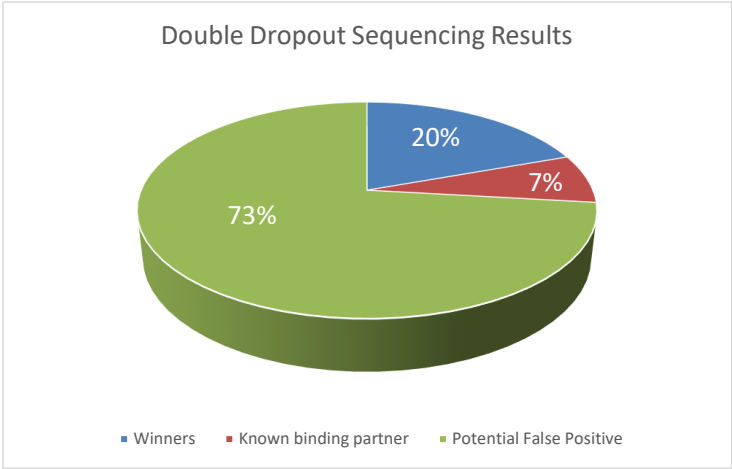
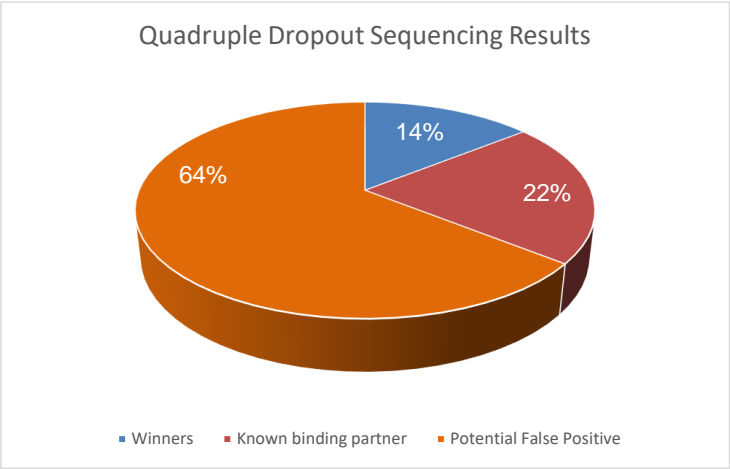


Figure 19
Breakdown of Possible New Binding Partners, Known Binding Partners, and Eliminated Proteins for Quadruple Dropout Sequencing Results



Interestingly, the breakdown between the DDO and QDO are similar with the number of “winners”, real potential binding partners, being around 14-20% of the proteins, which is surprising given the QDO should be the more stringent of the two. Table 1 and 2 show the winners from each yeast two hybrid screen. It’s interesting how widespread the functions of the proteins are but that in many cases the localization patterns are similar to Rad51. These could indicate additional functions that are yet unknown. Nonetheless, both screens yielded “winners” to explore further in the future.

CHAPTER 4: DISCUSSION

Telomerase Activity and the Alternative Lengthening of Telomeres in *T.vaginalis*

Telomerase is a ribonucleoprotein that is normally upregulated in 90% of cancer cells. The enzyme elongates telomeres in order to ensure that cancer cells are able to divide more than the normal amount. Along with this way of cancer cell maintenance, the ALT method is also used. This mechanism accounts for 10% of telomere maintenance in cancer cells and relies on a recombination method for elongation. This mechanism has been seen to be used in yeast that is lacking telomerase activity. The topic of telomeres and its maintenance has not been explored in *T. vaginalis* nor did its sequenced genome suggest that the telomerase enzyme exists. As our studies have successfully shown, telomerase is present in the *T.vaginalis* genome and has significant activity. Three strains of *T.vaginalis* were tested showing that the B7RC2 and G3 strain has higher levels of telomerase activity. This could potentially be due to those two strains being more virulent than the T1 strain, or just due to differences in the sample preparation. Given that the probe used for the TRAP assay was one for the HeLa cells, the telomere sequence could potentially be very close to the human sequence. Recently, the genome of *T.vaginalis* was re-sequenced and telomerase genes were shown to be present. Since *T.vaginalis* does express telomerase, the role of ALT as a primary mechanism of telomere maintenance is unlikely.

Whether ALT, and recombinases such as Rad51, play a role in antigenic variation or other similar processes, could still be addressed. To help show that *T.vaginalis* uses the ALT method we would need to create a telomerase negative environment and test to see how the telomeres are being affected or if some mechanism such as ALT could help them divide. The compound TMPyP4 (5,10,15,20-Tetrakis-(N-methyl-4-pyridyl)porphine) can stabilize G-

quadruplex structures and inhibit telomerase activity. When tested on *T.vaginalis* extract in the TRAP assay, it was shown to inhibit *T.vaginalis* telomerase activity. However, months of continuous treatment with this compound did not affect the growth of the organism (Wrischnik, personal communication), so these experiments were put on hold.

The Functional Role of TvRad51

In the event of a double strand break Rad51 has been seen to localize to the nucleus to help repair the break. DMC1 has been shown to help Rad51 perform strand invasion, and BRCA2 helps localize Rad51 (and DMC1) to the nucleus. Previous, studies in our lab have been done looking at the localization of Rad51 and DMC1 in *Trichomonas*. In *T.vaginalis* endogenous expression levels of Rad51 and DMC1 are very low and undetectable. Using TvRad51 antibodies generated against His-tagged, purified TvRad51, endogenous Rad51 levels were undetectable. Previous studies have been done in order to detect endogenous Rad51 using the G3 strain, which what has been heavily used in the past for experiments such as these. Methanol and paraformaldehyde fixation assays were done on these samples and visualized using a fluorescent microscope. Unfortunately, both methods yielded negative results with the problem being the primary antibody. The Rad51 primary antibody was affinity purified using a sulfolink coupling resin which was specific for the TvRad51. However, the antibody was not strong enough to detect endogenous Rad51 which is why an inducible clone was used.

Tetracycline inducible clones were used in order to see Rad51 and DMC1 expression levels. As previously mentioned the clones were induced with different concentrations of tetracycline and expression was visualized using an anti-HA antibody since the pTV clone has a HA tag and also an anti-Rad51 antibody was used as well. The western blots probing with anti-

HA had the better result whereas when using the anti-Rad51 the bands were not as strong. This is either could be a problem with the antibody or the sample.

While induced TvDMC1 has been seen to be in the nucleus in *T. vaginalis*, it was seen to be cycling between the nucleus and the cytoplasm; in contrast, Rad51 appeared primarily nuclear (Wrischnik Lab, personal communication). Expression of Rad51 was through an inducible promoter that induced very low levels of expression, so it could be that TvRad is also cycling but is at too low a cytoplasmic concentration to be visible. In order to collect more information on Rad51 localization, we looked at Rad51 expression in HeLa cells that were exposed to Bleomycin (a double strand break agent) at different time points. The data showed that regardless of exposure time to Bleomycin the Rad51 seemed to be primarily nuclear, which is expected for the cells that were treated with Bleomycin. However, the untreated seemed to also be nuclear, which suggests that without a double strand break event Rad51 could be there. Additionally, at each time point there seemed to be images showing fluorescence where Rad51 is coating the chromosomes. This is not typical for Rad51 and there was not another example of this in the literature suggesting that this was an alternative function of Rad51. The only other example of this was of mice spermatocytes during meiosis 1 when a synaptonemal complex was being formed. This staining is interesting and further experiments are going to be done using DMC1 to see if this recombinase behaves similarly. This finding could shed light on a new function of TvRad51 as well as more studies will be performed to look at how the localization of this protein is regulated.

Rad51 Interactions

Rad51 has been shown to interact with BRCA2 and DMC1 during homologous recombination (and DMC1 also interacts with BRCA2). In order to confirm these results are

conserved between the *Trichomonas* homologs, two separate experiments were done to examine these interactions: co-immunoprecipitation and yeast two-hybrid analysis. Before both studies could be performed corresponding clones had to be built for both. For DMC1, the gene was ligated into two different vectors, pGFP2 and pGBK, one being for co-IP studies and the latter for yeast two hybrid experiments. I made the pBAD AD-BRCA2 clone to be used along with the pGBK-Rad51 BD vector as well as the pGBK-DMC1 vector, and a pCMVflagtag-BRCA2 clone was previously made to be used in the co-IP studies. As the data showed anti-Rad51, anti-GFP, and anti-flag antibodies were tested to ensure that they were able to detect the protein after they were transfected into HeLa and Hek cells. While there were some issues seen with the anti-flag antibodies, the anti-GFP and anti-Rad51 antibodies worked well however the wrong band size was seen when an IP was performed. This could be an indication that the antibodies are not being able to get to the protein, possibly because it is stuck in the nucleus, suggesting that the lysis buffer being used should be stronger. Additionally, to confirm that the transfection worked properly the transfected samples were looked at under a microscope to ensure GFP fluorescence which was seen. This shows that the protein was present and normally at higher levels in the Hek cells. For future studies, Hek cells will be used due to better expression levels, and tests will be carried out to find a better method to extract Rad51 from the nucleus while in a native state (so it can still interact with potential binding partners).

The yeast two-hybrid screens is another way of showing protein-protein interactions. Here two proteins, one with a vector that has the Gal4 activating domain (AD) connected to your gene of interest and the other with the Gal4 binding domain (BD) connected to a gene you want to test for interactions. Only if there is an interaction between the proteins attached to the two Gal4 domains will there be transcription and translation of the target reporter genes. Gal4 helps

with selection due to its ability to activate expression via RNA polymerase, producing reporter genes such as β -galactosidase, so that a blue white screen can be performed, among other tests, to look for protein-protein interactions. As the data showed, a ONPG test was done measuring β -galactosidase reporter activity, using Rad51 or DMC1 and BRAC2 interacting domains. This showed that Rad51 and DMC1 were expressed and folded correctly in yeast, and could interact with a predicted binding partner. Additional experiments using the BRCA2 and Rad51 as well as the DMC1 clones can be performed to examine differences between Rad51 and DMC1, or between human Rad51 and *T. vaginalis* Rad51, to better understand how the *T. vaginalis* proteins are being regulated, especially in localization studies (where domain swaps can be carried out to evaluate effects).

I then examined the interaction of Rad51 and a variety of clones expressed from a HeLa cDNA library to search for other possible interactions that Rad51 could have, with known and, hopefully, novel binding partners. Several known interaction partners were identified, including M phase phosphoprotein 9 (MPHOSPH9) variant 1, BRCA2 region, Homo sapiens SWI5 homologous recombination repair protein, Homo sapiens fidgetin like 1, and Homo sapiens FKBP prolyl isomerase as well as some novel partners that will be investigated further.

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