The Impact of BRCA2 on Homologous Recombination and PARP Inhibitor Sensitivity Examined in *BRCA2* Heterozygous Cell Lines

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HÁSKÓLI ÍSLANDS

Áhrif BRCA2 á viðgerð með endurröðun og PARP hindrun í *BRCA2* arfblendnum frumulínum

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Ágrip

Erfðamengið verður stöðugt fyrir DNA skemmdum, bæði vegna efnaferla sem eiga sér stað í frumunni og líka vegna utanaðkomandi þátta. Allar frumur eru háðar öflugum DNA viðgerðarferlum til þess að hægt sé að gera við þessar skemmdir. Endurröðunarviðgerð er ein þessara viðgerðarleiða og brjóstakrabbameinsgenið *BRCA2* tjáir fyrir lykilpróteini í slíkri viðgerð. PARP1 er annað prótein sem tekur þátt í DNA viðgerð og sýnt hefur verið fram á að frumur með stökkbreytingar í *BRCA2* séu næmar fyrir PARP hindrum og fari í stýrðan frumudauða eftir slíka meðhöndlun. Vitað er að PARP hindrar hafa áhrif á krabbameinsæxli þar sem ekkert BRCA2 prótein er til staðar án þess að hafa áhrif á eðlilegan vef sem enn hefur báðar samsætur *BRCA2*. Hins vegar er lítið vitað um áhrif PARP hindra á *BRCA2* arfblendin æxli og á *BRCA2* arfbera. Á Íslandi er ein landnema stökkbreyting í *BRCA2* sem nefnist *999del5* og finnst hún í u.þ.b. 6-7% þeirra sem greinast með brjóstakrabbamein. Markmið þessarar rannsóknar var að nota *BRCA2* arfblendnar frumulínur til þess að skoða áhrif *999del5* stökkbreytingar á endurröðunarviðgerð og næmni fyrir PARP hindrum. Helstu niðurstöður rannsóknarinnar eru að frumulínur arfblendnar fyrir *BRCA2* eru ekki næmar fyrir PARP hindrun en virðast hafa minnkaðan hæfileika til að takast á við DNA skemmdir með endurröðunarviðgerð. Því er líklegt að frumur einstaklinga sem eru arfberar fyrir *999del5* stökkbreytingunni séu ekki næmar fyrir PARP hindrum þó svo að hæfileikinn til viðgerðar sé hugsanlega eitthvað minni. Hins vegar verður að hafa í huga að galli í endurröðunarviðgerð getur orðið þess valdandi að stökkbreytingar safnist upp á löngum tíma, sem hugsanlega skýrir hvers vegna þessir einstaklingar eru í aukinni áhættu á því að fá krabbamein.

Abstract

The genome is under constant assault from exogenous and endogenous forces that can cause aberrations within the DNA. Cells depend on competent DNA repair systems to deal with these aberrations, one of these being homologous recombination. BRCA2, a protein long implicated in breast cancer, plays a key role in carrying out successful homologous recombination. PARP-1, another protein involved in DNA repair, has recently become the target of cancer therapy by exploiting the synthetic lethality observed when PARP1 is inhibited in the absence of BRCA2. This treatment has shown to be effective in treating tumors that are completely deficient in BRCA2 and having little impact on normal tissue containing two functional copies of *BRCA2*. However, little is known about the effect of PARP inhibitors on heterozygous tumors and normal tissue of *BRCA2* mutation carrier. In Iceland, a founder mutation in *BRCA2* called *999del5* is found within the population and is present in a significant amount of breast cancer patients. This study looked at the impact being a carrier for the *999del5* mutation has on the ability to respond to a PARP inhibitor and the capability for successful homologous recombination. This was done through the use of a *BRCA2* heterozygous epithelial breast cell line. The results show that cells heterozygous for *BRCA2* are tolerant to PARP inhibition and display competent yet diminished ability for homologous recombination. In conclusion, cells from individuals heterozygous for *999del5* are likely to be tolerant to PARP inhibition, despite slightly impaired HR. However, a minor impairments in HR over a lifetime might result in the accumulation of mutation, possibly explaining the increased risk of tumor formation in these individuals.

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List of Abbreviations

A176 ....……….. Epithelial Breast Tissue cell line containing Icelandic Founder mutation *999del5*, refered to literature as cell line *BRCA2-999del5-1N*

ATR... Ataxia Telangiectasia and Rad3 related protein

ATRIP... ATR Interaction Protein

BER……………………………………………………………….Base Excision Repair

BRCA1...Breast Cancer 1, Early Onset

BRCA2... Breast Cancer 2, Early Onset

CldU………………………………………………….......5-Chloro-2′-deoxyuridine

DDR………………………………………….......……… DNA Damage Response

DMEM…………………………………………Dulbecco's Modified Eagle's Medium

DNA.. Deoxyribonucleic Acid

DSB………………………………………………………… Double-Stranded Break

FBS...Fetal Bovine Serum

G1...Growth 1 phase of cell cycle

G2...Growth 2 phase of cell cycle

ICL..Interstrand-Crosslinks

IdU...5-Iodo-2′-deoxyuridine

IF..Immuno-Fluoresence

HR……………………………………......……….……… Homologous Recombination

HU……………………………………………………………………..Hydroxyurea

MRN Complex.............Protein Complex Composed of MRE11, RAD50 and NBS1

NHEJ …………….…….……………………… Non-Homologous End Joining

PAR.. Poly(ADP-ribose)ylation

PARP…………………………………………….Poly ADP ribose polymerase

PARPi…………………………………………………………….PARP Inhibition

PBS...Phosphate Buffered Saline

RNA…………………………………………….…………Ribonucleic Acid

RNAi……………………………………………………………..RNA interference

RPA …………………………………………………………… Replication Protein A

RPMI..Roswell Park Memorial Institute

S-Phase..Synthesis phase of cell cycle

shRNA...short-hairpin RNA

SSB………………………………………...…………………Single-Strand Break

XRCC1…………………………………X-ray repair cross-complementing protein 1

γH2AX………………………………………….……...Phosphorylated Histone 2AX

1 Introduction

One of greatest threats to cell survival is genomic instability, which is characterized by accumulation of mutations that can lead to the formation of a disease such as cancer. There is an abundance of ways cancers can form, and many are a result of mutations within DNA. How these mutations occur can vary wildly. Therefore, cancer is not simply one disease, but many with different origins. For example, it can be hereditary, where mutations are being passed down to the offspring from the parents. They can appear spontaneously through imperfections in normal cell processes such as DNA repair or DNA replication. They can also be acquired by interaction with our environment, with mutagenic agents existing in the food we eat or the air we breath. DNA damage is usually repaired by using highly sophisticated repair mechanisms found in all cells (Hoeijmakers, 2009). However, if these repair mechanisms are not functioning properly DNA alterations are able to persist, possibly leading to mutations. If mutations exist within so-called oncogenes or tumor suppressor genes, they may cause the proteins produced by these genes to malfunction. Losing function in tumor suppressor genes could mean that cellular growth becomes unchecked and a tumor may form that could ultimately result in the death of the individual. It is therefore of paramount importance to repair DNA damage before cells divide. In order to deal with DNA damage, the cell has developed a multitude of pathways in order to repair the DNA. Homologous recombination (HR) has become recognized as a key pathway of DNA repair in mammalian cells (Beneke et al, 2000). HR has key role in the repair of some of the most serious forms of DNA damage, one of which comes in the form of double stranded breaks (DSBs). The process of DSB repair and more specifically HR involves a multitude of proteins, each with distinct roles.

This thesis examines the role of a hereditary mutation within *BRCA2,* a gene essential for HR, and what possible consequences result from it. The aim is to use cells from individuals with one functional *BRCA2* allele and examine their ability to efficiently perform HR and study how they respond to certain treatments. These findings could be important for designing more effective and safe treatment for *BRCA2* mutation carriers.

1.1 Cancer

Cancer is the leading cause of death worldwide, accounting for 8.2 million deaths in 2012 (World Health Organization, "Breast Cancer: prevention and control", 2015). Breast cancer specifically, was responsible for 521,000 of those deaths and is the most common cancer among women. In Iceland alone, more than 200 women are diagnosed with breast cancer each year. This accounts for about 30% of all cancer diagnoses in Icelandic women (Krabbameinsskrá, "Brjóstakrabbamein", 2012). The formation of breast cancer can be the result from a multitude of factors, one of which is hereditary mutations. *BRCA1* and *BRCA2* are genes that have been highly linked to hereditary breast cancer. One *BRCA2* founder mutation is found in the Icelandic population. This mutation accounts for 6-7% of all breast cancer patients and 40% of males with breast cancer in Iceland (Thorlacius et al, 1997; Tryggvadottir et al, 2006). A mutation within *BRCA2* results in a decreased HR ability, possibly allowing any DSBs that occur to persist or the DNA to be repaired by more error prone methods.

1.2 Double-Stranded Breaks

DSBs are an incredibly lethal form of DNA damage that cause severe genomic instability. Merely a couple of unrepaired breaks may lead to cell death (d'Adda, 2008). DSBs are particularly harmful because they increase the likelihood of large scale genomic rearrangements such as chromosome translocations, deletions or insertions. When the DNA is improperly repaired, the breaks may leave behind a mutation that can potentially aide in the formation of a cancer by methods such as inactivation of a tumor suppressor gene or the activation of an oncogene that can potentially aide in the formation of cancer. Because of this, accurate and quick repair is needed. The primary processes involved in this DSB repair are HR and non-homologous end joining (NHEJ) that can both be error free and error prone (Betermier et al, 2014). However before any repair pathways can take effect, the cell needs to recognize the presence of a DSB within the DNA.

1.2.1 Detection of Double-Stranded Breaks and DNA Damage Response

There are multiple pathways used for the detection and the transduction of the signal to activate DNA repair proteins in response to DSBs. This detection, signal transduction and repair are collectively known as DNA damage response (DDR). The primary method of detecting DSBs is through the MRN complex, which is composed of MRE11, RAD50, and, NBS1 (MRN). The MRN complex is responsible for initially recognizing the presence of a DSB within the DNA and then recruiting the Ataxia Telangiectasia Mutated (ATM) kinase that phosphorylates several other key proteins triggering the DNA damage checkpoint in the cell cycle (Paull & Lee, 2005). The 5' ends of the DSB are resected resulting in the formation of a 3' single-stranded overhangs that are coated by Replication Protein A (RPA). RPA is recognized by BRCA2, which initiates HR, as well as ATR Interaction Protein (ATRIP) that recruits Ataxia Telangiectasia and Rad3 related protein (ATR). Activated ATR phosphorylates Chk1 that causes a signal cascade leading to arrest of the cell cycle (Zou & Elledge, 2003). It is of paramount importance to stop the cell cycle when DNA damage is present. This allows the cell to repair the DNA damage and if the damage is extensive undergo programmed cell death or apoptosis(Figure 1). When DDR or cell cycle checkpoints malfunction the damage is allowed to persist within the DNA, which could be detrimental to the cell and the host (Polo & Jackson, 2011).
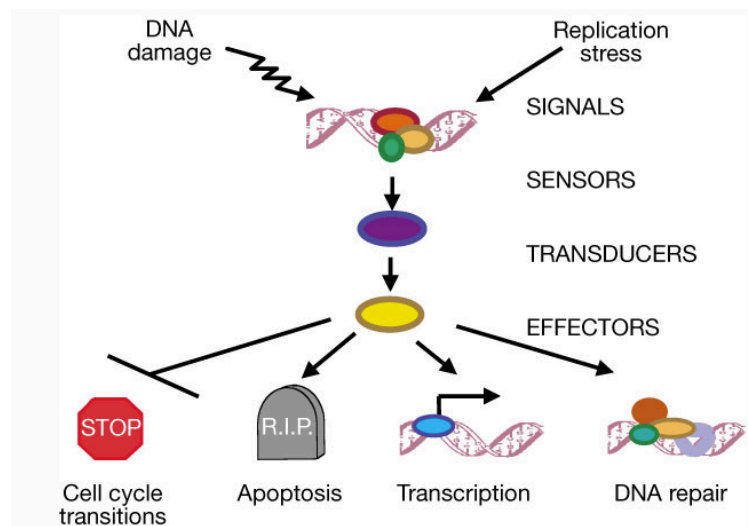
Figure 1: Possible outcomes of DNA damage response. DNA damage or replication stress causes a signal cascade with multiple potential outcomes including cell cycle arrest, apoptosis, transcriptional changes and DNA repair. Modified from Zhou & Elledge, 2000.

Along with checkpoint proteins, ATM phosphorylates histone H2AX on serine 139, changing it into the phosphorylated γH2AX. While phosphorylated, γH2AX will spread to the chromatin around the DSB (Rogakou et al, 1999). This amplifies the response to the DSB. Functionally γH2AX is believed to play a role in the recruitment of repair factors to the sites of DNA damage (Celeste et al, 2002). γH2AX is an important protein because it is frequently used as a marker for the presence of DNA DSBs.

1.2.2 Repair of Double-Stranded Breaks

DSBs are primarily repaired by two pathways; HR and NHEJ. During NHEJ non-homologous ends of the DNA are simply ligated together. In contrast to HR, NHEJ is frequently associated with small deletions due to nucleases processing prior to the ligation of the DNA ends, resulting in loss of genetic information (Kass & Jasin, 2010).

Recently an alternative NHEJ repair pathway was described but its mechanism of repair is not fully understood yet and is seen as the tertiary method should the other two fail. The classical NHEJ typically uses microhomologies present on single-stranded overhangs present at the DSB. Similarly to HR, alternative NHEJ is characterized by resection of the of the DNA ends at the break, revealing short homologous sequences between the two chromosome ends. This will result in small deletions and therefore this repair pathway is considered to be error prone (Ceccaldi et al, 2015; Mateos-Gomez et al, 2015). Cells in the G1 phase of the cell cycle use NHEJ due to the lack of a sister chromatid. However, HR is believed to be error-free and is therefore the "preferred" method by cells in late S to G2 phase when a sister chromatid is available. It involves invasion of the broken chromosome into corresponding homologous sequence on the intact sister chromatid and using that sister chromatid as a template for the repair of the damage sequence.

Should HR fail or be unable to occur, the cell will turn to other forms of DSB repair, typically NHEJ. Because of the error prone nature of the alternative NHEJ method it is critical that HR is intact in mammalian cells.

1.3 BRCA2 and RAD51 in Homologous Recombination

Following a DSB, the single-stranded 3' overhangs are coated by RPA, forming a nucleoprotein filament. RPA is responsible for preventing the DNA both from forming potentially harmful secondary structures and from degradation by single stranded nucleases (Chen et al, 2013). BRCA2 recognizes RPA and binds to the end of the single strand and via protein protein interactions bringing along the recombinase RAD51, a key protein in HR. BRCA2 then facilitates the loading of RAD51 onto the DNA, displacing RPA. The RAD51 nucleoprotein filament, along with other proteins, then facilitates the pairing of the broken chromosomal end with undamaged sister chromatid and stimulates the invasion

into the sister chromatid followed by DNA repair synthesis, using the intact sister chromatid as a template (Wilson & Elledge, 2002). This process is shown in Figure 2.
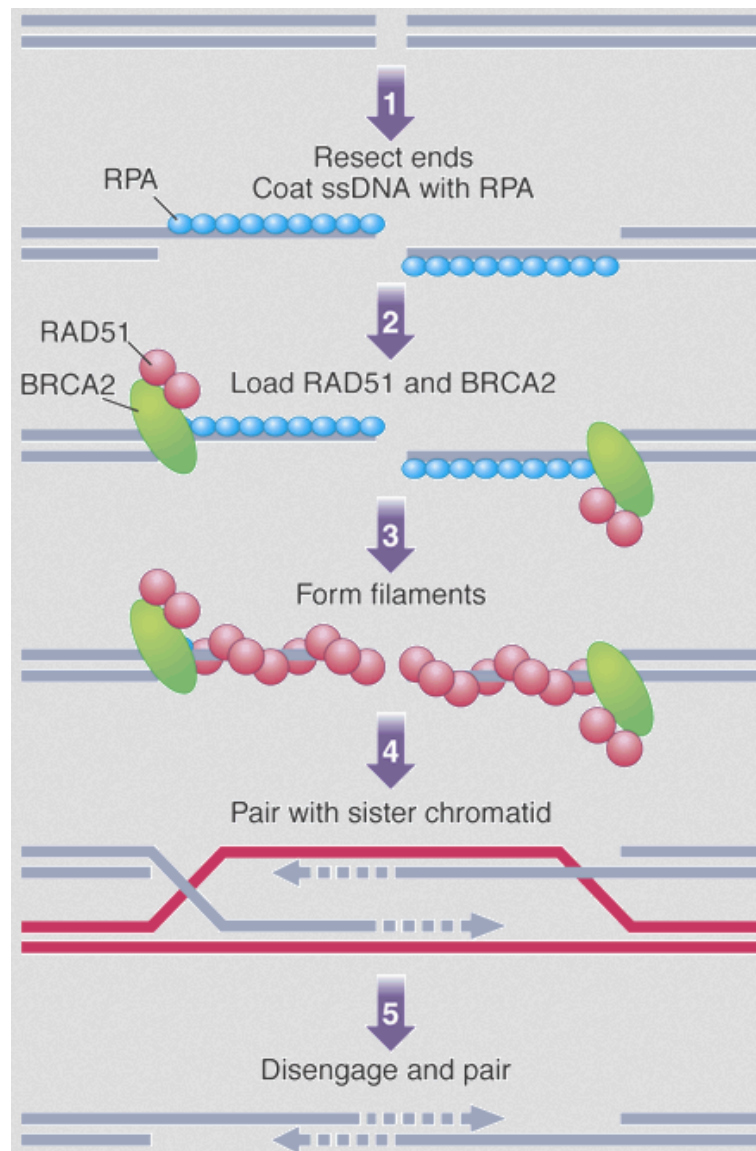
Figure 2: Illustration of homologous recombination. RPA first binds to the resected ends of DNA. RPA is then recognized by BRCA2, which loads RAD51 onto the nascent strands. RAD51 then mediates the pairing and invasion of the sister chromatid followed by DNA repair synthesis. Modified from Wilson & Elledge, 2002.

RAD51 is entirely dependent on BRCA2 for its nuclear import (Jeyasekharan et al, 2013) and loading onto the DNA. BRCA2 contains 8 BRC repeats within the protein (figure 3). These BRC repeats are where RAD51 binds to the protein and is then brought with BRCA2 into the nucleus for loading onto the single stranded tails. In the case of a mutation within the BRC repeats RAD51 is unable to efficiently bind to BRCA2 and the nuclear import of RAD51 is decreased. Similar process is

described in figure 4. In the absolute abscence of functional BRC repeats, which could be caused by loss of function mutations or mutations leading to the degradation of BRCA2, the cell is unable to form RAD51 nucleoprotein filaments and HR can not take place (Jeyasekharan et al, 2013; Jensen et al, 2010). The question is if this same principle applies when a heterozygotic *BRCA2* mutation causes its degradation, meaning there will be less BRCA2 to import RAD51. Will a single allele allow for sufficient RAD51 binding?
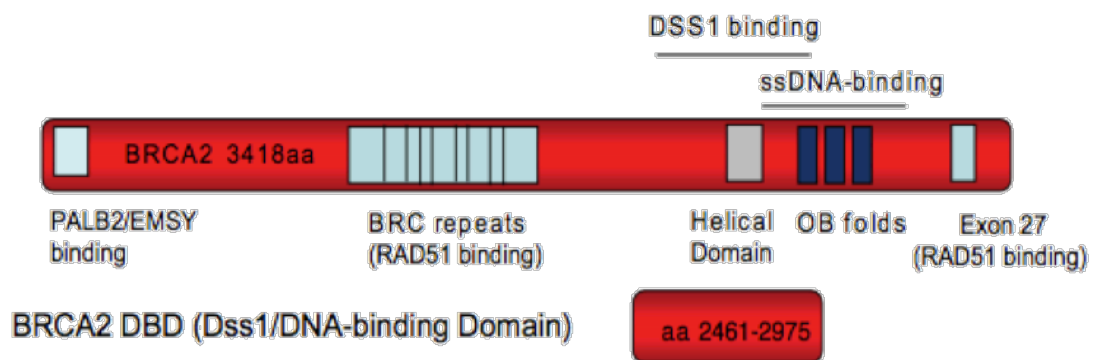
Figure 3: The BRCA2 protein and it's binding domains. BRC repeats are where RAD51 is bound for import into the nucleus. Modified from Jeyasekharan Supplementary, 2013.
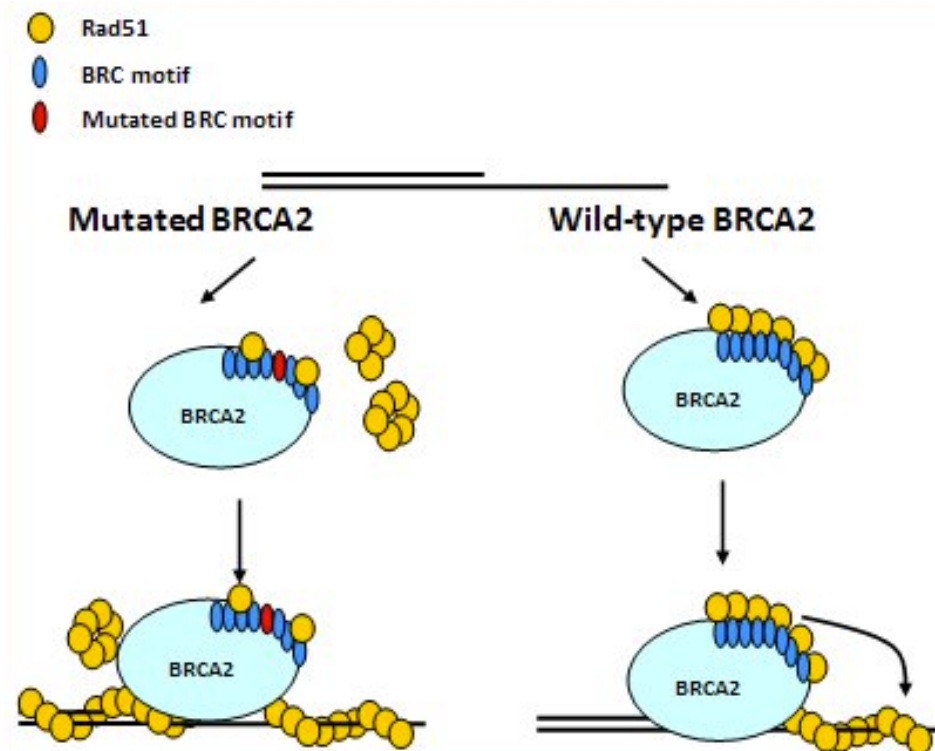
Figure 4: Nuclear import and loading of RAD51. A schematic showing that mutations within BRCA2 lead to decreased loading of RAD51 onto the DNA due to decreased import. This mutation lies within the BRC repeats which cause less RAD51 binding. Modified from Tal et al, 2009.

After exposure to ionizing radiation or other DNA-damaging agents RAD51 re-localizes within the nucleus to form distinct foci where repair takes place. By examining these foci one is able to determine cells ability to undergo HR. Because of the close link between BRCA2 and RAD51, we can use RAD51 foci formation to measure the impact of a mutation in BRCA2 on the cell's ability to perform HR (Tarsounas et al, 2003).

BRCA2 is also involved in the protection of the replication fork when stalled following cellular or replicative stress. When the replication fork is stalled, it may reverse in an attempt to restart leaving nascent DNA strands exposed. MRE11, an endonuclease and exonuclease, will degrade these nascent DNA strands should they be left unprotected. BRCA2 ensures that RAD51 filaments are loaded onto the nascent strands to ensure they are not degraded following a stalled replication fork (Schlacher et al, 2011). Due to its function in HR and protection of the replication fork, BRCA2 is crucial for maintaining chromosomal stability. The fiber assay is a method used to study the cells ability to replicate their DNA. One can incorporate nucleotide analogs and measure the ability of cells to restart replication following stalling induced by drugs such as Hydroxyurea (HU) that slows down replication. After cell lysis, the DNA is then spread on a coverplate and fluorescent antibodies against the analogs can be used to then measure replication ability using a microscope (Schlacher et al, 2011).

In addition, cells deficient in BRCA2 have been shown to suffer from faulty cell division. Improper division can cause chromatid breaks which can lead to chromatid exchanges such as translocations (Gretarsdottir et al, 1998). Lack of BRCA2 can also lead to aneuploidy with examples of 4N DNA content and aneuploidy found in cancer cells from *BRCA2* mutation carriers, suggesting BRCA2 has a role during mitotic progression (Jonsdottir et al, 2012; reviewed in Venkitaraman, 2014).

1.4 Icelandic Founder Mutation *BRCA2 999del5*

Like mentioned previously, one founder mutation in *BRCA2* is found in the Icelandic population. This mutation is a five base pair deletion (*999del5*) in exon 9 leading to an early truncation of the BRCA2 protein (Thorlacius et al, 1996). This truncated protein is degraded within the cell (Mikaelsdottir et al, 2004). Previously, cell lines derived from breast epithelial tissue and of breast cancer patients carrying the *999del5* mutation were established (Rubner-Fridriksdottir et al, 2005). One of these cell lines derived from adjacent normal epithelium of a tumor, A176, was selected for use in this study to examine the effects of a PARP inhibitor (olaparib). This cell line was selected due to its competence in growth and capability to be used for immuno-fluoresence (IF) studies.

1.5 PARP-1 in Repair

Poly(ADP-ribose)polymerase 1 or PARP-1 has been implicated to be involved in multiple forms of DNA repair including single strand breaks (SSBs), DSBs, interstrand crosslinks (ICLs), alternative NHEJ and protection of stalled replication forks (Beck et al, 2014). PARP proteins are known to be responsible for Poly(ADP-ribose)ylation (PARylation), which is the covalent modification of proteins with ADP-ribose polymers (PAR) (Hakme et al, 2008). Following DNA damage, PARP-1 is recruited to the site of the lesion where it catalyzes PARylation. This is in turn responsible for recruiting many other proteins, including those involved in SSB repair (Haince et al, 2007). After forming PAR, PARP-1 then loses affinity and dissociates from the DNA. These PAR chains are detected by XRCC1, a SSB repair protein through base excision repair (BER), and PARG. The XRCC1 protein accumulates at SSBs only when PARP-1 is present in a cell and does not accumulate in its absence (Fisher et al, 2007). PARG is thought to be acting as an inhibitor of XRCC1 binding preventing over-accumulation of XRCC1 and blocking efficient SSB repair (Fisher et al, 2007). In the absence of PARP-1, cellular PAR is reduced to nominal levels, decreasing binding of PARG and XRCC1 and therefore greatly hindering SSB repair via PARP-1.

PARP-1 has also been shown to play a role in other DNA repair processes. PARP-1 and PARylation were found to be important in DSB repair with PARP-1 shown to bind to and be activated by DSBs (Audebert et al, 2004). Recently, PARP-1 has been implicated as a key protein in alternative NHEJ and HR (Ceccaldi et al, 2015).

1.6 Knudson Two-Hit Hypothesis

This theory was initially proposed by Carl Nordling in 1953 and improved upon by Alfred Knudson in 1971 with regards to Retinablastoma. The basic principle behind the theory is that an inactivating mutation within one gene will count as a "hit" against the cell. This mutation could either be acquired during the lifetime of the cell or inherited from a parent. Another "hit" to the same gene on the second allele will cause the gene to be knocked out completely and may then be directly responsible for the formation of a cancer. This is described in figure 5.

In the case of the *BRCA2 999del5*, mutation carriers are heterozygous for the mutation and according to the Knudsons two-hit hypothesis loss of the second allele would be needed for tumor formation. However, carcinogenesis is far more complicated than simply lacking a *BRCA2* allele since its absence does not mean the formation of cancer is certain. In fact, tumor formation can occur in heterozygous *BRCA2* mutation carriers without loss of the functional wild type allele (Skoulidis et al, 2010; Stefansson et al, 2011). However, whether or not these cancers result from deficiency in *BRCA2* or another gene is difficult to prove. This raises the question of whether or not gene expression from one functional *BRCA2* allele is sufficient to suppress tumor formation.
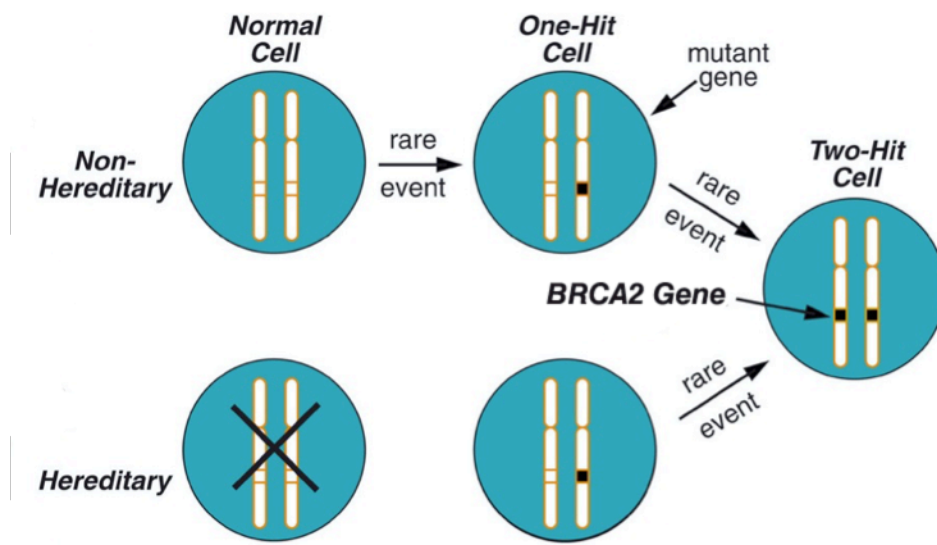
Figure 5: Knudson two-hit hypothesis illustrated. Mutations either are acquired or inherited (such as *BRCA2* mutation carriers) to form a cell with only one functional allele (one-hit cell). If a mutation occurs in the remaining functional allele, the cell will become a two-hit cell and this may lead to the formation of a disease. Modified from ("Knudson's Two-Hit Theory of Cancer Causation", 2015)

1.7 Haplo-sufficiency and Haplo-insufficiency

When a diploid cell has only a single functional copy of a gene without showing a phenotype it is considered to be haplo-sufficient. In this case the single functional allele produces enough protein to bring about wild-type conditions. Haplo-insufficiency, on the other hand, occurs when a single functional allele does not produce enough protein for the cell. Therefore, the function of the gene will be impaired, typically being detrimental to the cell.

In this thesis, cells heterozygous for *BRCA2* are used to examine how efficiently and effectively they perform HR when containing a non-functional allele of the *BRCA2* gene.

1.8 Synthetic Lethality and PARP Inhibition

Synthetic lethality is the concept that when a gene is knocked out, the knocking out of a second gene will prove lethal to the cell. In this case we are looking at the synthetic lethality between the genes *BRCA2* and *PARP-1*. BRCA2 is responsible for the loading of RAD51 on DNA that has undergone a DSB and go through HR. When a cell lacks BRCA2 it is unable to perform HR and can therefore no longer use this form of error-free DSB repair. When PARP-1 is inhibited, it allows for the accumulation of single stranded breaks that when allowed to remain may develop into DSBs. The synthetic lethal relationship between BRCA2 and PARP-1 can be seen in that inactivation of both pathways emerges in a synergistic effect on cellular survival (Farmer et al, 2005; Bryant et al 2005).

PARP inhibition is a target of cancer therapy due to the multitude of roles PARP plays in repair pathways. The effectiveness of PARP inhibitors is dependent on defects in DNA repair by HR repair (McCabe et al, 2006). This has been particularly looked into for individuals with tumors deficient in *BRCA1* or *BRCA2* as a method of synthetic lethality (Fong et al, 2009). Cells deficient for BRCA2 are hypersensitive to PARP inhibition; however, little is known about the sensitivity in cells heterozygous for *BRCA2*. This thesis will attempt to shed light on whether cells with a single functional copy of *BRCA2* are resistant or sensitive to PARP inhibition.

Olaparib is the PARP-1 inhibitor used in these experiments. Olaparib has been used in multiple phase I and phase II studies. It has shown promising effects in *BRCA2* deficient ovarian cancers (Ledermann et al, 2012; Ledermann et al, 2014; Oza et al, 2014; Clamp & Jayson, 2015) as well as in BRCA2 patients with advanced breast cancer (Tutt et al, 2010). In summary, tumor cells from *BRCA1* and *BRCA2* patients show synthetic lethality and are likely to help in the prevention of tumor progression following Olaparib treatment

By inhibiting PARP-1*,* we aim to exploit the synthetic lethality between *PARP-1* and *BRCA2*. By doing this within cell lines that display wild-type, knock-out and *999del5* carrier phenotypes, we can gain insight into how carriers for the BRCA2 mutation may be affected by treatment with this drug.

2 Aims

BRCA2 is essential for DNA repair through HR. BRCA2 deficient tumors are not capable of efficient HR repair and are sensitive to PARP inhibition. In Iceland, the founder mutation *999del5* in the *BRCA2* gene has been linked to breast and ovarian cancer. The minor allele frequency of this frameshift deletion is about 0.4% in the population that corresponds to nearly 2400 individuals being heterozygous for this mutation (Gudbjartsson et al, 2015). Carriers for the mutation are completely normal and appear to be haplo-sufficient with regards to the *BRCA2* allele. They do, however, have greatly increased risk of certain types of cancer. This might suggest they are haplo-insufficient to some extent and because of minor defect in HR might accumulate mutations over a lifetime due to channeling of DSBs into other error-prone pathways. Currently we do not know how efficiently cells from individuals with one functional *BRCA2* allele perform HR, or how they respond to PARP inhibition. The aim of this study is to examine the capacity of heterozygous *BRCA2* cell lines to repair DSBs by HR and to study the sensitivity towards PARP inhibitors. These findings could be important for designing more effective and safe treatment for *BRCA2* mutation carriers.

The specific aims of this thesis are therefore:

1. Establish a *BRCA2* knockdown variant of the A176 cell line, a heterozygous mammary cell line carrying the *999del5* mutation, using a Lentiviral system.
2. Observe the ability of the *BRCA2* heterozygous cell line in forming RAD51 foci following treatment with Olaparib compared to *BRCA2* wild-type and deficient cells, thus giving insight into its capability for HR.
3. See what effect treatment with Olaparib has on survival in the heterozygous cell line, with and without knockdown.
4. Try to determine if PARP inhibitors should be considered as a treatment option for carriers of mutated *BRCA2*.

3 Materials and Methods

3.1 Cell Cultures

The heterozygous cell line used in this study was derived from tumor adjacent mammary epithelium of a carrier of the Icelandic *999del5* mutation (as described in the introduction) in the *BRCA2* gene. This cell line, named A176 (*BRCA2-999del5-1N*), was E6/E7 transformed and shows epithelial characteristics (Rubner-Fridriksdottir et al, 2005). It was cultured in H14 media, which is composed of DMEM (Dulbecco's Modified Eagle's Medium) with insulin (250 ng/ml), transferrin (10 mg/ml), sodium selenite (2.6 ng/ml), estradiol (10^{10} M), hydrocortisone (1.4×10^{-6} M), prolactin (5 mg/ml), and epidermal growth factor (10 ng/ml). CAPAN1 is a metastatic pancreatic cancer cell line used as a negative control (-/-) for *BRCA2*. This cell line is lacking functional BRCA2 protein (Goggins et al, 1996). CAPAN1 was cultured in DMEM with 20% Fetal Bovine Serum (FBS), penicillin (100 U/mol) and streptomycin (1.5 μg/mL). MCF7 is a breast cancer cell line used as the positive control (+/+) for *BRCA2*. MCF7 was cultured at 37°C in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% FBS, penicillin (100 U/mol) and streptomycin.

3.2 Lentiviral Knockdown of BRCA2

From A176 two lentiviral lines were derived: A176 with a scramble vector shRNA and A176 with a *BRCA2* shRNA. This was done through the use of a lentiviral system (Thermo Scientific). Both lentiviral lines were grown in the aforementioned H14 with puromycin (1.5 ug/ml). Lentiviral oligos were ordered from Sigma Aldrich. Lentiviral vectors expressing short hairpin RNAs (shRNA) against *BRCA2* were synthesized as follows (table 1).

Table 1. *Primers used for synthesis of lentiviral shRNA*

Primer	Gene	Forward	Reverse
BRCA2 shRNA	*BRCA2*	5'- CCG GTA TAC AGG ATA TGC GAA TTA ACT CGA GTT AAT TCG CAT ATC CTG TAT ATT TTT G -3'	5'- AAT TCA AAA ATA TAC AGG ATA TGC GAA TTA ACT CGA GTT AAT TCG CAT ATC CTG TAT A -3'
Scramble shRNA	*Non-targeted*	5'- CCG GTC TAA GGT TAA GTC GCC CTC GCT CGA GCG AGG GCG ACT TAA CCT TAG GTT TTT G -3'	5'- AAT TCA AAA ACC TAA GGT TAA GTC GCC CTC GCT CGA GCG AGG GCG ACT TAA CCT TAG G -3'

Both the *BRCA2* and scramble sequences were cloned into a pGIPZ vector containing a puromycin resistance marker (figure 6). Table 2 shows the components of the lentiviral vector represented in figure 6. Instructions on the creation of the lentiviral vectors were followed from Thermo Scientific Open Biosystems Expression Arrest GIPZ Lentiviral shRNAmir manual.
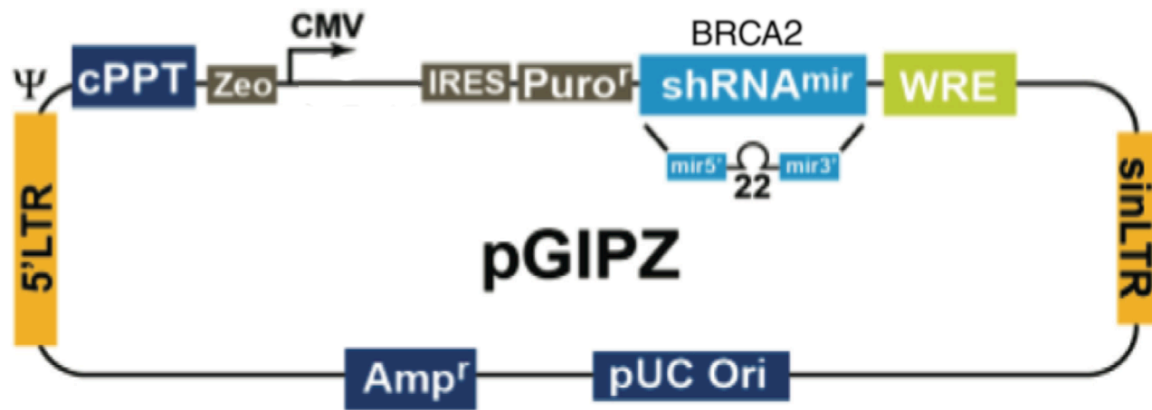
Figure 6: Schematic of the lentiviral vector containing *BRCA2* shRNA. Modified from Thermo Scientific Open Biosystems Expression Arrest GIPZ Lentiviral shRNAmir manual.

Table 2. *Components of the lentiviral vector.*

MV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
IRES-puro resistance	Mammalian selectable marker
Amp resistance	Ampicillin (carbenicillin) bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in *E. coli*
SIN-LTR	3' self inactivating long terminal repeat (Shimada, et al. 1995)
Zeo resistance	Bacterial selectable marker

3.3 PARP-1 Inhibition

We used PARP-1 inhibition drug called olaparib (AZD2281 from AstraZeneca; London, UK). The compound was diluted to a 10 mM stock solution using 99.7% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and stored at -20°C. Cells were treated with 5 µM and 10 µM concentrations of Olaparib for 96 hours without media change and compared with untreated cells incubated for the same period of time.

3.4 Immunostaining

Approximately 50,000 cells were plated on 12 mm cover slides and allowed to attach overnight. 6 slides for each cell line, thereof 3 were for untreated control and 3 for Olaparib treatment. The next day the cells' media was replaced with half of the media containing 5 µM Olaparib. The cells were then grown for 96 hours in their respective media. The cells were then fixed for 10 minutes in ice-cold methanol at -20°C and washed in PBS (phosphate buffered saline) twice. Cells were then permeabilized in 0.2% Triton x100 in PBS for 10 minutes and rinsed 3 times with PBS. Cells were then

blocked in immunofluorescent buffer (PBS + 2% FBS + 1% bovine serum albumin) for 10 minutes. The cover slides were then incubated with the primary antibodies for 3 hours. Finally, the slides were washed 3 times in PBS and incubated with the secondary antibodies for 45 minutes. The slides were then washed 3 more times in PBS followed by washing in dH$_2$O. Cover slides were allowed to dry overnight. The next day the cells were mounted to a slide with fluoromount and examined under confocal microscope (Zeiss LSM 5 Pascal). Two hundred cells from each cell line were counted three times for each condition within each cell line. Cells were counted as positive if they displayed >5 nuclear foci for the targeted protein. The experiment was repeated in triplicate. The primary antibodies used were γH2AX Mouse IgG1 (ab22551 from Abcam; UK) and RAD51 Rabbit IgG (ab63801 from Abcam; UK). Secondary antibodies were Alexaflour 488 anti-mouse IgG1 (A21121; Life technologies) and Alexafluor 546 Anti-rabbit IgG (A21434; Life technologies).

3.5 Cell Viability and Survival Assay

Approximately 5000 cells were seeded into 96 well plates overnight. Eight wells for each cell line. Cells were treated with the same conditions as before (see section on Immunostaining). Half of cells received refreshed media and the other half received media containing 5 µM or 10 µM Olaparib. After 96 hours, 20 µL of ProMega Cell Titer 96 was added. After three hours the wells were measured at 490 nm using a spectrophotometer (Spectramax Plus 384, Molecular Devices). The effects on survival are computed as ratio between the untreated and treated. Error bars were computed as the standard deviation of the ratio of two means.

$$Error = \sqrt{\frac{1}{\mu_u^2}\sigma_t^2 + \frac{\mu_t^2}{\mu_u^4}\sigma_u^2}$$

μ_u: Untreated Average Error

μ_t: Treated Average Error

σ_u: Untreated Standard Deviation

σ_t: Treated Standard Deviation

3.6 DNA Fiber-Assay

The DNA fiber-assay method is based on Schlacher et al, 2011, with slight modifications. Two million cells were seeded in a T-25 flask (BD Falcon). The next day three separate media containing either 50 mM 5-Chloro-2′-deoxyuridine (CldU, MFCD00006531, Sigma Aldrich), 50 µM 5-Iodo-2'-deoxyuridine (IdU, MFCD00134656, Sigma Aldrich) or 0.4M Hydroxyurea (HU, MFCD00007943, Sigma Aldrich) were made. Cells were first grown with the media containing CldU for 30 minutes, followed by three rinses with PBS. This was followed by 30 minutes to 6 hours of growth with media containing HU and then three rinses with PBS. The cells were then grown in the media containing IdU for 30 minutes and rinsed a final three times with PBS. The cells are then trypsinized and resuspended in PBS. 10 µl of the trypsinized cells were then spread onto a slide in a circular motion and placed on a hot plate at 40 °C. Immediately after drying, the slide was removed and placed onto the back of a Sequenza

coverplate. The combined coverplate and slide were then placed into a holder box. 150 μL of lysis solution (25 ml 0.07 M NaOH with 10 ml of 99.9% EtOH) was applied for 30 seconds followed immediately by 200 μl of methanol fixation for two minutes. The slide was then removed from the coverplate and allowed to air dry.

DNA was then denatured 2.5 N hydrochloric acid for 20 minutes at 20 °C. This was then neutralized with 8.0 pH PBS followed by three 5-minute washes of 7.4 pH PBS. The slides were then blocked with 10% FBS and 0.1% Tritonx100 in PBS for one hour. The slides were then incubated with the primary antibodies in blocking buffer for one hour. Primary antibodies were BrdU rat monoclonal IgG2a at a dilution of 1:100 (MA1-82088, Thermo Scientific) and Anti-IdU mouse monoclonal at a dilution of 1:100 (SAB3701448, Sigma Aldrich). This was followed by three washes in PBS over 15 minutes. Slides were then incubated with secondary antibodies for one hour. Secondary antibodes were Alexa Fluor® 488 Goat Anti-Mouse IgG_1 at a dilution of 1:250 and Alexa Fluor® 555 Goat Anti-Rat IgG at a dilution of 1:200. This was followed by three 5-minute washes with PBS and a final rinse with water and allowed to air dry. Coverslips were mounted with Fluoromount (Sigma Aldrich) and the slides were examined in a confocal microscope.

3.7 Statistical analysis

Wilcoxon rank sum test were performed in R, a statistical computing software, with the help of Dr. Ólafur Andri Stefánsson. All other statistical analysis and graphs were performed in Microsoft Excel. Statistical significance was determined to be at values less than 0.05.

4 Results

4.1 Cell Lines with Functional BRCA2 Form RAD51 and γH2AX Foci Following Treatment with Olaparib

The presence of RAD51 and γH2AX foci in the nucleus of a cell indicates effective HR repair and DSBs, respectively. BRCA2 is known to be an essential factor for recruitment of RAD51 to sites of DNA damage. It is, however, not known if the A176 cell line expresses sufficient amount of BRCA2 to support efficient RAD51 recruitment. In figure 7 we did an immunostaining for RAD51 and γH2AX to see the ability of each cell line to form foci before and after treatment with olaparib. These findings were then quantified, RAD51 in figure 8 and γH2AX in figure 10.

The *BRCA2* wild-type (MCF7) and heterozygous A176 cell lines show proficient formation of RAD51 and γH2AX foci (Figure 7a & b). The A176 with incorporated scrambled shRNA showed similar foci formation to the wild-type and parent A176 heterozygous cell line (Figure 7c). Following knock-down of the *BRCA2* gene in the heterozygous cell line A176, the formation of RAD51 foci was seen to be reduced (Figure 7d). The same effect was also seen in the BRCA2 deficient cell line (CAPAN-1) (Figure 7e).
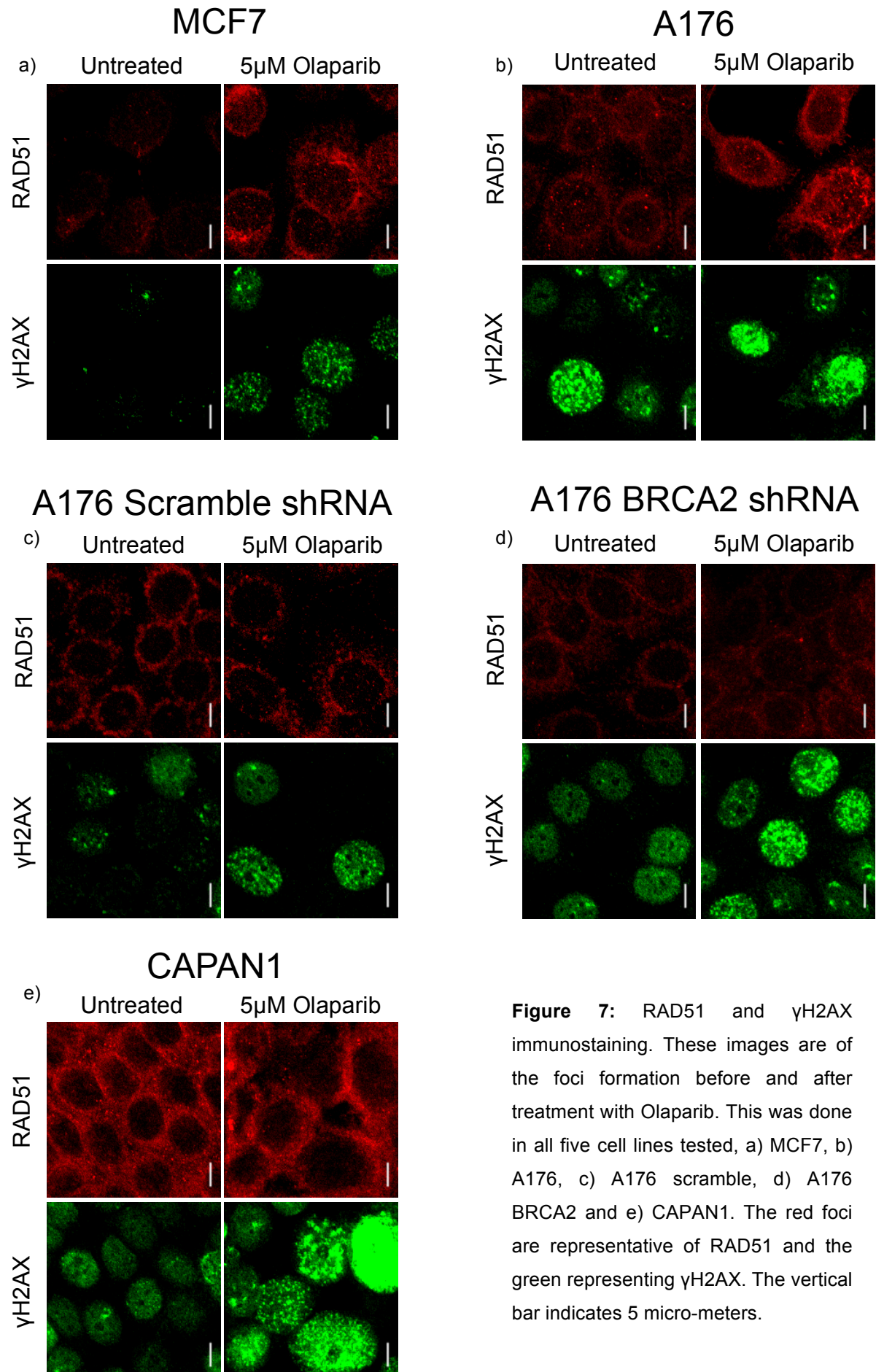
MCF7

A176

Figure 7: RAD51 and γH2AX immunostaining. These images are of the foci formation before and after treatment with Olaparib. This was done in all five cell lines tested, a) MCF7, b) A176, c) A176 scramble, d) A176 BRCA2 and e) CAPAN1. The red foci are representative of RAD51 and the green representing γH2AX. The vertical bar indicates 5 micro-meters.

4.1.1 A176 Shows Competent yet Decreased Ability to Form RAD51 Foci
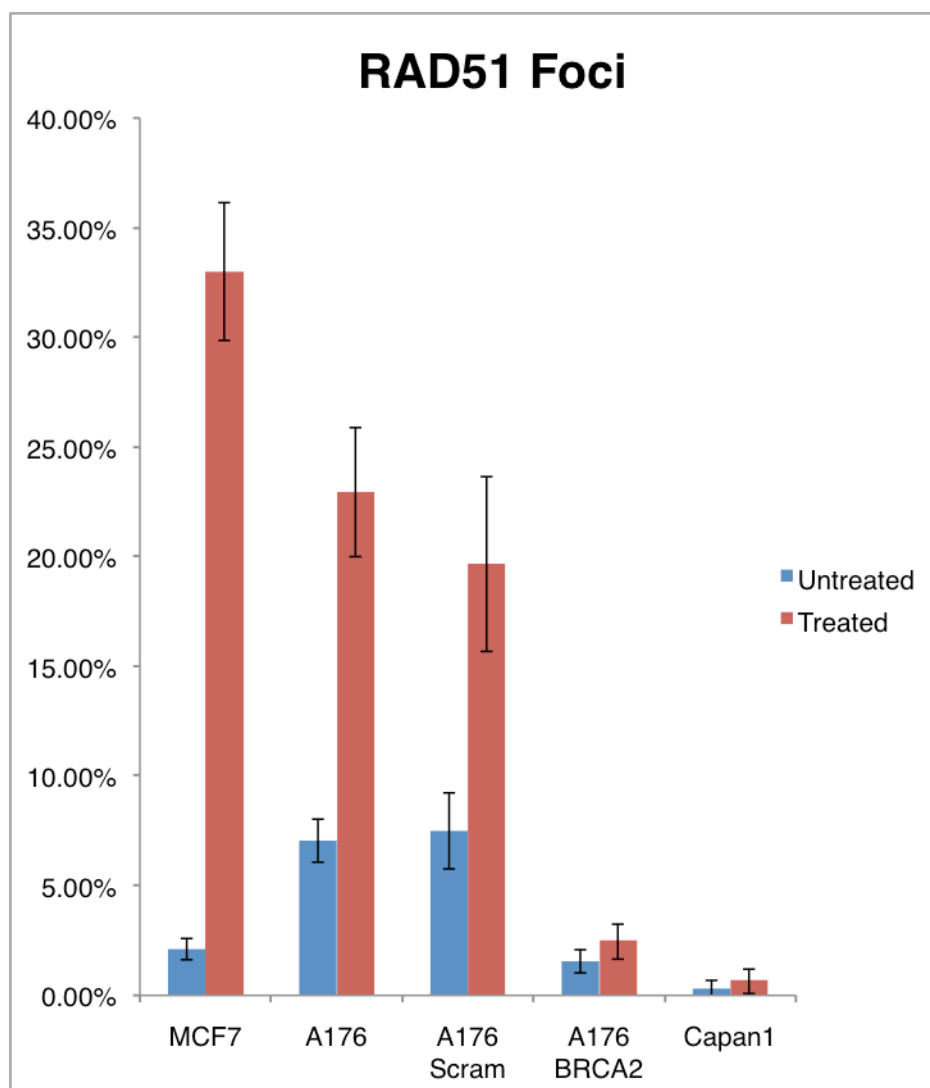
Figure 8: RAD51 foci quantification before and after treatment with 5µM olaparib. This figure is the result of counting 200 cells for RAD51 foci from the same samples shown in Figure 7. Cells were deemed positive for RAD51 if they displayed >5 nuclear foci. The counting was repeated in triplicate for each condition.

Foci formation without treatment; MCF7 2.1%, A176 7.1%, A176 scramble shRNA 7.5%, A176 BRCA2 shRNA 1.6%, CAPAN-1 0.31%. Following treatment with 5µM olaparib. MCF7 displayed the highest amount of RAD51 foci formation at 33.0%. A176 and A176 with Scramble lentivirus were at 22.9% and 19.4% respectively. A176 with *BRCA2* shRNA foci formation was comparatively less 2.5%. Capan1 at 0.64% with an error of 0.56%.

The *BRCA2* wild-type MCF7 displayed the greatest ability to form RAD51 foci indicating it is likely the most efficient in HR (figure 8). The heterozygous lines A176 and A176 with scramble shRNA displayed sufficient yet diminished ability to form RAD51 foci possibly indicating that lacking a functional *BRCA2* allele can have an impact on the cells ability to form RAD51 foci.

The differences before and after treatment showed P-Values < 0.05 for all cell lines tested except for Capan-1 which had a P-value of 0.118. However, the A176 with BRCA2 shRNA cells had a median value for foci formation of 3 before treatment, and 5 after treatment with Olaparib. This gives a statistically significant value but the biological significance is negligible.
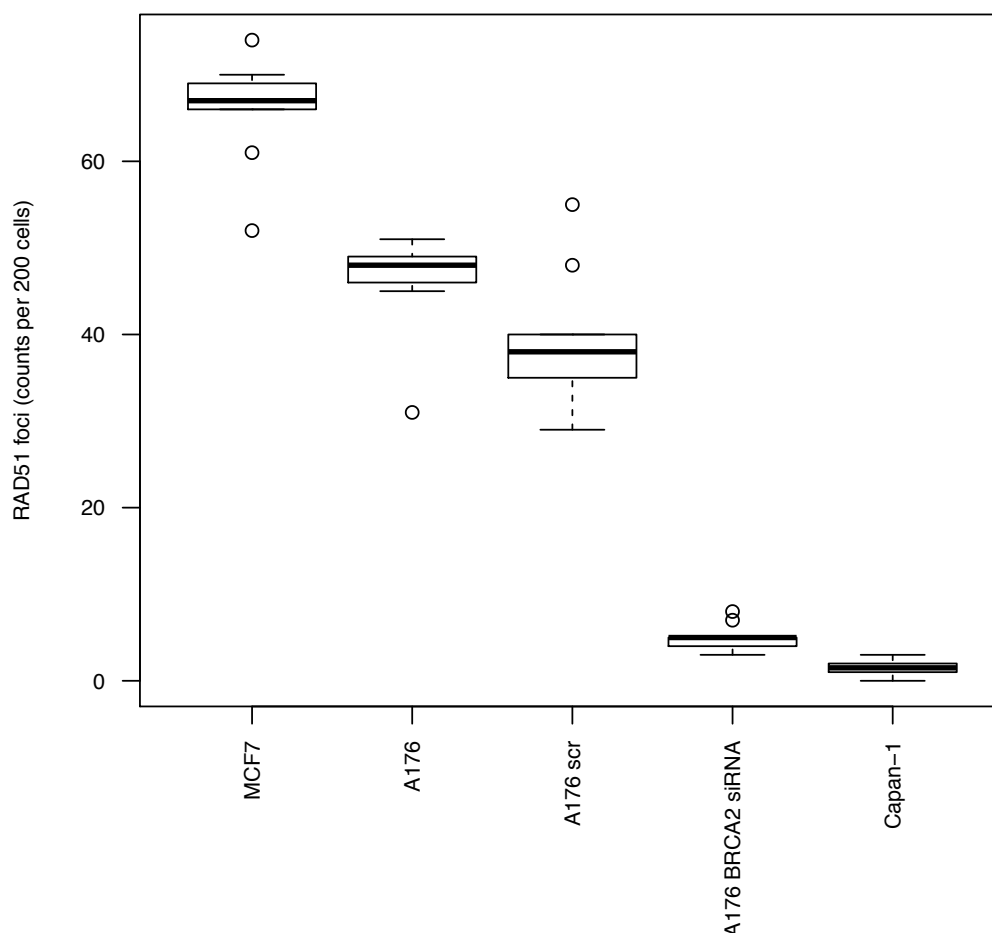
Figure 9: Wilcoxon rank sum test for RAD51 foci formation. We performed a Wilcoxon rank sum test with all cell lines compared against the A176 cell-line.

The heterozygous cell line (A176) where the *BRCA2* gene has been knocked down showed statistically significant reduction of RAD51 foci formation as compared with the parent A176 cell line following PARP inhibitor (PARPi) treatment (Figure 9). P Values for the RAD51 foci were seen as A176 with scramble shRNA at 0.076. MCF7, A176 with BRCA2 shRNA, and CAPAN-1 were all seen at P<0.01 (Figure 9).

4.1.2 γH2AX Foci Formation Consistent Among All Cell Lines

Phosphorylated histone H2AX is a signal for DSBs. γH2AX is used as a marker for DNA damage. This figure represents that all cell lines were undergoing DSBs and responded more or less equally after treatment with olaparib (figure 10).
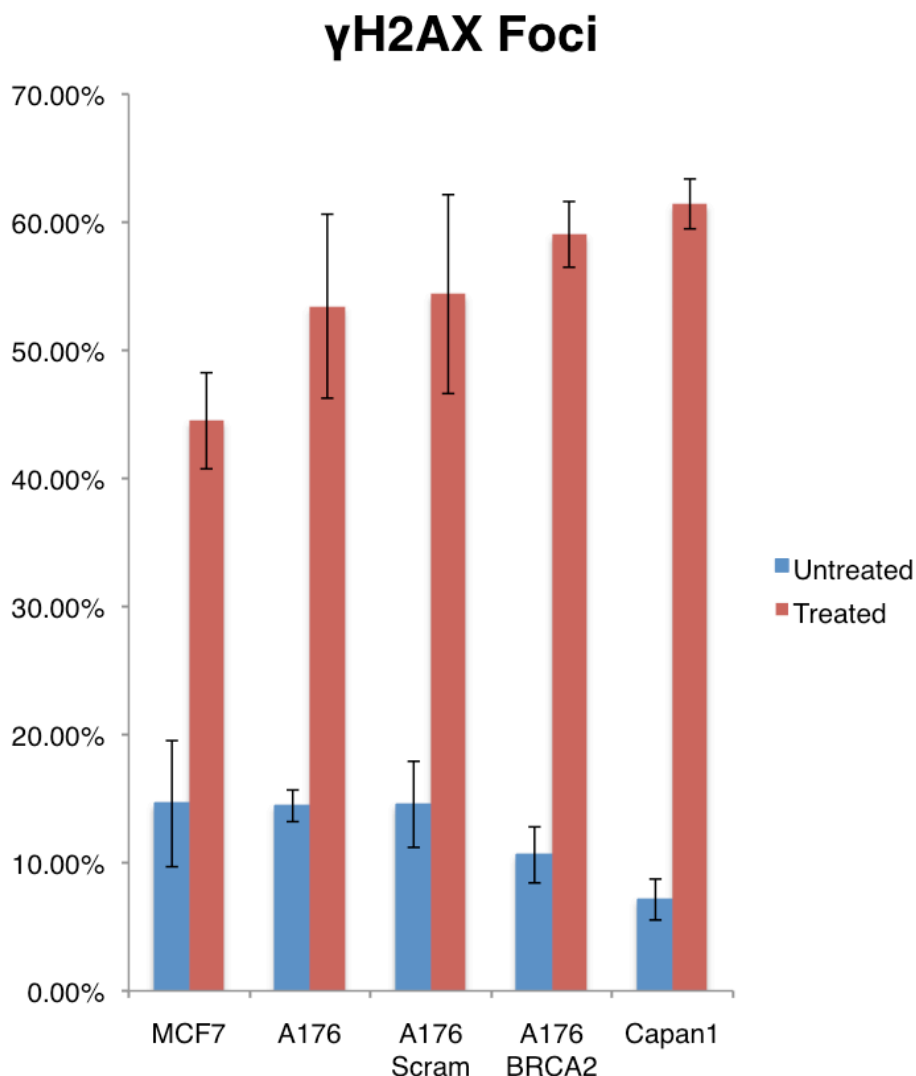
Figure 10: γH2AX foci formation before and after treatment with olaparib. 200 cells were counted for γH2AX foci. Cells were counted positive if displaying greater than five nuclear foci.

The formation of γH2AX foci was consistent between PARPi treated and untreated for all cell lines tested. Proportion of untreated γH2AX foci: MCF7 14.6%, A176 14.4%, A176 Scramble shRNA 14.6%, A176 BRCA2 shRNA 10.6%, CAPAN-1 7.13%. Olaparib Treated cell with γH2AX foci: MCF7 44.5%, A176 53.44%, A176 Scramble shRNA 54.39%, A176 BRCA2 shRNA 59.1%, CAPAN-1 61.43%. No significant difference was found between the A176 cell line and the scramble shRNA (P-value 0.723) and BRCA2 shRNA (P-value 0.079). MCF7 and CAPAN-1 showed statistically significant values at 0.019 and 0.020, respectively (Figure 11).
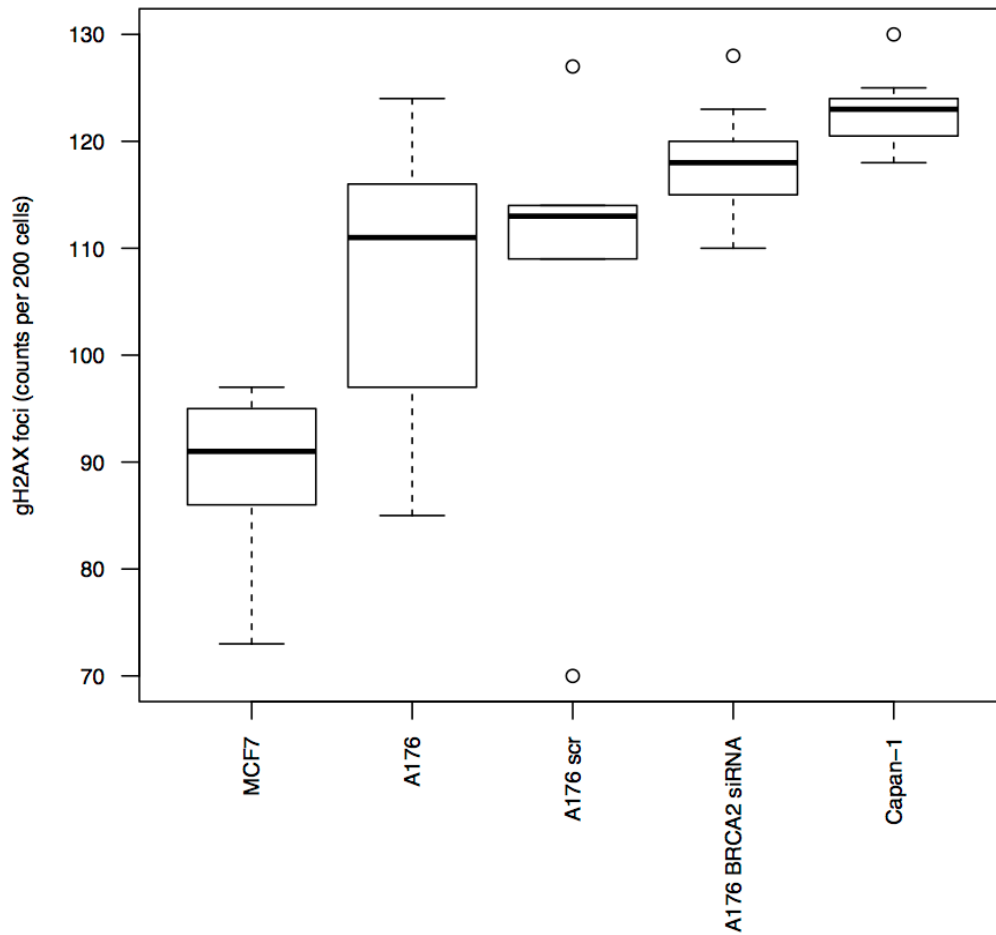
Figure 11: Wilcoxon rank sum test for γH2AX foci formation. Values are compared to A176 line.

The quantification of the γH2AX and RAD51 foci formation for each cell line with and without PARPi treatment showed statistically significant differences in the ability to form RAD51 foci following treatment (Figures 8 and 9). Collectively, these data show that one functional *BRCA2* allele is sufficient to promote RAD51 recruitment to sites of DNA damage, suggesting that HR repair is active. However, RAD51 foci formation is not as efficient, indicating that the heterozygous line might have a slight impairment in HR.

4.1.3 γH2AX and RAD51 Co-localize at DSBs Following DNA Damage

The presence of both RAD51 and γH2AX is indicative that RAD51 and γH2AX are colocalizing to sites of DNA damage (figure 12). This confirms that RAD51 foci are forming at the site of a DSB where γH2AX are also known to form foci.

Figure 12: γH2AX and RAD51 colocalization. This image is of MCF7 following treatment with 5 μM of Olaparib.

4.2 Cellular Survival Mirrors RAD51 Foci Formation

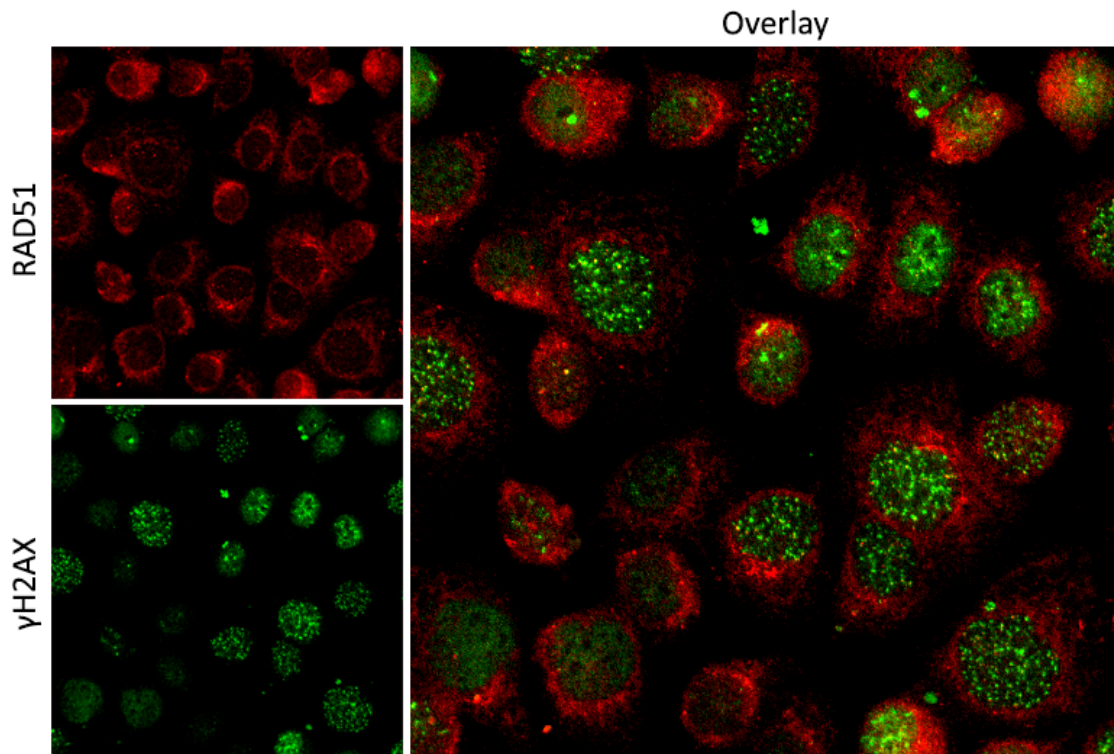When looking at cellular survival with and without PARPi treatment, the *BRCA2* wild-type (MCF7) and heterozygous A176 cell line displayed the least sensitivity (Figure 13). Loss of cellular proliferation capacity was observed following shRNA knock-down of the *BRCA2* gene in A176 (BRCA2+/-). Capan-1 displayed similar results for PARPi treatment as seen for the shRNA knock-down of the *BRCA2* gene in the heterozygous A176 cell lines (Figure 13).
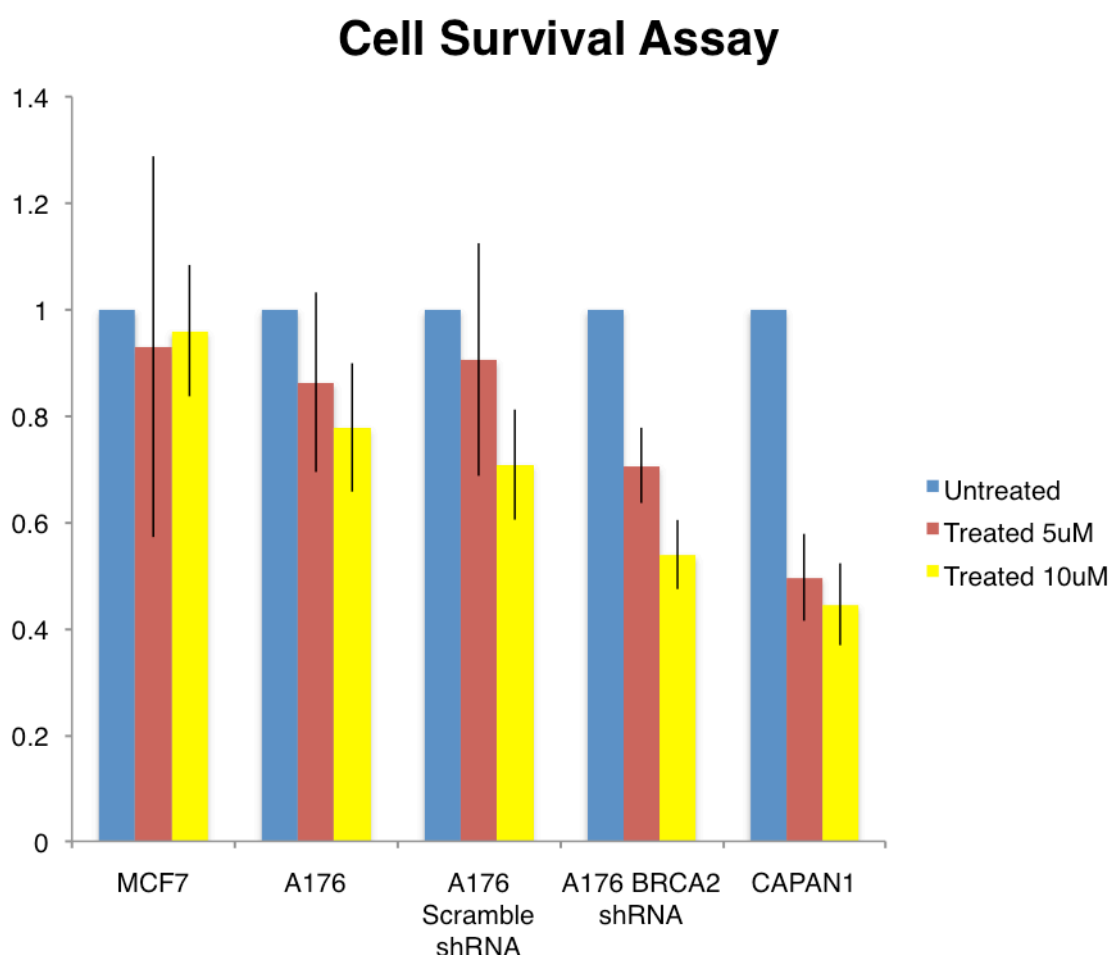
Figure 13: Cellular survival assay for cell lines after treatment with olaparib.

The Promega Cell Titer 96 Assay showed that MCF7 was the most resistant to treatment by olaparib for 96 hours with 93.0% survival at 5 µM and 96.0% at 10 µM. A176 86.3% at 5 µM and 77.8% at 10 µM. A176 Scram 90.6% at 5µM and 70.8% at 10 µM. A176 with BRCA2 shRNA virus 70.6% at 5 µM and 53.9% at 10 µM. Capan1 suffered the worst with treatment at 49.6% at 5 µM and 44.6% at 10 µM.

Following treatment with Olaparib, no matter what concentration or time period, cellular survival did not drop below 40%. It is possible that cells that are not killed by the olaparib may become senescent. At higher concentrations, the heterozygous cell line displayed impaired survival ability, indicating

increased sensitivity to PARPi. This is in line with the defect in HR seen in Figure 8 by RAD51 foci formation after treatment with olaparib.

4.3 Fiber-assay as Method to View Stalled Replication Forks

The DNA Fiber-assay is a method in which you can view a cells ability to restart replication following periods of replicative stress that cause the cell to halt replication. I was unsuccessful in making the Fiber-assay able to show discrepancy between the CldU and IdU. This is likely due to lacking a DNA plug to load the DNA with as well as a "comb" that allows for the spreading of fibers. Functional Fiber-assay would however be an excellent method in determining a cell line's ability to restart the replication fork following collapse. BRCA2 plays a role in this restarting and the impact of heterozygozity would be interesting to observe.
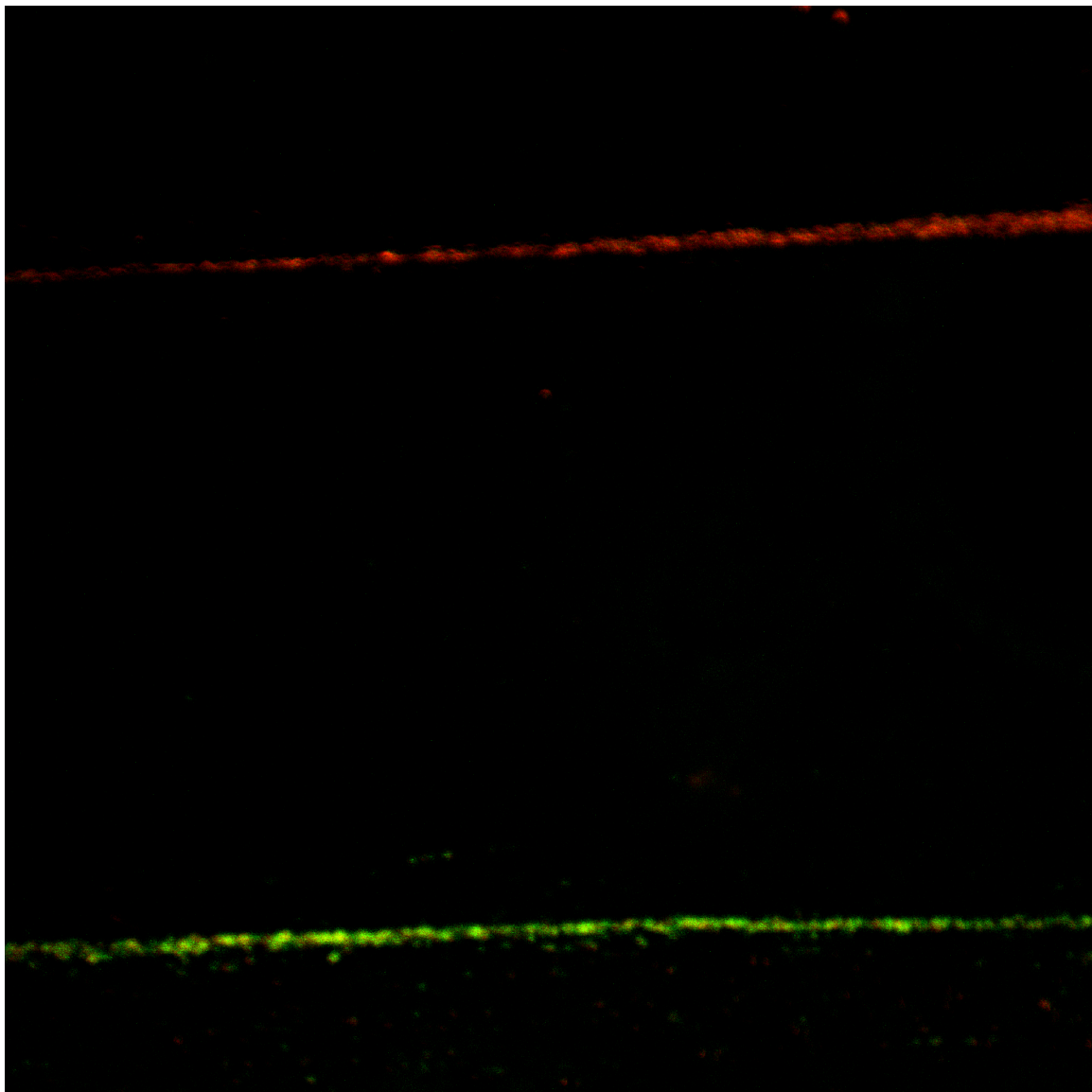

Figure 14: DNA fiber-assay. DNA Fibers stained for CldU and IdU in a confocal microscope. Red (Alexafluor 555) fibers represent CldU and Green(Alexafluor 488) represents IdU.

5 Discussion

5.1 General Discussion

In this study, we looked at a human breast epithelial cell line established from a carrier of the Icelandic *BRCA2 999del5* mutation and what effect a PARP inhibitor had on it. We then compared this cell line to a control cell line containing wild type *BRCA2* alleles and a cell line deficient for *BRCA2*. In the heterozygous line, RNAi in the form of a lentiviral vector was used to create a stable version of the heterozygous line that now was lacking the remaining functional allele of *BRCA2*. This was done to model the effects of loss of heterozygosity (i.e. loss of the remaining wild-type allele).

The results indicate that the *BRCA2* heterozygous cell line A176 is mildly affected, by treatment with a PARPi (olaparib) as seen by its ability to form RAD51 foci and proliferation capacity. The MCF7 breast cancer cell line is known to be proficient for HR repair demonstrated similar ability to form RAD51 foci and proliferate following treatment with PARPi. CAPAN-1, the cell line deficient for BRCA2 was unable to form RAD51 foci showing defective ability for HR and also performed poorly in the cell proliferation assay following PARPi.

The heterozygous cell line, following knockdown with incorporated lentiviral *BRCA2* shRNA, displayed similar ability to form RAD51 foci and proliferate following RNAi as the *BRCA2* deficient CAPAN-1. This suggests that loss of the wild-type allele results in the inability to form RAD51 foci indicating DNA repair deficiency.

MCF7(+/+) displays the greatest ability to respond and grow following treatment with Olaparib. The heterozygous lines, A176 and A176 with incorporated lentiviral scramble shRNA, showed they too were able to respond and thrive following treatment with Olaparib yet had a diminished ability at higher concentrations of the drug. This finding is in line with the cell lines' ability to form RAD51 foci. The *BRCA2* deficient lines, A176 with lentiviral *BRCA2* shRNA and CAPAN-1, clearly suffered following Olaparib treatment yet interestingly would never be fully killed following drug treatment. The cells appear to become senescent and stop dividing, however this has to be proven with additional tests. Yet when compared with the staining experiments these cells are clearly under duress from DNA damage and are incapable of responding through HR.

5.2 Carcinogenesis as a Result of Heterozygozity

While the *BRCA2* heterozygous cell lines displayed competent ability to form RAD51 foci, the amount of foci formed was slightly diminished when compared to the wild-type. This decrease in RAD51 foci is possibly indicative that the heterozygous cells have impaired ability to undergo HR. Decreased HR could mean that the cells survival is decreased or that the cell will now rely more on NHEJ to repair the DNA following any DSBs that occur. Due to NHEJ being much more error-prone method of repair, there is a higher chance that DNA is unrepaired or mutations are formed within the genome. Over time these mutations could accumulate. Should they lie in key genes, such as a tumor suppressor genes or oncogenes, they may lead to the formation of a cancer.

Should this be the case, Knudson's two-hit hypothesis may not applicable to all genes. The loss of heterozygozity for *BRCA2* may not be necessary for the initial formation of a cancer. This idea is not

without precedent as it has been shown to be the case with *BRCA1* and *BRCA2* (Pathania et al, 2014; Stefansson et al, 2011; Skoulidis et al, 2010)).

5.3 BRCA2 Dimerization

BRCA2 was recently shown to dimerize (Shahid et al, 2014). In the case *999del5* the BRCA2 protein is degraded and therefore does not have the ability to dimerize with the functional version of the protein. In a case where the mutated protein is not degraded, it could possibly dimerize with the non-mutated version and impair its ability to import RAD51 or the mutated version could dimerize with itself, which may cause issues as well. This could possibly cause a negative dominant phenotype. Cells with *BRCA2* mutations unlike the *999del5* mutation may potentially suffer much worse in a heterozygous state.

5.4 Future Directions

5.4.1 Senescences Test

When increasing the level of Olaparib for treatment, cells typically died at increasing levels. However, even at concentration of 25 μM the cells never dropped below 40% survival within the *BRCA2* deficient CAPAN-1 cell line. It is possible the cells have transferred into a senescent state at this point and will be unaffected by the drug since they are no longer replicating and will not undergo apoptosis as a result of the presence of DSBs due to arrest of the cell cycle. It would be interesting to perform a test for senescence markers in the cells remaining following treatment with Olaparib. This could be achieved by examining the cells for Sen-β-Gal, SAHFs, or by seeing low expression of Ki67 with high γH2AX (Lawless et al, 2010)*.*

5.4.2 CRISPR for Control

A major issue with this experiment is whether or not the effects seen are a result from discrepancies between cell lines or not. Cell lines are established by modifying the genome in order to immortalize them. This is not always done in the same way and can cause strange effects to be seen within the cell line making comparisons between different cell lines difficult and possibly inaccurate. The lentiviral system provided a good comparison for the negative control by removing the remaining *BRCA2* allele by introducing a *BRCA2* shRNA into the genome. It is however limited because you cannot create a positive control cell line that transcribes at the same rate as the cell normally would because lentiviral systems incorporate themselves randomly into the genome.

CRISPR has the added advantage that you can choose exactly where in the genome you want to modify. This could be used to create both negative and positive control versions of a cell line. In the A176 cell line, a mutation could be introduced into the remaining functional allele for the negative control. The 5 missing bases could be reintroduced into the non-functional allele to restore function and create a positive control. This system would provide much more accurate analysis of what is happening within the cell line as you would be able to compare it with itself and ignore off-target effects or other compensations the cell lines have developed.

5.4.3 *γH2AX Kinetics*

An interesting observation about the γH2AX foci formation is that the cell lines deficient for *BRCA2* did show visibly more foci than those with functional *BRCA2*. There was statistical significance seen in the CAPAN-1 line when compared to the heterozygous cell line, and this possibly suggests that repair is not only impaired in ability but possibly also impaired in speed. By viewing γH2AX at various time points following treatment with a DNA damage causing drug, we could measure the ability of cell lines to clear the DNA of damage once its occurred, giving a better indication of the actual ability to competently perform DSB repair.

5.4.4 Confirmation of *BRCA2* Expression Levels

I was unsuccessful being able to confirm expression of BRCA2 within the cell lines through Western Blot or qPCR. We obtained some data for qPCR but it was excluded due to experimental variation. The expression of BRCA2 needs to be confirmed in order to correlate the decrease in HR efficiency to BRCA2 expression and support the findings of this thesis.

6 Conclusion

The *BRCA2* heterozygous cell line proved to be effective for modeling the ability to undergo HR. It displayed competent ability to form RAD51 foci and survive treatment with a PARP inhibitor as compared to the negative control. However, differences in HR capability and survival at higher concentrations were seen when compared to the positive control. This could be indicative that BRCA2 heterozygous individuals may have impaired HR capabilities. Even minor impairments in HR over a lifetime could allow for mutations to accumulate, leading to the possible formation of a disease.

Further study with better controls and additional tests would provide a much clearer outlook on the status of individuals heterozygous for BRCA2.

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