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Investigation of the Cell-Cycle Dependent Activity of the BRCA1-Rbbp8 Complex for Homologous Recombination

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INVESTIGATION OF THE CELL-CYCLE DEPENDENT ACTIVITY OF THE BRCA1-RBBP8 COMPLEX FOR HOMOLOGOUS RECOMBINATION

by

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ABSTRACT

When cells undergo mitosis, they must replicate all six billion base pairs of DNA within the nucleus. With the sheer volume of information, it is impossible to replicate with 100% accuracy each time. Homologous recombination (HR) is one of many mechanisms the body has developed to correct and repair replication errors to DNA. HR is specific to double-stranded breaks to DNA, and it requires a sister chromatid to preserve the genetic code. BRCA1 and Rbbp8 interact to form a complex that is heavily involved in this process. Although there is a strong consensus about the involvement of these proteins in a cell cycle-dependent manner, there are discrepancies in the current literature regarding when homologous recombination repair occurs. The goal of this thesis is to elucidate the true activity of BRCA1-Rbbp8 complex in the HR process. First, a meta-analysis was performed to review current research to understand the various experimental protocols that led to the conflicting conclusions about cell-cycle activity of HR. Then, we measured the mRNA levels of BRCA1 and Rbbp8 during different phases of the cell cycle. In order to accomplish this, the cell cycle of the L cells was synchronized using thymidine and RO-3306 for the S and G_2 phases, respectively. The RNA was collected and converted to cDNA via reverse transcriptase. Next, RT-qPCR was performed to measure the expression of BRCA1 and Rbbp8. The levels of Rbbp8 are not significantly different between these phases, though there was a clear downward trend worth noting. Furthermore, a statistically significant increase in BRCA1 as the cells moved from S phase to G_2 phase was observed. When normalized to the housekeeping gene, GAPDH, the levels of mRNA for BRCA1 during the S phase were significant lower than the control group. These results suggest that HR occurs during both S and G_2, but BRCA1 and Rbbp8 interact only during G_2.

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INTRODUCTION

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Homologous recombination (HR) and nonhomologous end-joining (NHEJ) are recognized as the two methods of repairing double stranded DNA breaks (DSBs) within eukaryotic cells (Watson, 2008). NHEJ and HRR are essential to the preservation of the genetic code. The breast cancer early onset gene 1 (BRCA1) gene and retinoblastoma binding protein 8 (RBBP8) are heavily involved in the cell-cycle dependent activity of HR (Soria-Bretones et al., 2013). Previous research indicates that the activity of these proteins in HR is dependent upon the cell cycle; however, there are discrepancies about the stage of the cell cycle during which it is active. The aim of this project is to identify the time in the cell cycle in which BRCA1 and Rbbp8 are actively repairing double-stranded DNA breaks through homologous recombination.

Cancer

Approximately 38% of all people will be diagnosed with cancer at some point during their life, making it one of the top 10 causes of death in the world. It is a collection of different but related diseases, all characterized by unchecked growth of cells. Cancer can be caused by errors that occur during replication of the genome that are left uncorrected and cause the cells to divide uncontrollably. Mutations can occur in three different types of genes that can lead to cancer: proto-oncogenes, tumor suppressors, and DNA repair genes. When functioning normally, these genes are all involved in normal regulation of the cell cycle and cell division. Mutations in proto-oncogenes can result in oncogenes, and mutations can inactivate tumor suppressor genes.

These mutated genes are then unable to properly regulate cell growth and division, and as a result, cells continue to divide without repairing DNA or properly passing cell checkpoints. As the cells continue to proliferate unchecked, they can encroach on normal functions of the specialized cells and spread to other parts of the body.

There are many environmental and genetic components that interact to form the circumstances leading to cancer. It is possible to inherit copies of genes that already contain mutations and increase the likelihood of developing certain types of cancer. There are a number of other risk factors for different types of cancers, including age, diet, hormone levels, infections, and obesity. Also, carcinogenic substances, such radiation, tobacco smoke, ultraviolet rays, and alcohol, can increase the rate of cellular mutation and can lead to cancer (National Cancer Institute). There are over 100 different types of cancer, categorized by the type of cell that is undergoing unchecked division, the specific organ these cells are localized within, and the amount of damage or stage the cancer has reached.

Breast Cancer and the Genetic Component

The National Cancer Institute reports that breast cancer represents 15.2% of new cancer cases in 2018, giving it the highest rate of incidence of any type of cancer. One of the specific risk factors for this type of cancer is inheritance of a mutated BRCA1 or BRCA2 gene. In humans, the BRCA1 gene is located on the long arm of chromosome 17. The 24 exons that compose this gene code for a 1,863 amino acid protein. This tumor suppressor gene normally functions to repair damage to the DNA and is involved in transcriptional regulation and cell-cycle control. When mutated, this gene is unable to perform these functions and mutations to the

DNA may continue to accumulate. There are a number of different mutations that have been identified that alter the functionality of this gene, including over 750 mutations that cause the truncation of the protein (Antoniou et al., 2003).

Inherited mutations of the BRCA1 gene are associated with an increased risk of developing breast and ovarian cancer, increasing a person's chance of being diagnosed with breast cancer from 12% to 72% and ovarian cancer from 1.3% to 44%. Inheritance of a mutated copy of BRCA1 is also linked with other types of cancer, including tumors of the fallopian tube and peritoneum (National Cancer Institute).

Rbbp8 Background

The retinoblastoma binding protein 8 (Rbbp8), or in humans C terminal binding protein (CtBP) interacting protein (CtIP), is an important binding factor for BRCA1 in DNA damage repair. The Rbbp8 gene is found on chromosome 8 and contains 24 exons. This protein is part of the family of retinoblastoma binding proteins that help regulate cell proliferation. Activation of Rbbp8 is accomplished by the cell through phosphorylation, a crucial cyclin dependent kinase (CDK) contingent step (Prakash et al., 2015). Rbbp8 is important in critical processes of the cell as an endonuclease and in complex with other proteins.

Repairing Double Stranded DNA Breaks (NHEJ, MMEJ, and HR)

DNA Double stranded breaks (DSB) are lesions that can result from chemicals, radiation, or ultraviolet light. These breaks are extremely problematic and can have severe consequences if not repaired or if they are repaired incorrectly, such as chromosomal translocations, mutations,

and damage to the cell. When double stranded breaks occur during the process of genome replication, there are three different approaches the cell can pursue to repair the DNA. The first is non-homologous end joining (NHEJ). This type of repair is active throughout the cell cycle, but dominates in G_0 and G_1. NHEJ is used to directly ligate the broken ends of DNA, which leads to the loss of base pairs (Biehs et al., 2017). The change in DNA sequence observed with NHEJ can alter the downstream products, mRNA and proteins, formed from this sequence. For this reason, NHEJ is not the preferred method of repairing double-stranded DNA breaks. The second pathway is microhomology mediated end joining (MMEJ), which is also known as alternative nonhomologous end joining. MMEJ is even more deleterious than NHEJ because it utilizes microhomologous sequences to locally resect and align regions of complementary DNA (Yun & Hiom, 2009).

The main pathway of DNA repair that this paper will focus on is homologous recombination (HR). HR is a method of repairing DSBs by using the sister chromatid to correct the error in order to conserve the DNA sequence. Due to the requirement for a sister chromatid, HR can only occur after the DNA has already been replicated. This is the preferred method of correcting mistakes by the cells because it is able to conserve our genetic code more effectively than NHEJ or MMEJ. Beginning in S and G_2, CDK-dependent phosphorylation of serine 327 of Rbbp8 occurs, which activates this protein (Yun et al., 2009). Rbbp8 directly interacts with BRCA1 C-terminal domains (BRCT) to promote and perform end resection required of the DNA in order to effectively repair the DSB. Rbbp8 and BRCA1 also interact with the MRE11-RAD50-NSB1 (MRN) complex, which effectively creates single stranded DNA through its endonuclease activity. DNA end resection and formation of ssDNA are important steps at the

beginning of HR that allow the RAD51 protein to direct the homologous strand exchange of the sister chromatid in order to conserve the DNA sequence (Prakash et al., 2015).

Cell Cycle-Dependent Activity

Homologous recombination is the most accurate and preferred method of repairing DSBs due to its use of a sister chromatid as a template for repair. This also limits the activity of HR to phases of the cell cycle after genome replication has occurred and a sister chromatid is available, eliminating G_1. However, there have been discrepancies in the literature about whether the BRCA1-Rbbp8 complex is active during both the S and G_2 phase or only the G_2 phase.

Many scientists have found that the complex is active from S phase onward (Chen et al., 2008; Escribano-Díaz et al., 2013; Yun et al., 2009; Table 1). The techniques used in these experiments ranged greatly; however, they all concluded that homologous recombination occurs during both S and G_2. Two experiments did yield results that led the scientists to believe that the BRCA1-Rbbp8 complex was only active in repairing DSBs during the G_2 phase of the cell cycle (Sartori et al., 2007; Yu et al., 2004; Table 1). The experiment conducted by Sartori *et al* concluded that HR was active during both S and G_2 by measuring the hypersensitivity of the DNA to camptothecin and etoposide, topoisomerase inhibitors, in CtIP depleted cells. However, when conducting immunoprecipitation of CtIP during the S phase, they were not able to efficiently retrieve BRCA1 as its binding partner due to the activity of the complex being solely in G_2.

Yu *et al* focused on the activity of BRCA1 and CtIP during the G_2/M checkpoint vs the G1/S checkpoint. These scientists found that phosphorylation of CtIP only occurred during G_2,

rather than both S and G$_2$. Since interaction of CtIP with BRCA1 depends on its phosphorylation, these scientists concluded that these proteins did not interact during the S phase of the cell cycle. While they found sufficient evidence to indicate the importance of the complex at the G$_2$/M cell cycle checkpoint, they did not distinguish the presence of the BRCA1-Rbbp8 complex in S phase. A summary of the methods and conclusions found from all five of these studies can be found in the meta-analysis (Table 1).

Table 1: Meta-analysis of current literature to determine the activity of BRCA-Rbbp8 in HR.

Publication		Escribano-Díaz et al. (2013)	Chen et al. (2008)	Yun and Hiom (2009)	Sartori et al. (2007)	Yu and Chen (2004)
Gene		BRCA1	BRCA1/Rbbp8	Rbbp8	Rbbp8	Rbbp8
Cell Line		Avian B (DT40)	Human (T98G)	Avian B (DT40)	Human (U2OS)	Human (HeLa)
Synchronization		Centrifugal Elutriation	BrdU	Centrifugal Elutriation	Serum Starvation	Thymidine Treatment
Methods		Fucci Fluorescence Activated Cell Sorting (FACS)	siRNA transfection and immuno-precipitation	CtIP -/-/- mutants, immuno-precipitation, and western blotting	siRNA mediated depletion of CtIP	shRNA and immuno-precipitation
Phase when HR is active	S	X	X	X	X	-
	G$_2$	X	X	X	X	-
Phase when BRCA1-Rbbp8 interact	S	X	X	X		
	G$_2$	X	X	X	X	X

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The goal of this thesis is to increase our understanding of the way that our cells undergo homologous recombination to maintain the integrity of our DNA. In order to comprehend the true cell-cycle dependent activity of the Rbbp8-BRCA1 complex in homologous recombination, the levels of mRNA expression of these genes were measured during different phases of the cell cycle (Figure 1). Hopefully, this will allow us to better understand the expression of these genes when they are functional and how to correct mistakes when these mechanisms are not functional. Eventually, this knowledge may help us target therapies and treatments for the cancers that develop due to mutations in these genes.
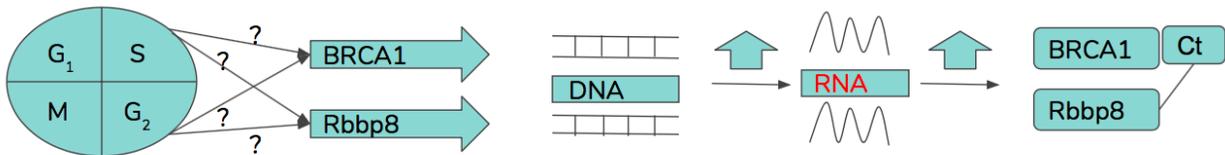

Figure 1: Hypothesized pathway of BRCA1 and Rbbp8 expression. Labelling in red indicates factor under investigation.

METHODS

Cell Culture

   Mus musculus L cells were used in this experiment, a strain of mouse areolar and adipose connective tissue. In this experiment, these cells were grown in media containing Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, and penicillin and streptomycin. Cell splitting occurred when confluency reached approximately 80% using trypsin.

Cell Synchronization

   The cells were synchronized to S or G_2 phase in order to determine the RNA concentrations during those points in the cell cycle. In order to synchronize the cells in S phase, the cells were grown to approximately 60% confluency before being treated with 100mM thymidine, which inhibits DNA synthesis by halting the pathway of nucleotide metabolism (Ma et al., 2011). This causes the cells to pause in the S phase, rather than move forward through the checkpoint into G_2.

   After an incubation period of 14 hours, the cells were washed with HBSS before an addition of growth medium and 24 μM deoxycytidine. After an incubation period of 9 hours, the cells underwent a second thymidine treatment (100mM). The purpose of the second thymidine treatment was to retrap the cells in the S phase, which allows for stronger synchronicity. Next, they were incubated for a period of 14 hours and then washed twice with PBS. The growth media with 24 μM deoxycytidine was added to the plate and the cells were harvested after three hours (Figure 2).
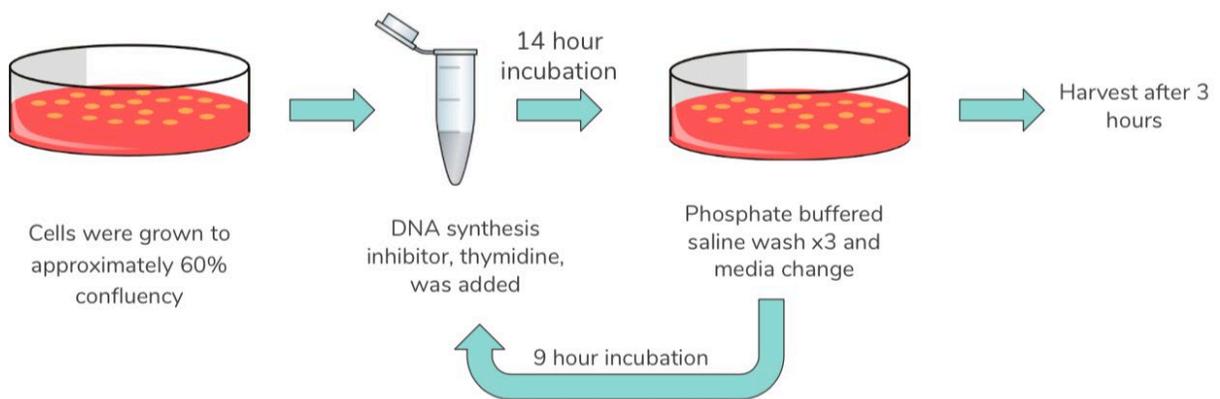
Figure 2: Synchronization of cells in S phase.

For synchronization in G_2 phase, the cells underwent the double thymidine block

procedure from the protocol for synchronization during S phase. After this, the cells were

incubated for approximately two hours before the addition of RO3306 (10 µM). This reagent is a

specific inhibitor of CDK1 activity which prevents the cells from moving past the next

checkpoint into mitosis. Then, the cells were incubated for an additional ten hours before

aspiration of the media and washing of the cells twice with PBS before being harvested (Figure

3). The control used was unsynchronized cells of the same passage grown through the
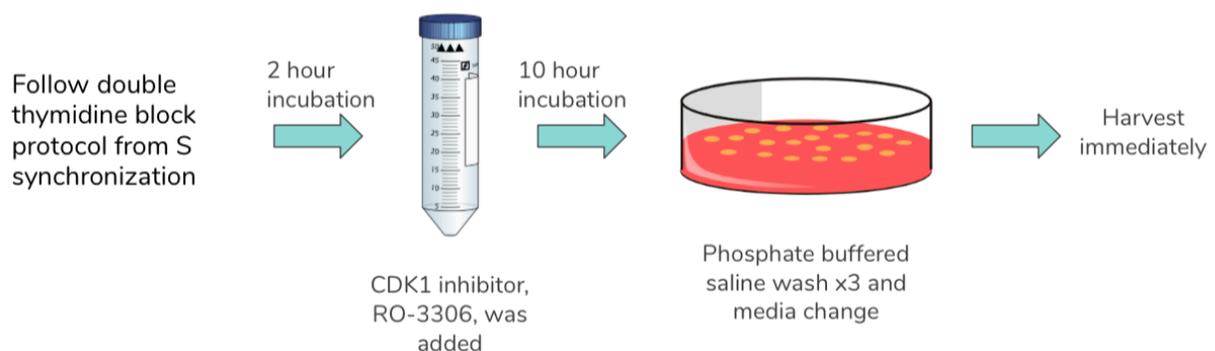
synchronization time period.

Figure 3: Synchronization of cells in G_2 phase.

Isolating RNA

For the technique of RNA purification, the Qiagen RNA Isolation RNeasy Mini Kit (Cat. No. 74104) protocol was followed. RNase-free water was used to elute the column twice. A Nanodrop Lite (ThermoFisher) was used to quantify the RNA that was purified in the sample.

RT-qPCR

For the real time quantitative polymerase chain reaction (RT-qPCR), the ThermoFisher protocol for the Applied Biosystems High Capacity RNA-to-cDNA kit and SsoFast EvaGreen Supermix was utilized. The DNA obtained from the reverse transcriptase reaction was diluted 1:2 with nuclease free water to a total volume of 60 μl. The reaction was run in the MiniOpticon Real-Time PCR System (BIO-RAD). The program ran the reaction mixture at 95°C for the first three minutes and then forty cycles of 95°C for three seconds and 60°C for thirty seconds. The primers used for qPCR are detailed in Table 1. Analysis of the results of quantitative PCR occurred through the $2^{-\Delta\Delta CT}$ method. T-tests were then performed to determine the statistical significance of the data obtained (SPSS).

Table 2: Primers used for quantitative real-time polymerase chain reaction (qPCR).
*Primers not used due to nonspecific binding.
#Primer not used because only one control housekeeping gene was necessary.

| Gene	Forward Primer Sequence	Reverse Primer Sequence
RBBP8-1*	CCTGTCTTCAAAACACCTCCATG	TGCAAGCTCACATCCTCCATGG
RBBP8-2	CCTCTAGTAAATTCTCTCCAGACCA	CACAAGAGAACCAGTACCCTTCA
BRCA1-1*	CCAGAAAAATGTCCGCGTAT	ACTGTCAGCCCATCTGCTCT
BRCA1-2	CGAGGAAATGGCAACTTGCCTAG	TCACTCTGCGAGCAGTCTTCAG
GAPDH	CTCATGACCACAGTCCATGC	TTCAGCTCTGGGATGACCT
B-ACTIN#	CCGTAAAGACCTCTATGCCAACA	CGGACTCATCGTACTCCTGCT

RESULTS

A.   B.
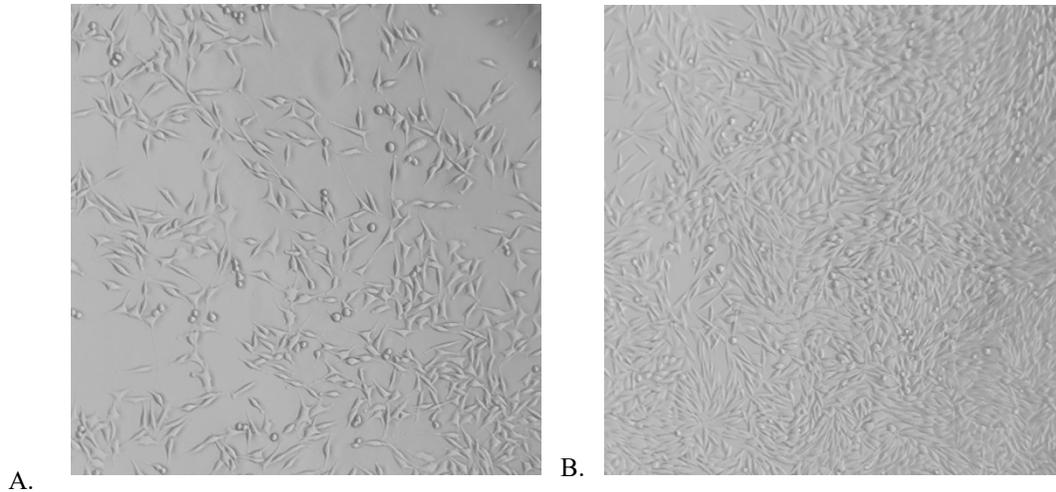
Figure 4: Images of L cells under microscope. A. Cells are at low confluency, approximately 40-50%. B. Cells are at high confluency ~100%.

After synchronization of the L cells to S and G_2 phases, collection of mRNA, and RT-qPCR to determine the relative expression levels, the $2^{-\Delta\Delta CT}$ method was used to analyze the data. First, the data of the two genes of interest, BRCA1 and Rbbp8, were normalized to the housekeeping gene, GAPDH. Then, the values obtained for the S and G_2 phases were compared to the control, unsynchronized cells. Results from the five trials were averaged and are displayed in Figures 1 and 2. A one-tailed Student's t-test was then performed comparing the levels during the S phase to G_2 and comparing each individual value to the control. mRNA levels of Rbbp8 during G2 phase and S phase were not statistically significant (t_4=-2.079, p=0.053), but there was a trend for less Rbbp8 expression in G2 phase. Further studies are needed to potentially resolve this trend. BRCA1 mRNA expression during S phase and the control was significantly less than control (t_4=-3.170, p<0.05). BRCA1 mRNA in S phase was also significantly less than G2 phase (t_4=-2.174, p<0.05).

Figure 5: Fold change in expression of Rbbp8 mRNA in S and G_2 phases. RT-qPCR results of five trials underwent $2^{-\Delta\Delta CT}$ analysis against unsynchronized controls, after being normalized to GAPDH.
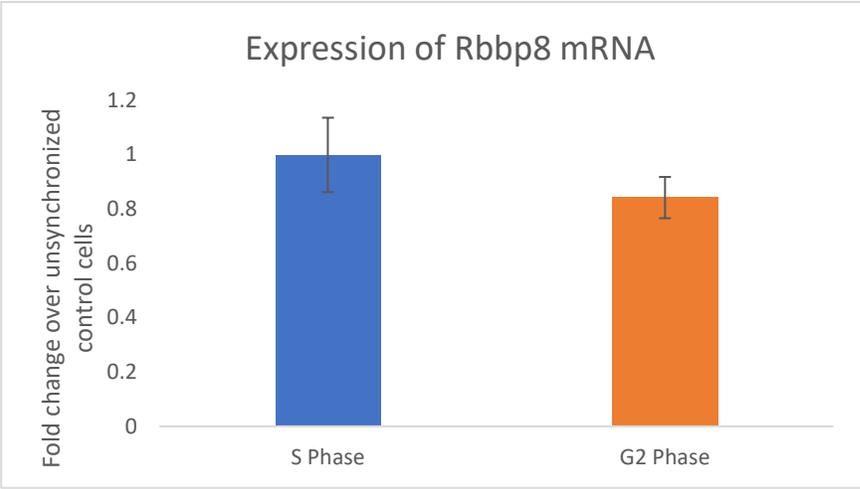

Figure 6: Fold change in expression of BRCA1 mRNA in S and G_2 phases. RT-qPCR results of five trials underwent $2^{-\Delta\Delta CT}$ analysis against unsynchronized controls, after being normalized to GAPDH.
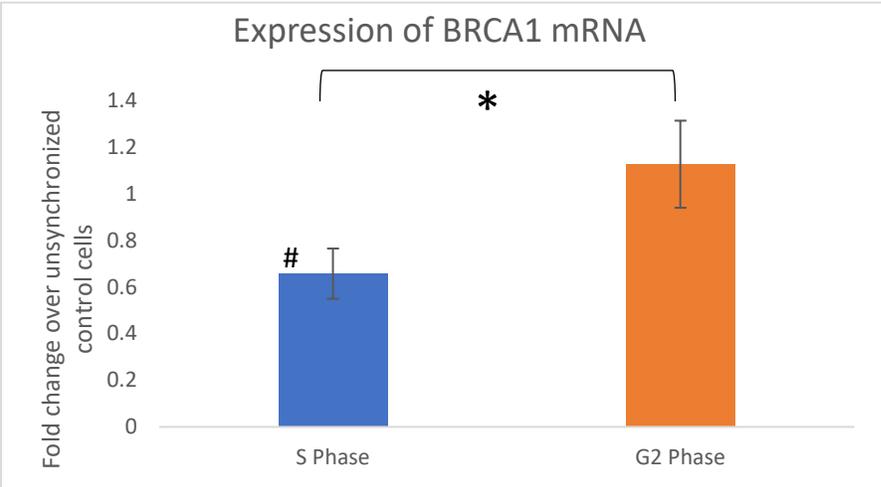# The amount of mRNA of BRCA1 during S phase is statistically less than 1 ($p<0.05$).
* the amount of BRCA1 mRNA in S phase was significantly less than in G_2 phase ($p<0.05$).

DISCUSSION

Homologous recombination is essential to the preservation of our genetic code and integrity of our DNA. In order to resolve the discrepancies in the current literature about the cell cycle-dependent complex of BRCA1 and Rbbp8 in HR, cells were synchronized and mRNA expression levels of both genes were measured, following conversion to cDNA and real-time quantitative polymerase chain reaction. After analysis with the $2^{-\Delta\Delta CT}$ method, the Rbbp8 RT-qPCR data indicate that the mean levels of this mRNA are fairly constant through S and G_2. In contrast, the mean mRNA levels of BRCA1 are significantly higher in G_2 phase than in S phase. We would expect the protein levels to also be higher during G_2, which supports the conclusion that the BRCA1-Rbbp8 complex is only active during the G_2 phase of the cell cycle. However, the constant levels of Rbbp8 suggests that it is active in the cell during S phase.

These results are consistent with the conclusions drawn by Sartori *et al.*, that homologous recombination repair occurs during S and G_2 phase, but interaction between BRCA1 and Rbbp8 is only possible during G_2. The consistent levels of Rbbp8 during both S and G_2, and the lack of difference between these levels and the control, show that the expression of this gene is fairly constant. The increase in expression of BRCA1 between S and G_2 shows that this gene is more actively transcribed during G_2. However, since the focus of this study was the mRNA expression levels of these genes, it is not possible to draw concrete conclusions about the protein levels of Rbbp8 and BRCA1.

According to the paper published by Yu and Chen, the BRCA1-Rbbp8 complex is only active during G_2, and is required for the G_2/M cell cycle checkpoint, in order for the cell to begin

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mitosis. The levels of Rbbp8 and BRCA1 mRNA dispute the claims made by Escribano-Díaz *et al*., Chen *et al*., and Yun *et al*. These researchers all concluded that following phosphorylation of Rbbp8 during S and G_2, the protein interacted with BRCA1 to perform end resection required for homologous recombination. The choice in this thesis to study mRNA expression of BRCA1 and Rbbp8 rather than protein levels was due to a few of the previous protocols that were referenced. While many of the publications referenced in Table 1 investigated protein levels using immunoprecipitation, others were found to be more feasible for the time and resources available. This study was interested in mRNA levels and we believe some inferences about protein levels can be made from these results. However, more experiments would need to be done to test the activity of other proteins involved homologous recombination and confirm the results from this study that support that BRCA1-Rbbp8 interaction occurs only during G_2.

This study was limited by the controls available. Due to insufficient resources, synchronization in G1 phase or mitosis (M) was not possible as a control. Ideally, the levels of BRCA1 and Rbbp8 in S and G_2 phases would have been compared to the levels of G1 or M. It is known that Rbbp8 participates in MMEJ during G1 (Escribano-Díaz et al., 2013). So, the levels of Rbbp8 are more difficult to gauge based on the unsynchronized control than BRCA1. Comparing to the unsynchronized cell mRNA levels is difficult because the cells are all in different places in the cell cycle and each have different expression levels of the genes.

Ideally, technology such as a cell cytometers or fluorescence activated cell sorting can distinguish and separate the cells based on their current phase of the cell cycle. In addition to these techniques, using a gene that has known and measured levels of expression during

the phases of the cell cycle as a positive control would have allowed confirmation that synchronization has occurred. Unfortunately, it was not possible to locate a reliable gene with these recognized and measured levels. However, the method of synchronization used for the L cells was adapted and optimized from a well-established method of synchronization used on HeLa cells with great success. For this reason, there is high confidence in the synchronization of cells in S and G_2 phases in this experiment.

Future studies could investigate if these results would apply to human cells also, by replacing the L cells with a human cell line, such as U2OS or HeLa cells. Conducting the same experiment to measure the levels of Rbbp8 and BRCA1 in human cells would validate these results and allow them to be applied in the future with a higher degree of confidence. Since this protocol only replicated one of the methods of synchronization of cells, conducting multiple experiments using the different methods of synchronization used may give insight to if the method of synchronization has an effect on the findings.

Also, investigation of the protein levels of BRCA1 and Rbbp8 would allow more concrete findings as to the activity of these proteins, as this study investigated expression of the genes. Measuring phosphorylation levels of serine 327 of Rbbp8 during the different phases of the cell cycle may confirm the interaction of this protein with the BRCA1 C-terminal domain (BRCT) during specific phases of the cell cycle.

An additional future project based on this research could examine how specific changes to the levels of BRCA1 and Rbbp8 affect the genome and progression of a cell through the cell cycle by downregulating the expression of these genes or creating knockouts. Tracking of the

cell through the cell cycle or sequencing of the genes to detect the rate of mutations would allow for a deeper understanding of the role that they play in the cell.

With a clearer understanding of the activity of BRCA1 in complex with Rbbp8 in HR, we would be able to understand the effects of the harmful mutations that affect the BRCT domain. In learning how these genes function to promote cell growth and division in healthy cells, we can gain a better understanding of the repercussions of what occurs when their function is impaired. Knowledge of these proteins function and activity can direct the search for more targeted therapies and treatments to correct the malfunctions when they may occur.
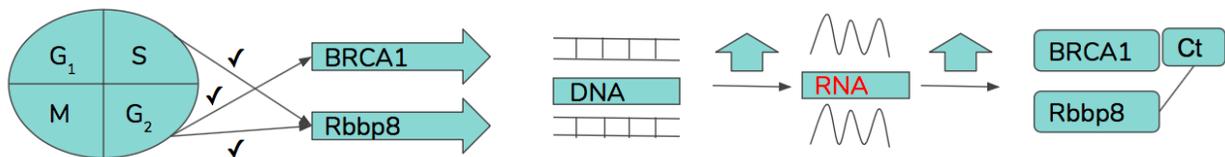

Figure 7: Confirmed pathway of expression of BRCA1 and Rbbp8.

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