THE ROLE OF UBL5 AND CT55 IN THE SURVIVAL OF BRCA2-DEFICIENT CELLS

by

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B.S. Psychology (University of Maryland, College Park) 2019

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

December 2022

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DEDICATION

This Master's thesis is dedicated to my family and mentors who have never doubted my abilities. To my mother, father, and brother as well as my mentors Satheesh, Kajal, and senior investigator Shyam, I would not be where I am today without you all. I will forever be grateful for your support, kindness, and guidance.

ACKNOWLEDGMENTS

I express my deepest gratitude to my thesis adviser and senior investigator Dr. Shyam Sharan. Thank you for giving me the incredible opportunity of learning under your mentorship at the National Cancer Institute. Beause of your guidance, I have found my true passion in research and I am thrilled to be applying to graduate programs to pursue a Ph.D. I hope that one day I can find the words to truly express my full gratitude to you.

I want to sincerely thank my mentor Dr. Kajal Biswas who has taught me the fundamentals of how to be a thorough scientist. I thank you for your kindness and patience as you guide me in becoming a better scientist. I am grateful to continue to learn from you and for our friendship as colleagues.

I owe my sincere gratitude to my mentor Dr. Satheesh Sengodan. I am grateful to learn from you every single day. Thank you for your continuous support in helping me become the best scientist that I can be. I can not thank you enough for your constant support in the completion of this thesis. You have helped me more than you know. Thank you for being a wonderful mentor and friend.

Next, I want to thank Arun for inspiring me in the lab on a daily basis. I sincerily thank you for everything you have taught me and for always encouraging me to continue to learn. I am lucky to learn from you. I also thank the rest of my labmates: Suhas, Sounak, Josey, Dylan, and Terry. You are all wonderful colleagues and have all helped me in more ways than one.

Lastly, I want to thank Dr. James Cherry for his guidance in this masters thesis as well as his unwavering support as I pursue my graduate career. Thank you for always being

a phone call away and for continuously supporting my dreams. I also thank Dr. Ann Boyd, Dr. Meredith Yeager, and the Graduate School at Hood College for providing me with the opportunity to embark on this thesis research as well as for guiding me through my masters education and toward my next steps in pursuing graduate school.

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ABSTRACT

BRCA2 is essential for genomic stability. BRCA2 deficiency results in severe proliferation defects and affects the viability of normal cells. Yet, loss of BRCA2 is known to lead to tumorigenesis. It is hypothesized that some cells acquire mutations in genes that allow the cells to survive in the absence of BRCA2. One such gene or genetic interactor of BRCA2 is *TP53,* which is mutated in many BRCA2-deficient tumors. Here, using a CRISPR activation screening, we identify CT55 and UBL5 as genetic interactors of *BRCA2.* Both CT55 and UBL5 rescue BRCA2-deficient mouse embryonic stem cells (mESCs). Though CT55's function and mechanism by which rescues BRCA2 lethality is yet to be discovered, CT55 shows nuclear localization in BRCA2 proficient and deficient cells. UBL5 has a role in the loading of cohesin by the cohesin cofactor Sororin. UBL5 has a significant effect on cell viability in with respect to BRCA2. Interestingly, loss of UBL5 is more lethal for BRCA2 proficient cells than BRCA2 deficient cells.

Also, UBL5 loss induced cell lethality is rescued by silencing Cohesion unloader, WAPL, suggesting the regulation between UBL5 and cohesion in affecting the cell viability. While we hypothesize, BRCA2 could be potential cohesion unloader, future experiments will explore these mechanisms to better understand the potential role of BRCA2 in the cohesin complex.

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LIST OF ABBREVIATIONS

BP	Base pairs
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
cDNA	Complementary DNA
CTA	Cancer testis antigens
CT55	Cancer Testes Antigen 55
DBD	DNA binding domain
DSBs	Double-stranded breaks
dsDNA	Double-stranded DNA
dCas9	Nuclease dead-Cas9
ES	Embryonic stem
FA	Fanconi Anemia
FP	Fork Protection
GOF	Gain-of-function
HD	Helical domain
HR	Homologous recombination
HSF1	Heat shock factor protein
ICL	Interstrand cross-linking
IR	Ionizing radiation
LOF	Loss-of-function
LTR	Long terminal repeats

mESCs	Mouse embryonic stem cells have
MSCV	Murine Stem Cell Virus
MRN	MRE11-RAD50-NBN
NHEJ	Nonhomologous end joining
OB	Oligonucleotide binding
PALB2	Partner-and-localizer of BRCA2
RPA	Replication Protein A
SAM	Synergistic Activation Mediator
sgRNA	Single-guide RNA
Smc1	Structural Maintenance of Chromosomes 1
Smc3	Structural Maintenance of Chromosomes 3
ssDNA	Single straded DNA
UBL5	Ubiquitin-like protein 5
WAPL	Wings apart-like homolog

INTRODUCTION

Breast Cancer

Breast cancer is one of the most common malignancies among women (Paul and Paul 2014) and is the most frequently diagnosed cancer in women worldwide accounting for 2.26 million cases in 2020. In the United States, breast cancer accounts for 29% of cancers among women (Łukasiewicz et al. 2021). Globally, breast cancer is the leading cause of cancer death among women and was responsible for 684,996 deaths in 2020 (Łukasiewicz et al. 2021). The likelihood of survival after developing breast cancer for women in high-income countries is much higher than for women in middle-income countries. Between 1990 and 2016, the incidence for breast cancer has more than doubled. Current projections predict that by 2030, breast cancer will affect 2.7 million women annually.

There have been several genetic mutations which have been reported to increase the risk of breast cancer. Mutations in *BRCA2* (BRCA2 DNA repair associated), a well-known tumor suppressor gene, significantly increases the risk of breast and ovarian cancers (Paul and Paul 2014). *BRCA2* is primarily linked to an increased risk of breast carcinogenesis. The mutations in these genes are typically inherited in an autosomal dominant manner but sporadic mutations are also commonly reported. The number of high-risk families with breast and/or ovarian cancers due to mutations in *BRCA1* or *BRCA2* varies among populations. Inherited predisposition to breast and ovarian cancer due to *BRCA1/2* mutation is highly prevalent among people of Ashkenazi ancestry. 2.5% of

people from Ashkenazi Jewish descent carry one of three founder mutations in *BRCA1* or *BRCA2* (185delAG or 538insC in *BRCA1* and 6174delT in *BRCA2*). A recent population study found that 29% of Jewish women with ovarian cancer carried one of these three founder mutations. In 220 high risk Ashkenazi breast cancer families, a founder BRCA mutation was found in 44% (Kauff 2002).

BRCA2

BRCA2 was discovered in 1995 and is intrinsic to all human beings. BRCA2 is expressed in a wide variety of tissues and localizes in the nucleus. BRCA2 is located on the long arm of chromosome 13q12, consists of 27 exons and codes for a protein of 3,418 amino acids. Several structural domains of BRCA2 have been identified in the last two decades (Carreira and Kowalczykowski 2011). Xia et al (2006) discovered a major interactor of BRCA2 and named it partner-and-localizer of BRCA2 (PALB2). PALB2 was mapped to the PALB2-binding domain of the extreme N-terminus, specifically, amino acids 21-39. Exon 11 of human BRCA2 is the largest and consists of 4932 base pairs (bp) and contains eight BRC repeats (Andreassen et al. 2021). BRC repeats consist of eight conserved motifs of about 35 amino acids (Fradet-Turcotte et al. 2016). BRCA2 recruits RAD51 to sites of DNA damage through the interaction of the BRC repeats (Carreira and Kowalczykowski 2011). All vertebrates have eight BRC repeats that are arranged in the central region of BRCA2 spanning over 1000 amino acids, specifically 1002-2085 in human BRCA2. Not all organisms have eight BRC repeats. BRCA2 homologs in *U. maydis* and *C. elegans* have a single BRC repeat. Still, relatively little is known about the BRC repeats including the reason for requiring all eight repeats (Andreassen et al. 2021).

The DNA binding domain (DBD) is comprised of a long helical domain (HD) and three oligonucleotide binding (OB) folds as well as the C-terminal domain (Fradet-Turcotte et al. 2016). The C-terminal domain of BRCA2 can bind to the DSS1 protein and includes three elements which lead to its DNA-binding properties. These include a helical domain and three oligonucleotide binding (OB) folds. OB folds are seen in most single straded DNA (ssDNA) binding proteins. OB2 and OB3 directly interact and bind ssDNA. The distal end of OB2 mediates binding to double-stranded DNA (dsDNA). This is important because both ssDNA and dsDNA are present at double-stranded breaks (DSBs) following end resection of the DNA strands after homologous recombination (Andreassen et al. 2021). BRCA2 plays a crucial role in maintaining genomic integrity because of its well-established role in double stranded DNA break repair by homologous recombination as well as in the protection of stalled replication forks. BRCA2 deficiency is known to lead to the accumulation of DNA:RNA hybrids known as R-loops which are a source of replication stress and genomic instability as they lead to broken or stalled replication forks (Santos-Pereira and Aguilera 2015). BRCA2 is also known to be required for cell cycle regulation (Roy et al. 2012).

Homologous Recombination

Double-stranded breaks (DSBs) threaten genomic integrity. The two main pathways which can repair DSBs are nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ repairs broken DNA ends and ligates broken ends together without considering homology. This can result in deletions or insertions due to the lack of regard for exact homology (Mao et al. 2008). Hence, it is considered an error prone

mechanism of DNA repair in mammalian system. On the other hand, homologous recombination is a mechanism which utilizes an intact identical DNA molecule, typically the sister chromatid, to restore a homologous DNA sequence to the site of a DSB. Whether HR or NHEJ is used for repair of DSBs is determined by the phase of the cell cycle. HR occurs when sister chromatids are readily available during the S and G_2 phases of the cell cycle prior to the cell entering mitosis (M phase). Sister chromatids are the ideal template for HR because they have an exact copy of a given chromosome (Chaffey et al. 2003). HR is essential for cell division in eukaryotes. HR repairs DSBs caused by ionizing radiation (IR), DNA-damaging chemicals, and cell intrinsic threats such as stalled replication forks and R-loop accumulation. Unrepaired damage can result in the large-scale rearrangement of chromosomes in somatic cells which can lead to neoplastic transformation (Khanna and Jackson 2001).

As shown in Figure 1, DSB's are initially sensed by the MRE11-RAD50-NBN (MRN) complex which loads onto DSBs to begin 5'-3' double-stranded DNA end resection. Single-stranded DNA (ssDNA) overhangs are protected by Replication Protein A (RPA) coating. ATR localizes to RPA-coated ssDNA and turns on the ATR-Chk1 DNA damage checkpoint which arrests the cell cycle and protects stalled replication forks. Upon cell cycle arrest BRCA2, and PALB2 colocalize to load RAD51 onto ssDNA and replaces RPA from 3' overhangs. Then, the RAD51 nucleofilament probes the sister chromatid for homology and invades the homologous dsDNA causing a displacement loop (D-loop). Using the intact sister chromatid as a template, both resected ends undergo DNA synthesis and ligation. The Holliday junctions which link sister chromatids are resolved and HR

repair is completed (Toh and Ngeow 2021). Decreased rates of HR causes inefficient DNA repair which can lead to cancer. Hence, mutation or loss of BRCA2 significantly increases the risk for breast and ovarian cancer (Powell and Kachnic 2003).

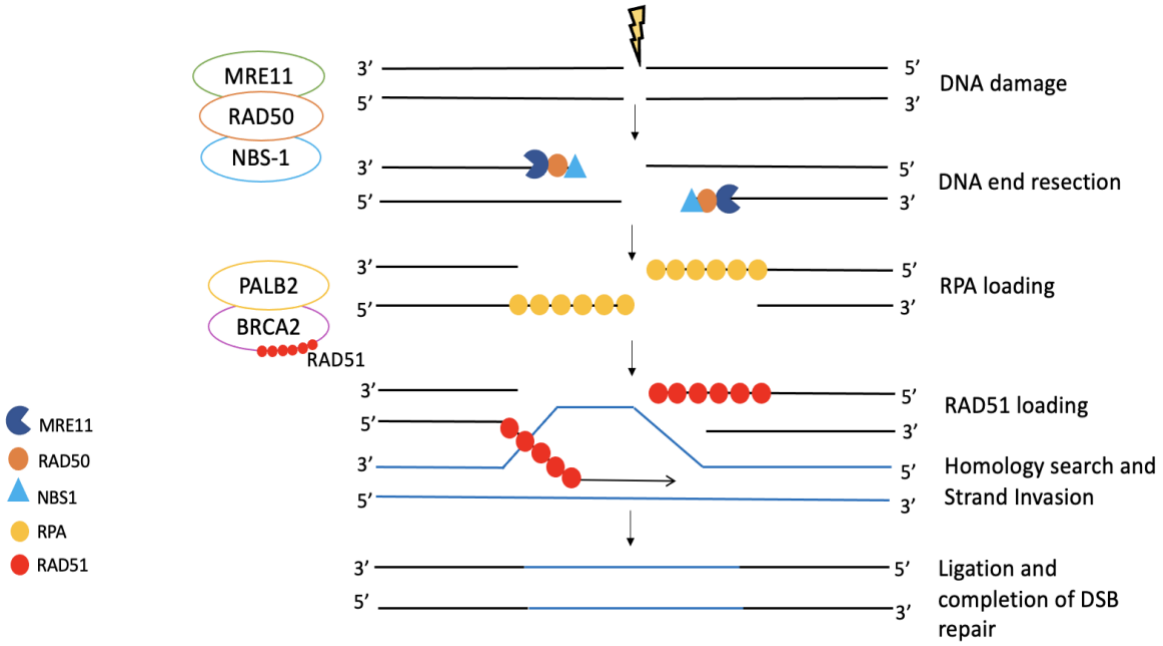


Figure 1. The role of BRCA2 in Homologous Recombination. Schematic showing the process of the resolution of a DSB by homologous recombination.

Fork Protection

Apart from HR, BRCA2 also has a role in protecting stalled replication forks from nucleolytic degradation. Fork Protection (FP) is a BRCA2-mediated process which contributes to genomic integrity. Several replication stress-inducing agents such as topoisomerase inhibitors, DNA interstrand cross-linking (ICL) agents, DNA synthesis inhibitors, and UV-radiation all increase replication fork reversal. Also, cells undergoing rapid proliferation slow replication fork progression and undergo fork reversal to protect against genomic instability from replicative stress (Feng and Jasin 2017). Upon replicative stress, replication forks stall and are remodeled into a four-way junction which is known as a 'reversed fork' or 'chicken foot' structure. This is completed by the re-annealing of parental strands while the unwinding and annealing of newly synthesized DNA forms a regressed arm. Fork reversal may allow time for repair machineries to resolve perturbations and prevent the progression of DNA synthesis across breaks which may result in DSBs (Tye et al. 2021).

As shown in Figure 2, under replicative stress, nascent DNA strands at stalled forks are susceptible to degradation by nucleases like MRE11. BRCA2 prevents the degradation of nascent strands and protects the stalled replication forks (Feng and Jasin 2017). The reversal of stalled replication forks is promoted by RAD51 as well as other DNA translocases such as SMARCAL1, ZRANB3, and HLTF. In the absence of BRCA2, fork degradation occurs as regressed arms are entry points for MRE11 degradation. Independent of BRCA2, RAD51 is required to promote the reversal of stalled replication forks while RAD51 and BRCA2 are required to protect the reversed fork from degradation (Rickman

and Smogorzewska 2019). Replication fork reversal is necessary for fork degradation in BRCA2-defective cells. Overall, the reversal of stalled replication forks is important in preventing chromosomal breakage in BRCA2-defective cells (Mijic et al. 2017).

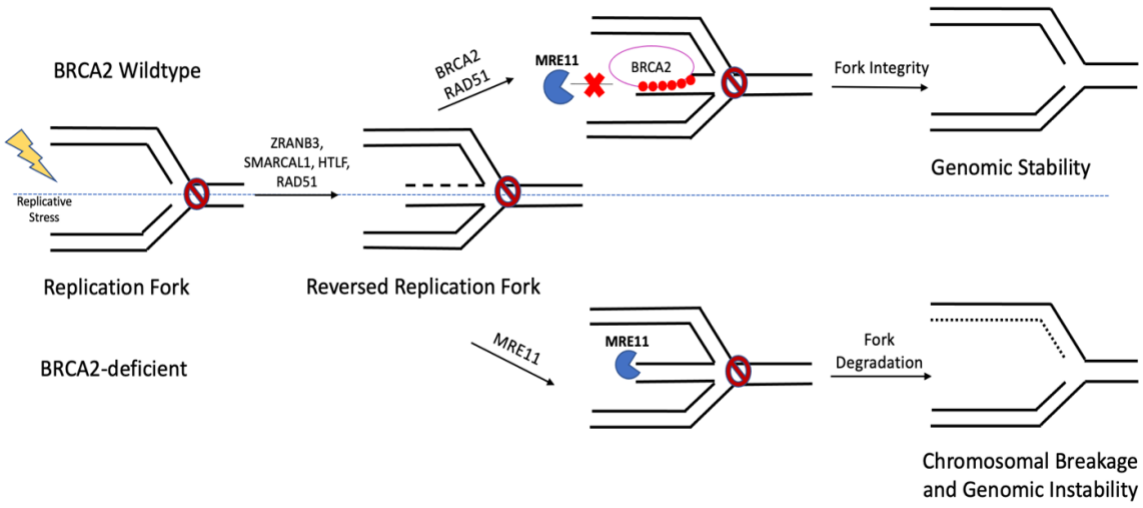


Figure 2. The Role of BRCA2 in protecting stalled replication forks. Schematic showing the role of BRCA2 in the protection of stalled replication forks in BRCA2-proficient and BRCA2-deficient conditions.

Hypothesis: Origin of BRCA2-deficient preneoplastic cells.

Though BRCA2 deficiency results in severe proliferation defects and affects the viability of normal cells, its loss of function is known to lead to tumorigenesis. How some normal cells survive in the absence of functional BRCA2 and develop into tumors remains unclear (Ding et al. 2017). It is hypothesized that some cells acquire mutations in other genes that allow the cells to survive in the absence of BRCA2. One such gene or genetic interactor of BRCA2 is *TP53,* which is mutated in many BRCA2-deficient tumors (Roy et al. 2012). Mouse embryonic stem cells have (mESCs) been successfully used as a model system to identify genetic interactors of BRCA2. Prior studies have generated $Brca2^{cko/ko}$ mouse embryonic stem cells (PL2F7) which have a functionally null and conditional allele of *Brca2*. When the conditional allele of *Brca2* is deleted in PL2F7 cells by Cre-mediated recombination, no viable $Brca2^{ko/ko}$ cells are obtained. However, some genetic interactors of *BRCA2* may confer a survival advantage in a context dependent manner to sustain viable $Brca2^{ko/ko}$ cells. Such genetic interactors may be involved in DNA repair and cell cycle regulation or contribute to the protection of stalled replication forks (Biswas et al. 2018). Using a Murine Stem Cell Virus (MSCV) based approach, we previously identified *BRE* (Biswas et al. 2018) and *GIPC3* (Xia et al. 2017) as genetic interactors of *BRCA2*. As shown in Figure 3, when transducing PL2F7 cells with MSCV which express *Cre* recombinase (MSCV-Cre), cell death is induced because of the deletion of the conditional allele of *Brca2* (Biswas et al. 2018). However, viral long terminal repeats (LTR) of MSCV can upregulate the expression of genes which may rescue $Brca2^{ko/ko}$ cells. Potential genetic interactors can be identified by examining their expression in the

rescued *Brca2*$^{ko/ko}$ mESCs and testing their ability to rescue lethality of *Brca2*$^{ko/ko}$ mESCs. However, identifying potential targets using this approach is a great challenge as it is known to upregulate genes several kilobases apart from the integration site. Hence, we adopted a CRISPR activation screening to identify potential genetic interactors of *BRCA2*.

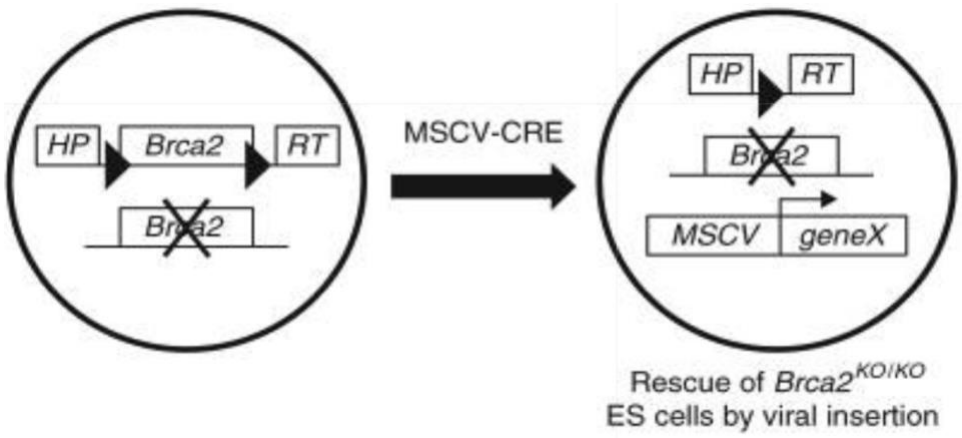


Figure 3. Schematic of MSCV interstional mutagenesis in Brca2 conditional mouse ES cells. In PL2F7 mESCs, mutagenesis by MSCV-CRE generates viable *Brca2*$^{ko/ko}$ ES cells by the upregulation of target genes (Biswas et al. 2018).

Synergistic Activation Mediator

The Synergistic Activation Mediator (SAM) system is a powerful transcriptional activator protein complex. This system has been proven to be highly effective in screening essential genes for drug resistance (Zhao et al. 2019). Achieving genome wide perturbations in biological systems is critical for understanding gene function. Genetic perturbations can be classified as loss-of-function (LOF) or gain-of-function (GOF). While there have been several genome wide loss-of-function screening methods which have been utilized, genome-wide GOF screening approaches have been limited to the use of complementary DNA (cDNA) overexpressing systems. Capturing complex transcript variants using these libraries as well as cloning large cDNA sequences into size limited viral expression vectors are limitations which must be overcome to enable efficient genome wise GOF perturbations.

The generation of custom DNA binding proteins have allowed for engineering synthetic transcription factors for regulating gene expression. Among these DNA binding domains, Cas9 is most apt in facilitating genome wide perturbations. Cas9 nuclease can be converted into an RNA-guided DNA binding protein nuclease dead-Cas9 (dCas9) by inactivating its two catalytic domains and fused to transcription activation domains. dCas9 targeted to the promoter region of endogenous genes can then regulate gene expression without cutting the DNA because it is endonuclease dead. Despite being able to achieve up-regulation of transcription, the amount of upregulation achieved by a single-guide RNA

(sgRNA) is low to ineffective. Therefore, targeting a promoter with several sgRNAs produces a more robust transcriptional activation.

Here, we used a CRISPR-dCas9-mediated SAM library to identify potential targets which can rescue the *Brca2*-null crisis. In this system, three unique sgRNAs were designed to target the promoter of the 23, 439 genes of the mouse genome. As shown in Figure 4, the stem and tetra-loop of the sgRNA library was modified to RNA hairpin aptamers which were designed to bind MS2 bacteriophage coat proteins which were fused with the transcription factors p65 and heat shock factor protein (HSF1). This works in combination with nuclease dead Cas9 (dCas9) which is fused with transcription factor VP64. Together, these form a complex which bind to the promoter region of each candidate gene to increase transcription of the gene (Konermann et al. 2014). Using this CRISPR activation screening, we were able to examine if the upregulation of candidate genes rescues the lethality of BRCA2-null cells.

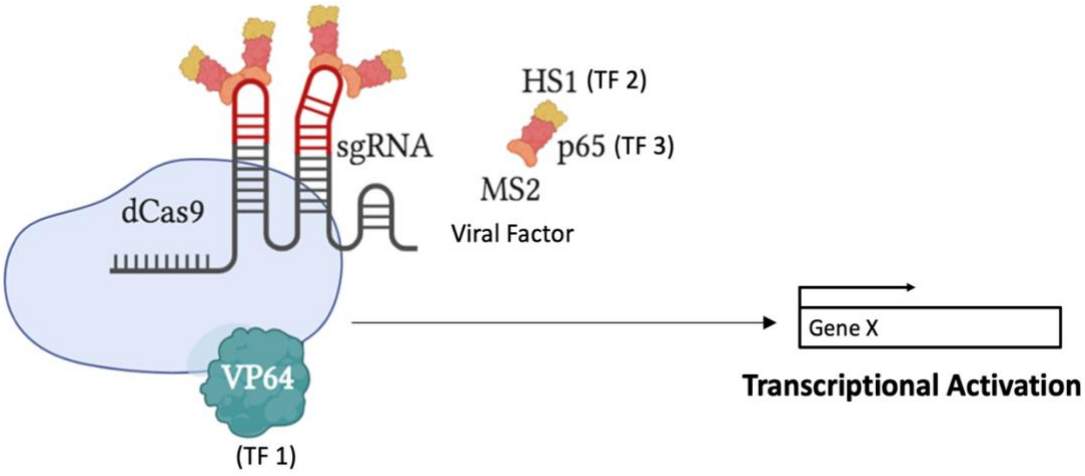


Figure 4. The Multiprotein Complex of the Synergistic Activation Mediator System. Schematic shows the CRISPR-activation screening system used in this study to target the promoter and upregulate the transcription of candidate genetic interactors of BRCA2. Figure derived from Addgene.

Ubiquitin-like protein 5 (UBL5)

By CRISPR activation screening, we identified 189 genes which rescues BRCA2 null cells. One of the 189 genes which were identified by the CRISPR activation screening is UBL5. Ubiquitin-like proteins are a family of proteins which are involved in post-translational modification of other proteins in the cell and typically play a regulatory role in cell growth and the stress response. Ubiquitination is the conjugation of the 76-amino-acid polypeptide ubiquitin to other proteins through a reversible isopeptide bond formed between the C-terminal glycine of the ubiquitin and the ε-amino group of lysine/methionine residue of the targets. Ubiquitin is known for its role in the proteasome-dependent degradation of many intracellular proteins (Chanarat 2021).

UBL5, known in yeast *Saccharomyces cerevisiae* as Hub1, is an evolutionarily conserved 73-amino-acid protein in the UBL family. UBL5 is unique among the UBL family because it lacks the C-terminal glycine that is used for covalent conjugation to target proteins. UBL5 being highly evolutionarily conserved suggests its importance for cellular function. UBL5 is required for sister chromatid cohesion maintenance in human cells (Chanarat 2021). UBL5 is largely associated with spliceosomal proteins and UBL5 depletion results in a decreased pre-mRNA splicing efficiency causing an enhanced intron retention. UBL5-deficiency results in retaining of the first intron of the cohesion cofactor Sororin. This first intron retention results in the loss of the Sororin protein and decreases the load of cohesion onto chromatitn which leads to premature sister chromatid separation.

Overall, UBL5 is required for prevention of premature sister chromatid separation by promotion of the correct pre-mRNA splicing and expression of Sororin.

After DNA replication, sister chromatids are tightly connected by cohesion. Cohesin is a multi-protein complex that has an important role in maintaining chromosomal segregation. As shown in Figure 5, Cohesin contains four subunits. Structural Maintenance of Chromosomes 1 and 3 (Smc1 and Smc3) are long polypeptides that fold on themselves and contain a hinge domain at one end and an ATPase head at the other end. The hinge domains of Smc1 and Smc3 bind together tightly while the Smc ATPase heads are connected by SA1/SA2 (Scc1) and Rad21. Scc3, or SA1 or SA2 in vertebrate somatic cells, interacts with the central region of Rad21. Pd35, Wings apart-like homolog (Wapl), and Sororin are regulatory factors of cohesion. PDS5 has two opposite functions based on its binding partner. When PDS5 is bound to WAPL, it promotes cohesion dissociation. On the other hand, PDS5 stabilizes cohesion when bound with Sororin (Morales et al. 2020). Sororin functions by protecting cohesion during the S and G2 phases by blocking the cohesion resolution factor WAPL (Oka et al. 2014). This allows the sister chromatids to be held together which is critical because cohesion cannot be reestablished once sister chromatids are separated. During mitosis, Sororin is phosphorylated which results in its dissociation from cohesion allowing WAPL to remove cohesion from the DNA.

Prior literature has shown that silencing UBL5 resulted in a significant impact on cellular survival. Cell cycle profiles of UBL5 knockdown showed an increase in the proportion of mitotic cells suggesting that UBL5 loss may impair chromosome alignment in mitosis (Oka et al. 2014). Time-lapse microscopy showed a significant delay to anaphase

in UBL5-deficient cells. To further understand this effect, metaphase chromosome spreads were prepared. UBL5 knockdown led to the premature loss of sister chromatid cohesion in the majority of mitotic cells. Taken together, these results concluded that UBL5 plays a key role in sister chromatid cohesion and cell proliferation in human cells (Oka et al. 2014).

Though cohesin is well-known for its role in sister chromatid separation, cohesin also has many proposed roles in replication and maintaining replication fork stability. Cohesin has been found to accumulate at stalled replication forks, possibly to fix damaging consequences that may arise as a result of DNA fork stalling. Stalling of the replication fork is known to result in long ssDNA which then becomes coated by RPA. The RPA coating may act as a template for cohesin loading. Keeping the sister chromatids tightly paired allows cohesin to facilitate template switching to repair DNA and promote efficient fork restart after stalling. Replication fork stalling can cause stretches of RPA-coated ssDNA that may be able to activate the replication checkpoint and are thought to promote cohesin recruitment. The stalled fork undergoes remodeling involving fork reversal to help fork restart. Cohesin may be involved in protecting stalled replication forks and in template switching to repair DNA lesions. PDS5 has been shown to recruit BRCA2 and RAD51, which protect reversed forks against excessive nuclease processing. Repair of the replication fork and fork restart depend on WAPL-mediated cohesin mobilization, possibly by increasing cohesin turnover at stalled forks such that replication can be resumed while cohesin levels are decreased (O'Neil et al. 2013).

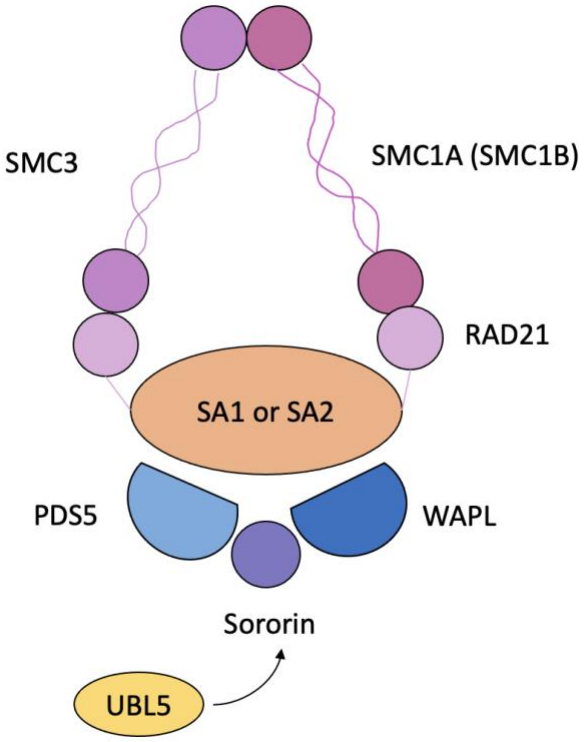


Figure 5. The Cohesin Ring. Schematic shows the four subunits of the cohesion complex SMC1A, SMC3, RAD21, and SAI/SA2 as well as its three regulatory cofactros PDS5, Sororin, and WAPL.

Cancer Testis Antigen 55 (CT55)

Another gene among the 189 genes discovered in the CRISPR screening is cancer testis antigen, CT55. Cancer testis antigens (CTA) are a type of tumor antigen which are known for their high immunogenicity. CTA expression is normally restricted to the testis and is otherwise silenced by promoter hypermethylation. CTA genes are frequency demethylated and re-expressed in various cancers with the potential of significantly altering the host immune response. This expression pattern coupled with its strong immunogenicity results in CTA being an important target for immunotherapeutic treatment of cancers. While the role of CTA in breast cancer is not well known, there is growing evidence to show that the expression of CTAs may have a role in tumorigenesis by regulating the proliferation of cancer cells and apoptosis (Mahmoud 2018). CTA's have been observed in several types of cancers such as melanoma, lung cancer, neuroblastomas, liver cancer, and bladder cancers (Simpson et al. 2005). Thus far, 70 families of CTA's with more than 140 members have been identified (Almeida et al. 2009). Most CTAs are expressed during spermatogenesis. CTAs involved in cancer therapy include MAGE-A1, MAGE-A3, MAGE-A4, NY-ESO-1, PRAME, CT83 and SSX2 (Fratta et al. 2011).

Though not much is known about CT55 specifically, it was found that expression of CT55 promotes cell growth. Also, CT55 has been found to be involved in the inhibition of apoptosis when induced by DNA damage. Therefore, it is speculated that CT55 may be a therapeutic target for treating breast tumors. Prior literature has shown that CT55 interacts with a highly conserved portion of BRCA2. Specifically CT55 has been shown to bind with BRCA2, specifically amino acids 121-235. The binding of CT55 and BRCA2 is

modulated by M-phase specific phosphorylation of BRCA2 which may be responsible for its function (Tomiyoshi et al. 2008). While the function of CT55 in DNA repair is unknown, its proposed role in carcinogenesis is related to cell proliferation and inhibition of apoptosis.

AIMS & RATIONALE

<u>Aim I:</u> To identify and clone genetic interactors of *BRCA2.*

Candidate genes discovered by the CRISPR activation screening such as *Ct55* and *Ubl5* were cloned and overexpressed using the retroviral vector (Murine Stem Cell Virus) MSCV. MSCV was used to activate candidate genes in embryonic stem (ES) cells by taking advantage of its viral long terminal repeat (LTR) which can drive high levels of target gene expression in ES cells. Confirmation of successful cloning of the gene of interest in MSCV will be confirmed by Sanger Sequencing.

<u>Aim II:</u> To examine the ability of UBL5 and CT55 in rescuing *Brca2* deficient ES cells.

BRCA2-null cells are embryonically lethal or exhibit severe defects in proliferation. BRCA2 deficiency is also known to lead to tumorigenesis. The first two genes which were chosen for further study were CT55 and UBL5. Therefore, the aim is to check if the individual CT55 and UBL5 overexpression rescues BRCA2-null ES cells with respect to the control vector. Once confirmed that CT55 and UBL5 overexpression leads to the rescue of BRCA2-null ES cells by Southern blot with respect to the control vector.

<u>Aim III</u>: To investigate UBL5 and CT55-mediated rescue of BRCA2-deficient cells.

Because BRCA2 localizes to the nucleus and studies show both nuclear and cytoplasmic localization of CTAs, we will perform a nuclear and cytoplasmic extraction to better

understand the localization of CT55. Because UBL5 is known to have a role in cohesion and in sister chromatid separation, we will explore the role of UBL5 in cohesion and in precocious sister chromatid separation with respect to BRCA2. A Colony Formation Assay will be performed to understand the effect of UBL5 on cell proliferation and cell viability with respect to BRCA2 status. We will observe the effect of silencing UBL5 on sister chromatid separation in BRCA2-proficient and deficient conditions.

MATERIALS & METHODS

Southern blot analysis. *Eco*RV-digested DNA was electrophoresed on a 1% agarose gel in 1×TBE (0.1 M Tris, 0.1 M Boric acid, 2 mM EDTA, pH 8.0) and transferred to a nylon membrane. A DNA probe for the conditional *Brca2* allele (*cko*, 4.8 kb) and *Brca2* knockout allele (*ko*, 2.2 kb) was labeled by [α-32P]-dCTP by Prime-It II Random Primer Labeling Kit (Agilent Technologies) and hybridized with Hybond-N+ nylon membrane (GE Healthcare) at 65° overnight. The membrane was washed twice with SSCP buffer containing 0.1% SDS in and exposed to a phosphor image screen overnight and developed in a Typhoon image scanner.

Cloning gene of interest in MSCV. PCR was completed with DNA to be amplified using the X PCR KIT. After PCR, gel extraction was completed using the gel extraction kit (Qiagen). The PCR product was then purified using the PCR purification kit. Ligation of the gene of interest into MSCV was completed using the NEBuilder HiFi DNA Assembly Cloning Kit. The products were electroporated and plated on 10 cm ampicillin LB-Agar plates and grown overnight at 4^0 C. Following this, six colonies were chosen from each plate and were grown in 5 mL LB culture overnight at 37^0 C for plasmid isolation. Following plasmid isolation, sequence PCR reactions were completed and confirmation of successful cloning of the gene of interest into the vector was confirmed by Sanger Sequencing.

Cell viability assay/Colony formation assay. Cells will be seeded at 10,000 cells per well in 96-well gelatinized plates. A few wells will be left empty with only feeder cells as a background control. After 24 hours, the plates will be washed twice with PBS, trypsinized with 200 µl trypsinEDTA for 15 minutes at 37 ˚C, and 200 µL M15 media will be added and pipetted vigorously up and down 20 times to achieve a single cell suspension. Cells will be counted using a 200 µL aliquot with a Coulter counter. The procedure will be repeated with the second and third set of cells on the second and third day after seeding. To estimate cell proliferation, the average feeder cell count will be subtracted from each value and the resulting numbers will be expressed as multiples of the average cell number (or a concentration) recorded at the first day after seeding.

Nuclear Cytoplasmic Extraction. 450 µl of CHIP cell lysis buffer and 50 µl of protein inhibitor cocktail were combined and 100 µl of the solution was added to each pellet and incubated on ice for 10 minutes and centrifuged at 4^0 C for 5 minutes at 10,000 RPM. The supernatant was removed into fresh tubes labeled with the cytoplasmic fraction and kept on ice. The nuclear lysis buffer was made using 450 µl of CHIP nuclear lysis buffer and 50 µl of protein and 75 µl of nuclear lysis buffer was added to the nuclear portion and kept on ice for 30 minutes and sonicated for 15 cycles and centrifuged at top speed for 5 minutes. 10 µl of protein mixed with 10 µl of dye was used for loading the cytoplasmic fraction and 15 µl of protein and 10 µl of dye was used for the nuclear fraction.

Cytogenic Analysis. To visualize sister chromatid separation upon silencing UBL5 and WAPL, transfection was completed by silencing UBL5 and WAPL in both BRCA2-

proficient and deficient DLD1 cells. After silencing for 48 hours, cells were arrested in the metaphase stage of mitosis by using colcemid. Metaphase spreads were then stained with Giemsa and visualized under the microscope.

RT-qPCR. For knockdown of UBL5 and WAPL, siRNAs against human UBL5 and WAPL were purchased from Dharmacon along with the control. Transfection of siRNAs was carried out using Liofectamine RNAiMAX transfection reagent (Life Technologies). Cells were silenced for 48 hours in all conditions.

RESULTS

Identification and cloning of genetic interactors of *Brca2.*

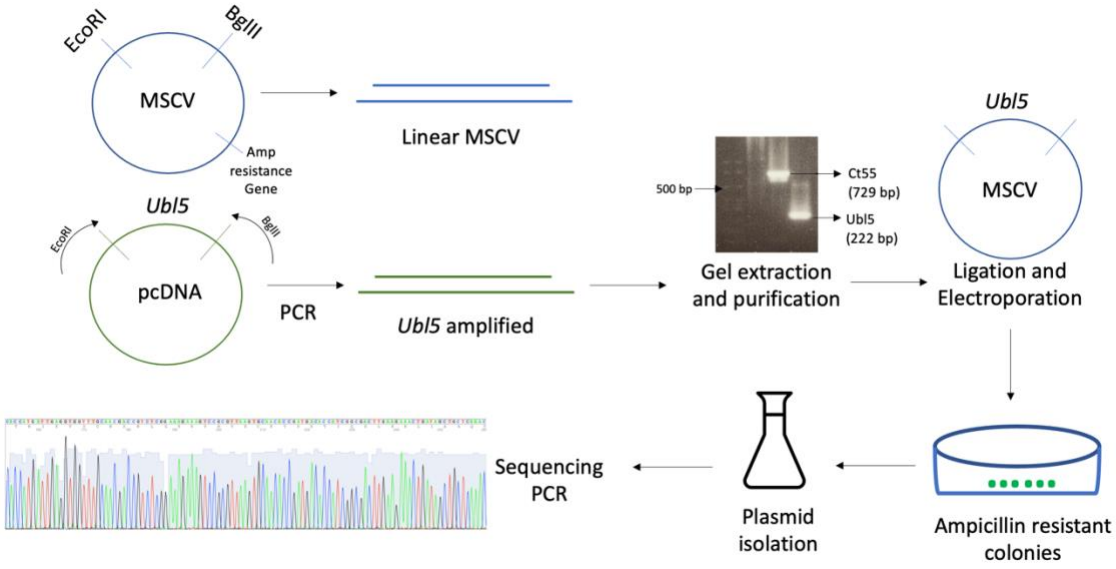
To identify genetic interactors of *BRCA2*, PL2F7 mESCs were used which carry a conditional (*cko)* allele of Brca2 and a functional null (*ko*) allele of BRCA2. PL2F7 cells stably expressing dCas9, and MS2 were transduced with the sgRNA library which was generated to target the 23, 439 genes of the mouse genome with three sgRNAs (69, 225 gRNAs total) used to target the promoter of each gene. Then, the sgRNA transduced PL2F7 cells were selected with puromycin resulting in sgRNA transduced puromycin resistant cells. In PL2F7 cells, the conditional allele of *Brca2* is flanked by the 5' and 3' ends of the minigene *HPRT*. Therefore, these cells were selected for the generation of a functional *HPRT* minigene. After Cre-mediated recombination, the conditional allele of BRCA2 is deleted. Any cells which survive after Cre-mediated deletion will be as a result of the sgRNAs as it is known that BRCA2-deficient cells do not survive. These cells will also be resistant to hypoxanthine-aminopterin-thymidine (HAT) media. Surviving clones were genotyped and analyzed by Southern blot for the presence of $Brca2^{ko/ko}$ clones. As shown in the Southern blot in Figure 7, there were 189 clones which showed the presence of $Brca2^{ko/ko}$ clones indicating that the sgRNAs were leading to the survival of *Brca2*-lethality. Finally, the surviving clones were sequenced to identify which sgRNAs were leading to the survival. Table 2 in the appendix shows the complete list of potential genetic interactors of *Brca2* identified by CRISPR-activation screening. We next investigated the KEGG pathway database to check if any of the identified genes cluster in any specific

pathway. Enrichment of specific pathways were not identified by pathways analysis or gene ontology terms. Hence, we scelected the potential genetic interactors of BRCA2 based on certain criteria such as 1) known role in DNA repair, 2) known interaction with BRCA2 and 3) multiple hits in screening for further evaluation. Table 1 shows the list of genes (n=16) selected for further analysis.

In the present study, we have cloned the cDNA of all 16 genes in the MSCV vector to further confirm whether individual over expression of these genes can rescue the BRCA2 null ES cells. Stable UBL5 and CT55 over expressing cells were generated by transducing the PL2F7 mESCs with UBL5 and CT55 lenti viral particles and selected with puromycin for 5 days. The MSCV system was used because of its powerful 5' LTR which acts as a promoter to upregulate the transcription of a gene of interest. MSCV was digested using the restriction enzymes EcoRI and BglII. The pcDNA containing our gene of interest was amplified using forward and reverse primers which included the corresponding restriction site sequence at the 5' and 3' end. Following the amplification of our gene of interest, we performed a gel extraction and purification and ligated our gene of interest into the MSCV system. Electroportation was completed using electrocompetent cells to increase the number of ligated plasmids. We plated these cells on LB-Agar ampicillin plates and picked six colonies for plasmid isolation. By sequence PCR, we confirmed by Sanger sequencing if our gene of interest was correctly inserted into the vector. By following this cloning procedure depicted in Figure 6, we successfully cloned 16 genes out of the 189 genetic interactors identified by the CRISPR activation. Of the 16 genes shown in Table 1, CT55 and UBL5 were selected for further study. UBL5 was selected because of its role in the FA

pathway and pre-mRNA splicing and CT55 was selected because of its physical interaction with BRCA2.

a.



b.

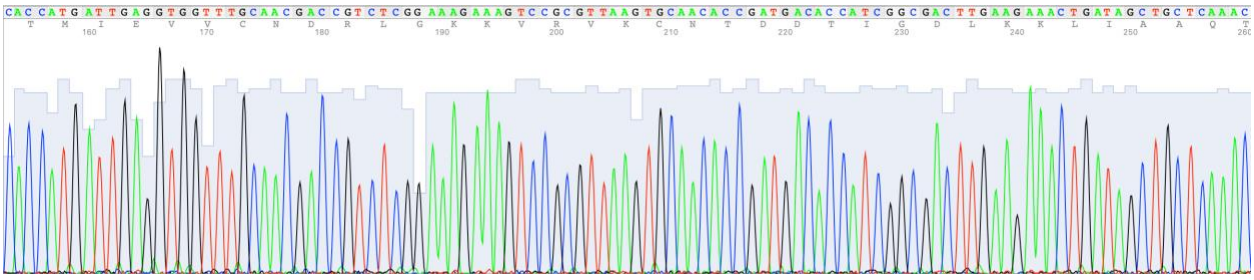


Figure 6. Cloning of genetic interactors of *Brca2* in MSCV. a. Schematic shows an overview of the protocol used for cloning which was followed in the present study. b. Image from Sanger sequencing which shows the successful integration of UBL5 in the MSCV vector.

Gene	Status
Ct55	Confirmed by Sequencing
Ubl5	Confirmed by Sequencing
Olfr884	Confirmed by Sequencing
Ndfip	Confirmed by Sequencing
Scyl3	Confirmed by Sequencing
Taar6	Confirmed by Sequencing
Wdr83	Confirmed by Sequencing
Armh1	Confirmed by Sequencing
Srd5a1	Confirmed by Sequencing
Cers2	Confirmed by Sequencing
Dnajb8	Confirmed by Sequencing
Triap1	Confirmed by Sequencing
Trim30a	Confirmed by Sequencing
Grk6	Confirmed by Sequencing
Dhx30	Confirmed by Sequencing
Eme1	Confirmed by Sequencing

Table 1. List of genetic interactors which were selected for further analysis and successfully cloned in MSCV. List of genes successfuly cloned in the MSCV vector and further confirmed by Sanger Sequencing.

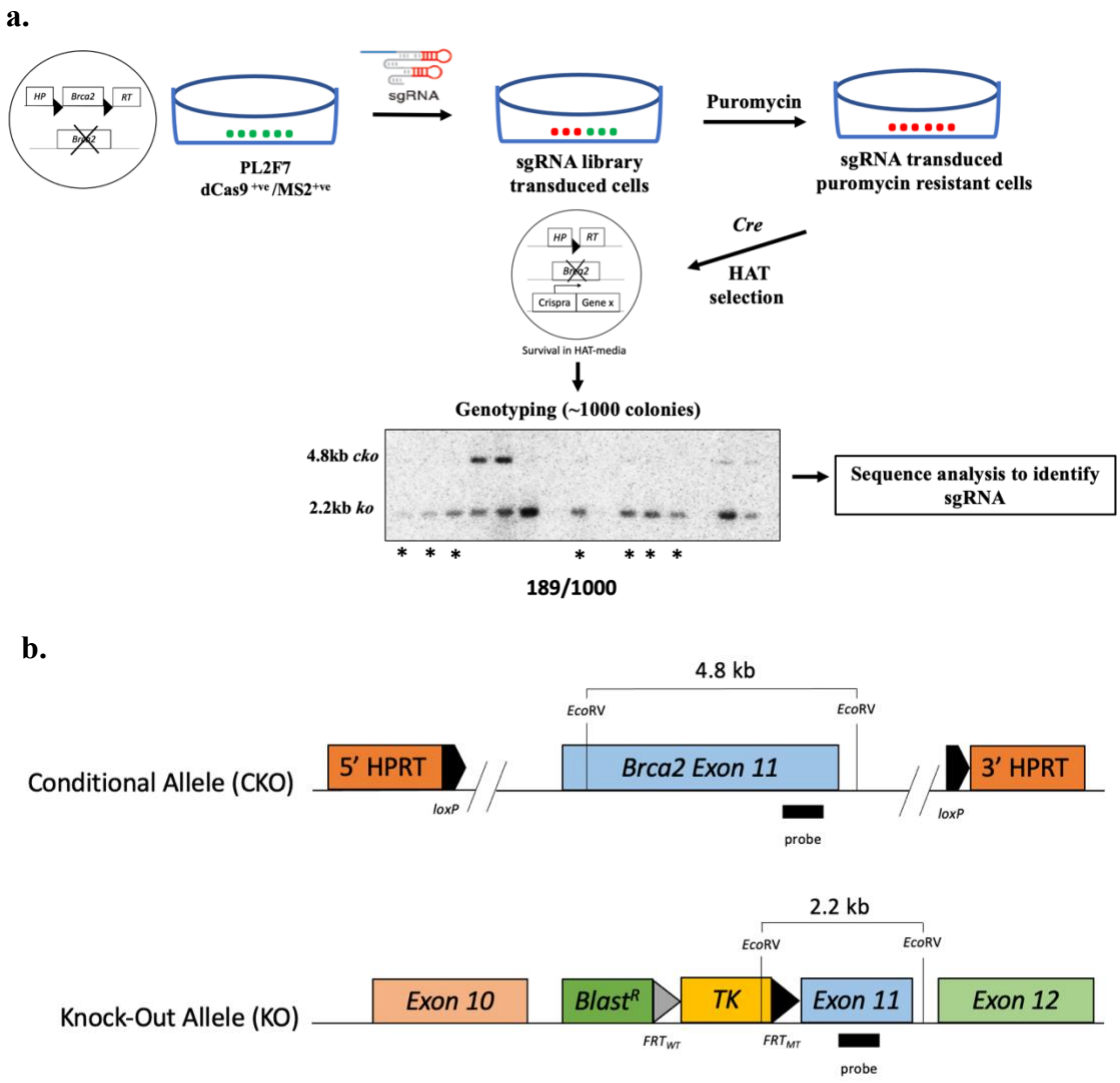


Figure 7. Model depiciting the CRISPR rescue assay. A stable cell line was generated using PL2F7 mESCs, dCas9 and MS2. The sgRNA library was transduced into the stable cell line and selected with puromycin. After Cre-mediated recombination, the conditional allele of *Brca2* is deleted leaving the cell *Brca2*-deficient. Surviving HAT-resistant colonies were observed by Southern blot. 4.8 kb band represents the conditional knock out allele of *Brca2* and the 2.2 kb band represents the knock out allele of *Brca2*. 189 clones were observed by southern to survive in the absence of *Brca2* and these clones were further analyzed by sequence analysis to identify the sgRNA leading to the survival of the *Brca2*-deficinet mESCs. b. Schematic shows the genotyping of HAT-resistant colonies obtained after Cre-mediated recombination in PL2F7 cells. Schematic shows the targeting of exon 11 of *Brca2*. *Eco*RV was used to digest genomic DNA and the position of the probe is marked by the thick black line. Schematic derived from Biswas et al. 2018.

Ubl5 and *Ct55* overexpression rescues the lethality of *Brca2* in mESC

To determine if the individiaul overexpression of *Ubl5* and *Ct55* rescues *Brca2* lethality, we carried out a rescue experiment wherein the *cko* allele of *Brca2* was deleted using *Cre*-electroporation in *Ubl5* and *Ct55* over expressing PL2F7 ES cells. Empty vector tranduced cells were used as control. The recombinant clones were selected in HAT media and HAT resistant colonies were analyzed for $Brca2^{ko/ko}$ clones by southern blotting. No $Brca2^{ko/ko}$ clones were obtained from cells which were transduced with the empty vector. However, *Ubl5* and *Ct55* overexpressing clones resulted in several $Brca2^{ko/ko}$ clones. Taken together, these results show that *Ubl5* and *Ct55* overexpression can rescue *Brca2*-loss induced cell lethality. Next, we investigated the mechanism of *Ubl5* mediated rescue of $Brca2^{ko/ko}$ clones.

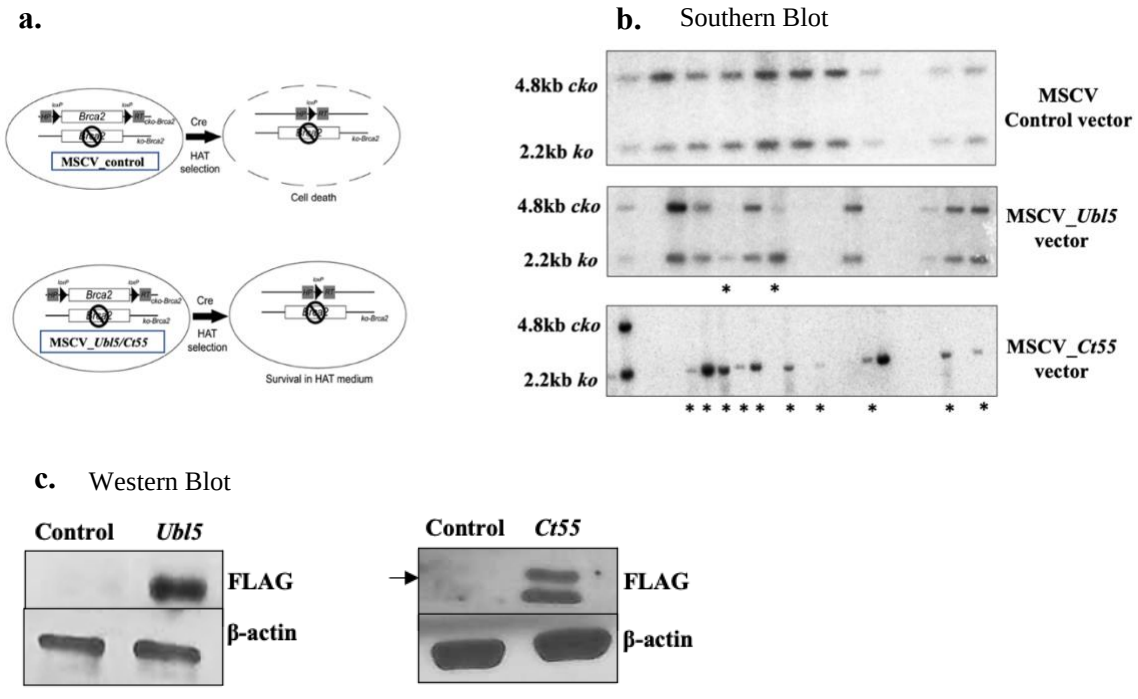


Figure 8. Overexpression of *Ct55* and *Ubl5* rescues *Brca2*-lethality. a. Model depicts PL2F7 cells individually overexpressing *Ct55* and *Ubl5* and an empty vector as a control. Schematic shows *Cre*-induced loss of the *cko* allele of *Brca2*. b. Southern blot analysis of HAT-resistant clones which were genotyped to identify any $Brca2^{ko/ko}$ clones. No $Brca2^{ko/ko}$ clones were detected in the empty vector. MSCV *Ubl5* vector and MSCV *Ct55* vector showed several $Brca2^{ko/ko}$ clones. c. Western blot which shows the overexpression of both *Ubl5* and *Ct55* compared to the control.

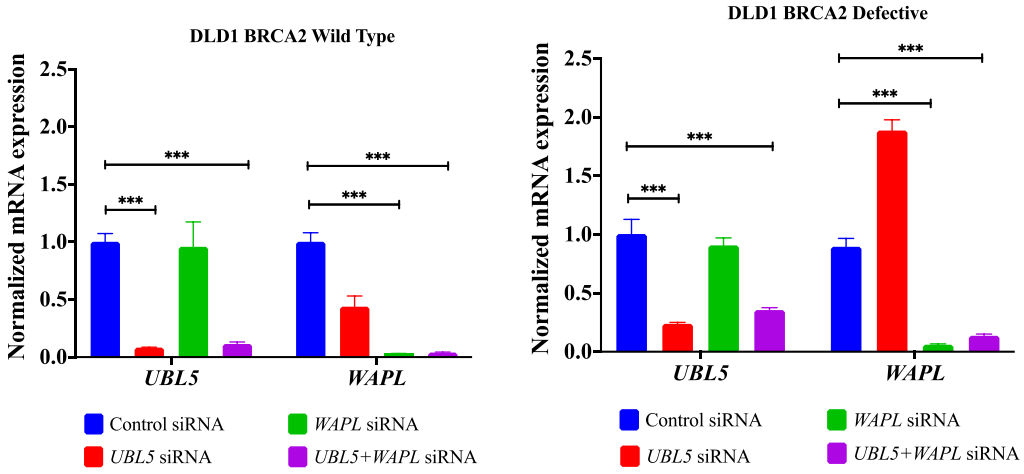
UBL5 affects cell viability with respect to BRCA2

UBL5 is known for its role in pre-mRNA splicing. In the context of DNA repair, UBL5 promotes the function of the Fanconi Anemia (FA) pathway for the repair of DNA interstrand crosslinks. This process is mediated by w specific interaction with a central FA pathway component known as FANCI. The maintenance of chromosome stability after interstrand crosslinks becomes compromised when the interaction between UBL5 and FANCI is inhibited in either protein (Oka et al. 2015). Cohesins also play an important role in DNA repair as UBL5 is reported to affect the pre-mRNA splicing of sororin, a cohesion factor, which is critical for loading and stabilization of cohesions on chromatids. Here, we investigated the effect of UBL5 with respect to BRCA2 status in DLD1 cells. An isogenic pair of BRCA2 proficient (DLD1) and BRCA2 deficient (DLD1 BRCA2-ve) were used and cell viability was analyzed by silencing UBL5 (Figure 9a). Silencing of UBL5 in DLD1 drastically reduces the cell viability (Figure 9b). Interestingly, the reduction in cell viability was not as dramatic in BRCA2 deficient DLD1 cells upon UBL5 knockdown. To understand if cohesions are involved in this process, we analyzed WAPL, a well-known cohesion release factor (Oka et al. 2014). Surprisingly, knockdown of WAPL partially (~20%) rescues cell viability in BRCA2 proficient DLD1 cells, whereas the rescue was more profound (~40%) in BRCA2 deficient DLD1 cells. These observations suggests that cohesin loss on chromatids could be one of the reasons for cell lethality observed under UBL5 deficient condition. Morever, WAPL knockdown alone induced the cell proliferation in both BRCA2 proficient and deficient DLD1 cell with the effect more pronounced in BRCA2 deficient cells. Silencing both UBL5 and WAPL in combination

showed a decrease in cell viability overall but an increase when compared to silencing uBL5 alone. This may be because of other cohesion cofactors which are unaffected by UBL5 silencing which may be leading to loading of cohesin, though at a decreased efficiency. Despite the impaired level of cohesin loading, the cohesin that has been loaded will remain loaded as a result of WAPL silencing. These few cells will be able to overcome precocious sister chromatid separation and will have equal distribution of sister chromatids into daughter cells and therefore resulting in an increased cell viability.

To reconfirm the observations with respect to BRCA2, we next silenced UBL5 in combination with BRCA2 silencing in the osteosarcoma cell line U2OS. As shown in Figure 9c, silencing of BRCA2 alone in U2OS cells causes a reduction in cell viability because of BRCA2's role in genomic stability. While UBL5 silencing alone is lethal as expected, silencing UBL5 and BRCA2 in combination results in a increase in cell viability when compared to silencing of UBL5 alone in U2OS cells. These results suggests that cohesion retention on chromatid is important for cell survival and the level of cohesion could be modulated by BRCA2. These observations were further validated in two independent cell lines, human breast cancer cell line, MDAMB231 and mouse mammary cancer cell line KB2P1.21(data not shown). We propose that BRCA2 may have a role in the unloading of cohesin, similar to that of WAPL, and leads to an increase in cell viability when silencing UBL5.

a.


DLD1 BRCA2 Wild Type
Normalized mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
UBL5
WAPL
Control siRNA
WAPL siRNA
UBL5 siRNA
UBL5+WAPL siRNA
DLD1 BRCA2 Defective
Normalized mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
***
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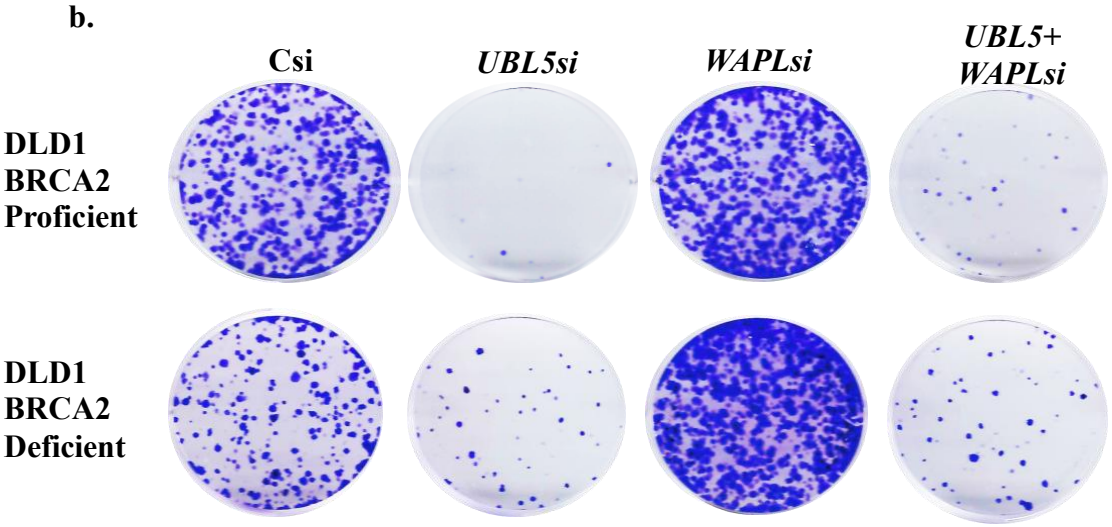
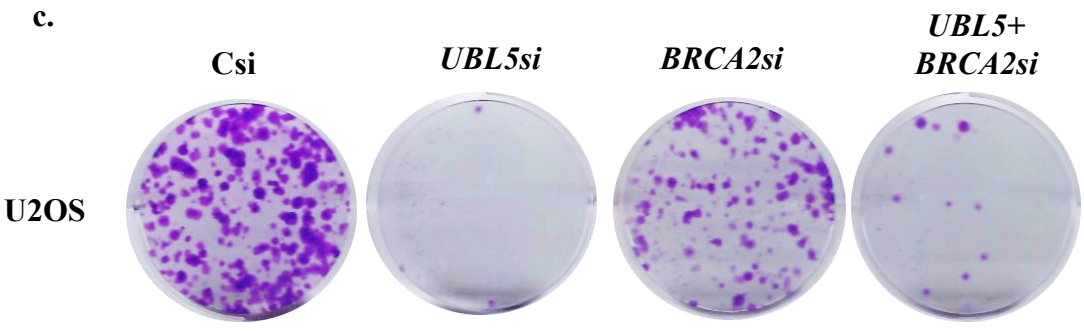
UBL5
WAPL
Control siRNA
WAPL siRNA
UBL5 siRNA
UBL5+WAPL siRNA


b.
Csi
UBL5si
WAPLsi
UBL5+
WAPLsi
DLD1
BRCA2
Proficient
DLD1
BRCA2
Deficient


c.
Csi
UBL5si
BRCA2si
UBL5+
BRCA2si
U2OS

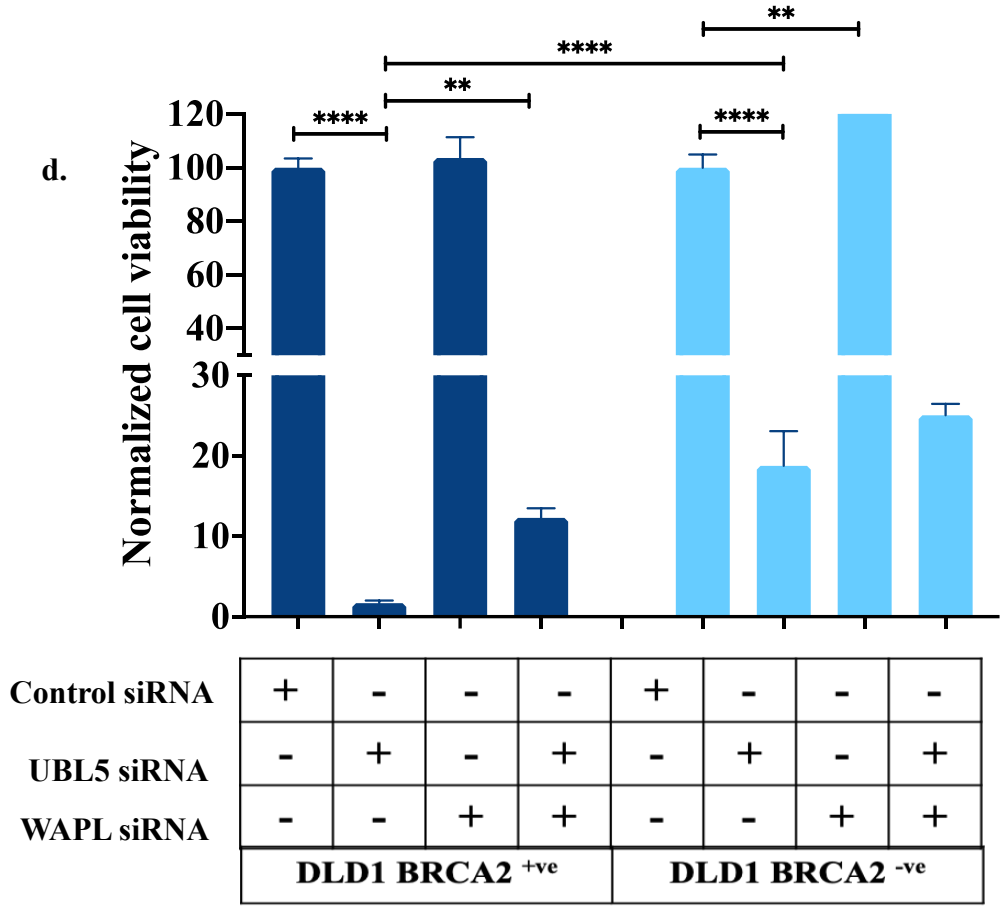


Figure 9. UBL5 affects cell viability with respect to BRCA2. a. RT-PCR. Result to show confirmation of silencing of UBL5 and WAPL. b**.** In BRCA2 proficient DLD1 cells, a significant reduction in cell viability is observed upon silencing UBL5. An increase in cell viability is observed upon silencing WAPL. Silencing uBL5 and WAPL in combination results in an increase in cell viability compared to silencing UBL5 alone. c. In U2OS cells, a similar decrease in cell viability is observed upon silencing UBL5. Silencing BRCA2 resulted in a decrease in cell viability and silencing both UBL5 and BRCA2 in combination appears to rescue cell viability as compared to silencing UBL5 alone. d. Graphical representation showing normalized cell viability upon silencing UBL5 and WAPL in BRCA2-proficient and deficient conditions.

UBL5 silencing results in a mild cohesin defect in sister chromatid separation

To understand the effect of BRCA2 on cohesion with respect to UBL5 status, we analyzed the metaphase chromosomes for any cohesion defects in BRCA2 proficient and deficient DLD1 cells. UBL5 deficiency has been known to result in precocious siter chromatid separation (Oka et al. 2014). To understand the effect of silencing UBL5 on sister chromatid segregation in BRCA2 proficient and deficient cells, cells were arrested in metaphase using Colcemid and stained with Giemsa. Metaphase spreads showed that silencing of UBL5 in BRCA2 proficient DLD1 cells resulted in a cohesin defect, marked by early separation of chromatids compared to the BRCA2 proficient controls (Figure 10). However, in BRCA2 deficient DLD1 cells, silencing of UBL5 results in an equal distribution of sister chromatids with a cohesin defect as well as sister chromatids with no cohesin defect (Figure 10). The half of the metaphase spreads which show no cohesin defect may have a better chance of proper sister chromatid segregation and an increased chance of cell survival. Silencing both UBL5 and WAPL in BRCA2 proficient DLD1 cells shows a mild cohesin defect compared to in BRCA2-deficient conditions where there is no cohesin defect. These results further suggests that similar to WAPL, BRCA2 may have a role in unloading of cohesin. In BRCA2-deficient conditions, there may be a partial retention of cohesin on the chromatin which may lead to an increase in cell survival. Taken together, these cytogenic results show that the effect of UBL5 loss on cohesion is more severe in BRCA2 proficient cells than in BRCA2 deficient cells, further suggesting the possible interaction between BRCA2 and cohesion.

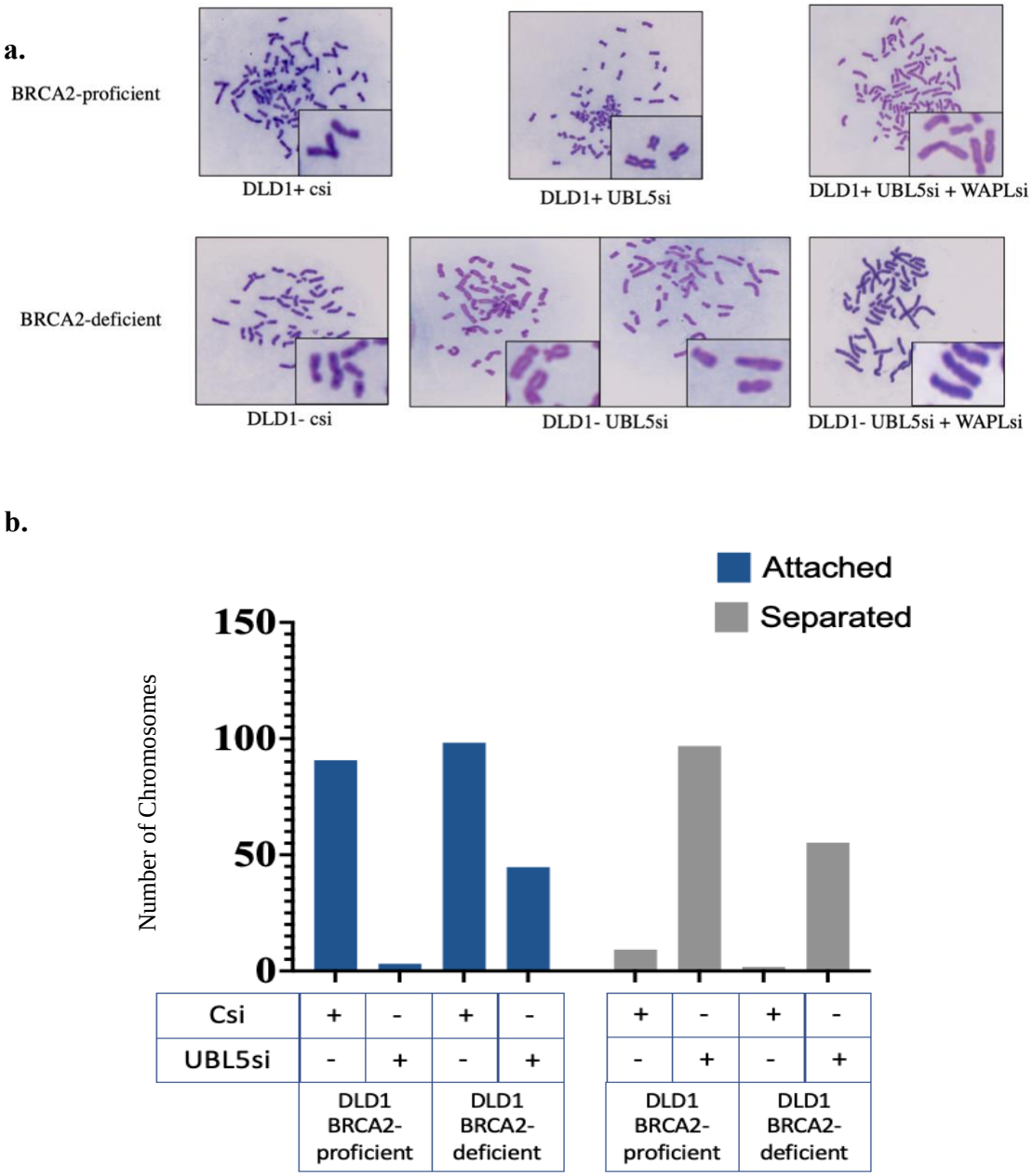


Figure 10. UBL5 silencing results in a mild cohesin defect in sister chromatid separation. a. BRCA2-proficient DLD1 cells showed a mild cohesin defect upon silencing UBL5. BRCA2-deficient cells showed a combination of sister chromatids with a mild cohesin defect as well as normal sister chromatids. b. Graph depicting the results shown in cytogenic analysis.

Nuclear and Cytoplasmic localization of CT55

While CTAs with surface localization have been identified, CTA expression is mainly intracellular. Currently, little is known about the function and localization of CTAs in normal testis or tumor cells. However, BRCA2 is known to localize to the nucleus. While CT55 might harbour several unknown functions outside of the nucleus similar to CTA's, we intended to understand if CT55 has any nuclear functions, and particulary its role in DNA repair. To better understand the interaction between BRCA2 and CT55, we performed a nuclear and cytoplasmic extraction of CT55 in BRCA2 proficient and BRCA2 deficient cell lines. By doing this, we found that CT55 does indeed show nuclear localization as well as cytoplasmic localization. As shown in Figure 12, CT55 expression is greater in the cytoplasm than the nucleus.

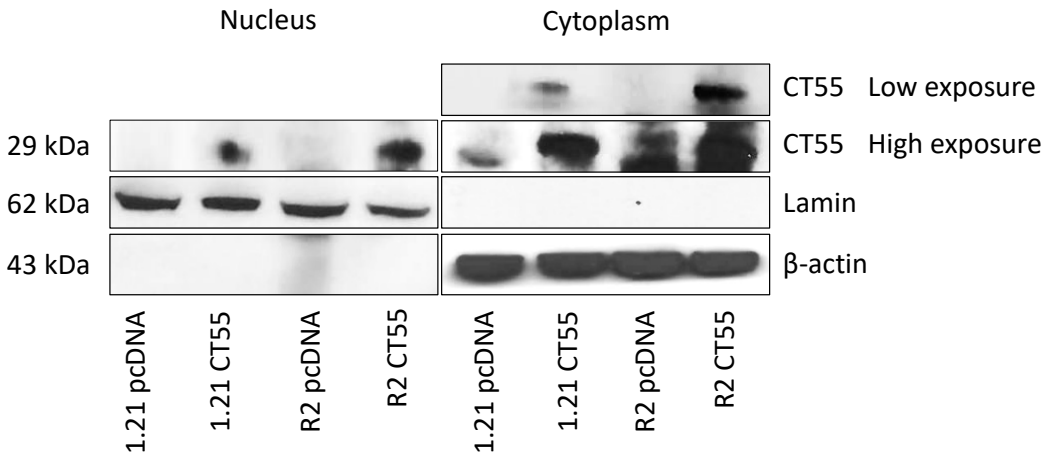


Figure 12. Nuclear Localization of CT55. Western Blot shows the presence of CT55 in the nucleus as well as in the cytoplasm. In the nuclear fraction, CT55 is present in both 1.21 (BRCA2-deficient) and R2 (BRCA2-proficient) cell lines. For the cytoplasmic fraction, CT55 is also present in both BRCA2 deficient and proficient cell lines.

DISCUSSION

In this study, we have utilized a CRISPR activation screening to upregulate the transcription of candidate genenes in the mouse genome to identify potential genetic interactors of BRCA2. Through this screening, we have idenfiied 189 genetic interactors which rescue the lethality of BRCA2. Out of these 189 candidate genes, we have successfully cloned 16 genes in the MSCV vector and have chosen *Ct55* and *Ubl5* for future study. We chose to study CT55 because previous literature has shown that CT55 physically interacts with BRCA2. There are many CTA's which localize to the cell surface such as CT83, SP17, SLCO681, and PLAC1. SPAN-X (sperm protein associated with the nucleus mapped to the X chromosome), and CTp11 (cancer/testis-associated protein of 11 kDa) are examples of a CTA that shows nuclear localization (Westbrook et al. 2001). Here, we have shown nuclear and cytoplasmic localization of CT55. Future work will focus on CT55 and its interaction with BRCA2 by confirming that CT55 physically interacts with BRCA2. We will also examine the role of CT55 in the DNA damage response as well as in cell viability.

We chose to study UBL5 because of its role in the FA pathway in the repair of ICL's as well as its role in Cohesin. Here, we have shown that UBL5 affects cell viability with respect to BRCA2. Silencing UBL5 is known to lead to retention of the first intron of the cohesion co-factor Sororin resulting in reduced loading of cohesion onto chromatin. Without cohesion, sister chromatids become separated before chromosomes attach to both

poles of the spindle. Therefore, an equal distribution of sister chromatids into forming daughter cells would not be possible. Because of this, cell viability is significantly reduced when UBL5 is silenced. Upon silencing WAPL, cohesion remains on chromatin which likely prevents premature sister chromatid segregation allowing the chromatin to properly align during metaphase and ensures equal distribution of sister chromatids into forming daughter cells. Though UBL5 is known to lead to proper cohesin loading onto chromatin through its interaction with Sororin, it may not be the only cofactor leading to this effect. Further, because UBL5 is a part of the pre-mRNA splicing complex, its silencing may be affecting several other genes as well. Interestingly, the dramatic reduction in cell viability was not as pronounced upon silencing UBL5 in BRCA2-deficient conditions as compared to in BRCA2-proficient conditions. Taken together, we propose that similar to WAPL, BRCA2 may have a role as a cohesion unloader. Further study will focus on uncovering the potential novel role of BRCA2 as a cohesion unloader. Cohesin is also known to be present at replication forks. Therefore, future study will also focus on UBL5 and its role in replication fork dynamics as well as in the cohesin complex with respect to BRCA2.

Identifying genetic interactors of *BRCA2* by MSCV-insertional mutagenesis has proven difficult as a result of the upregulation of genes that are far apart form the integration site. Here, we used a CRISPR activation screening to globally identify several genetic of *BRCA2*. By utilizing this method, we were able to identify 189 potential genetic interactors of *BRCA2*. Further, we have successfully cloned 16 of these potential genetic interactors into the MSCV vector. By further study of these genetic interactors and by examining the mechanism by which they resuce the lethality of *Brca2*-deficient cells, we

may be able to understand new potential functions of BRCA2. Understanding these functions may impact the treatment of patients suffering from various BRCA2-deficient cancers.

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APPENDIX

Gene name	NCBI ID	Gene Full Name
Sdr16c6	NM_001080710	Short-chain dehydrogenase/reductase family 16C member 6
Zfp775	NM_173429	zinc finger protein 775 (Zfp775)
Nipsnap3a	NM_028529	Protein NipSnap homolog 3A
Gpr137b	NM_031999	G protein-coupled receptor (GPCR)-like protein
Cib2	NM_019686	Calcium and integrin-binding protein 2
Bmpr1b	NM_007560,	bone morphogenetic protein receptor, type 1B (Bmpr1b)
Gm11564	NM_001100614	Predicted gene 11564
Ptpn23	NM_001081043	protein tyrosine phosphatase, non-receptor type 23 (Ptpn23)

Tmem19	NM_133683	transmembrane protein 19 (Tmem19)
Bmpr1b	NM_007560	bone morphogenetic protein receptor, type 1B (Bmpr1b)
Naif1	NM_194335,	nuclear apoptosis inducing factor 1 (Naif1)
Sez6l	NM_001253916	Seizure 6-like protein
Tmem19	NM_133683	transmembrane protein 19 (Tmem19)
Ccdc32	NM_199310	Coiled-Coil Domain Containing 32
Slc43a2	NM_001199283	solute carrier family 43, member 2 (Slc43a2)
Vmn1r238	NM_001167539	Vomeronasal type-1 receptor 238
Grk6	NM_001286063	G protein-coupled receptor kinase 6
Snx9	NM_025664	Sorting nexin-9
Kcnt1	NM_001145403	Potassium channel subfamily T member 1

Vmn1r203	NM_134236	Vomeronasal type-1 receptor 203
Jph1	NM_020604	Junctophilin 1
Kcnt1	NM_001145403	Potassium channel subfamily T member 1
Lce1f	NM_026394	late cornified envelope 1F
Zfp788	NM_023363	Zinc finger protein 788
Csta3	NM_001082542	Cystatin A family member 3
Ndufa1	NM_019443	NADH:Ubiquinone Oxidoreductase Subunit A1
Krtap4-8	NM_001085547	keratin associated protein 4-8
Vmn1r123	NM_001166707	vomeronasal 1 receptor 123 (Vmn1r123)
Bard1	NM_007525	BRCA1 Associated RING Domain 1
Pradc1	NM_028505	Protease Associated Domain Containing 1
Zdhhc16	NM_023740	zinc finger, DHHC domain containing 16

Spns2	NM_153060	spinster homolog 2
Arid4a	NM_001081195	AT rich interactive domain 4A (RBP1-like)
Mafb	NM_010658	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)
Abhd4	NM_134076	abhydrolase domain containing 4
Gm11992	NM_001037928	predicted gene 11992
Trim30a	NM_009099	Tripartite motif-containing protein 30A
Tubgcp2	NM_133755	tubulin, gamma complex associated protein 2
Gm20877	NM_001199332a	predicted gene, 20877
Ct55	NM_029142	cancer/testis antigen 55
Rwdd3	NM_028456	RWD domain containing 3
Smr3a	NM_011422, NM_001252680	submaxillary gland androgen regulated protein 3A, submaxillary gland androgen regulated protein 2

Srd5a1	NM_175283	steroid 5 alpha-reductase 1
Wapl	NM_001004436	WAPL cohesin release factor
Pcdhgb5	NM_033577	protocadherin gamma subfamily B, 5
Slc30a5	NM_022885	solute carrier family 30 (zinc transporter), member 5
Prss32	NM_027220	protease, serine 32
Mgat4a	NM_001290801	mannoside acetylglucosaminyltransferase 4, isoenzyme A
Pip5k1b	NM_008846	phosphatidylinositol-4-phosphate 5-kinase, type 1 beta
Olfr365	NM_146662	olfactory receptor 365
Plekhg3	NM_153804	pleckstrin homology domain containing, family G (with RhoGef domain) member 3
Pld5	NM_001195816,	phospholipase D family, member 5 (Pld5)

Alkbh8	NM_026303	alkB homolog 8, tRNA methyltransferase (Alkbh8)
Mrpl55	NM_001302335,	mitochondrial ribosomal protein L55 (Mrpl55),
Pip5k1b	NM_008846	phosphatidylinositol-4-phosphate 5-kinase, type 1 beta (Pip5k1b)
Nbea	NM_030595	neurobeachin
Gtf2h3	NM_181410	general transcription factor IIH, polypeptide 3
Triap1	NM_026933	TP53 regulated inhibitor of apoptosis 1
Actl9	NM_183282	actin-like 9
Chsy1	NM_001081163	chondroitin sulfate synthase 1
Eif3a	NM_010123	eukaryotic translation initiation factor 3, subunit A
Olfr821	NM_146776,	olfactory receptor 821 (Olfr821)
Prkcz	NM_008860	protein kinase C, zeta

Gabrg2	NM_177408	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 2
Slamf8	NM_029084	SLAM family member 8
Cypt2	NM_173436	cysteine-rich perinuclear theca 2
Rhob	NM_007483	ras homolog family member B
Vmn1r104	NM_001166738	vomeronasal 1 receptor 104
Pcgf2	NM_001163308	polycomb group ring finger 2
Rnh1	NM_145135	ribonuclease/angiogenin inhibitor 1
Asxl1	NM_001039939	ASXL transcriptional regulator 1
Btbd6	NM_001145900	(POZ) domain containing 6
Taf7l	NM_028958	TATA-box binding protein associated factor 7 like
Epb41	NM_183428	erythrocyte membrane protein band 4.1
Lpin2	NM_001164885	lipin 2

Ptpn23	NM_001081043	protein tyrosine phosphatase, non-receptor type 23
Csl	NM_027945	citrate synthase like
Sart3	NM_016926	squamous cell carcinoma antigen recognized by T cells 3
Nacad	NM_001081652	NAC alpha domain containing
Cetn3	NM_007684	centrin 3
Ubl5	NM_025401	ubiquitin-like 5
Snx5	NM_024225	sorting nexin 5
Syn3	NM_001164495	synapsin III
Kmt5c	NM_146177	lysine methyltransferase 5C
Pabpc4l	NM_001101479	poly(A) binding protein, cytoplasmic 4-like
Phb2	NM_007531	prohibitin 2
Ehf	NM_007914	ets homologous factor

Nqo2	NM_001163242	N-ribosyldihydronicotinamide quinone reductase 2
Henmt1	NM_025723	HEN1 methyltransferase homolog 1
C77080	NM_001285867	sequence C77080
Dnajb8	NM_019964	DnaJ heat shock protein family (Hsp40) member B8
Il25	NM_080729	interleukin 25
Cdr2	NM_007672	cerebellar degeneration-related 2
Vpreb2	NM_016983	pre-B lymphocyte gene 2
Mfap2	NM_008546	microfibrillar-associated protein 2
Sdr39u1	NM_001082975	short chain dehydrogenase/reductase family 39U, member 1
Cldn14	NM_001165925	Mus musculus claudin 14 (Cldn14), transcript variant 2, mRNA

Atp6v0b	NM_033617	Mus musculus ATPase, H+ transporting, lysosomal V0 subunit B (Atp6v0b), mRNA
Mucl1	NM_009268	Mus musculus mucin-like 1 (Mucl1), transcript variant 1, mRNA
Tmem69	NM_177670	Mus musculus transmembrane protein 69 (Tmem69), mRNA
Rmnd1	NM_025343	Mus musculus required for meiotic nuclear division 1 homolog (Rmnd1), transcript variant 1, mRNA
Olfr739	NM_146668	Mus musculus olfactory receptor 739 (Olfr739), mRNA
Sostdc1	NM_025312	Mus musculus sclerostin domain containing 1 (Sostdc1), mRNA

Scyl3	NM_028776	Mus musculus SCY1-like 3 (S. cerevisiae) (Scyl3), transcript variant 1, mRNA
Slc25a32	NM_172402	Mus musculus solute carrier family 25, member 32 (Slc25a32), mRNA; nuclear gene for mitochondrial product
3110082J24Rik	NM_001256263	Mus musculus RIKEN cDNA 3110082J24 gene (3110082J24Rik), mRNA
Nfyb	NM_010914	Mus musculus nuclear transcription factor-Y beta (Nfyb), mRNA
Psme2b	NM_001281472	Mus musculus protease (prosome, macropain) activator subunit 2B (Psme2b), mRNA

Wdr83os	NM_001001493	Mus musculus WD repeat domain 83 opposite strand (Wdr83os), mRNA
Syk	NM_011518	Mus musculus spleen tyrosine kinase (Syk), transcript variant 1, mRNA
Ank	NM_020332	Mus musculus progressive ankylosis (Ank), mRNA
Wdr17	NM_028220	Mus musculus WD repeat domain 17 (Wdr17), transcript variant 1, mRNA
Cers2	NM_029789	Mus musculus ceramide synthase 2 (Cers2), transcript variant 1, mRNA
Slc18a1	NM_153054	Mus musculus solute carrier family 18 (vesicular monoamine), member 1 (Slc18a1), mRNA
Rmdn1	NM_025476	regulator of microtubule dynamics 1 (Rmdn1)

Fli1	NM_008026	Mus musculus Friend leukemia integration 1 (Fli1), mRNA
Bfar	NM_025976,NM_025653	Mus musculus bifunctional apoptosis regulator (Bfar), transcript variant 1
H2ac19	NM_178212	Mus musculus H2A clustered histone 19 (H2ac19), mRNA
Eme1	NM_177752	Mus musculus essential meiotic structure-specific endonuclease 1 (Eme1), transcript variant 2, mRNA
Zkscan17	NM_001130529	Mus musculus zinc finger with KRAB and SCAN domains 17 (Zkscan17), transcript variant 2, mRNA
Arrdc5	NM_029799	Mus musculus arrestin domain containing 5 (Arrdc5), mRNA

Taar6	NM_001010828	Mus musculus trace amine-associated receptor 6 (Taar6), mRNA
Purg	NM_001098233	Purine-rich element binding protein G
Gpr63	NM_030733	Probable G-protein coupled receptor 63
Nat3	NM_008674	Mus musculus N-acetyltransferase 3 (Nat3), mRNA.
Epo	NM_007942	Mus musculus erythropoietin (Epo), transcript variant 1, mRNA.
Zfat	NM_198644	Mus musculus zinc finger and AT hook domain containing (Zfat), transcript variant 2, mRNA
Rai14	NM_001166408	Mus musculus retinoic acid induced 14 (Rai14), transcript variant 2, mRNA

Tnfrsf17	NM_011608	Mus musculus tumor necrosis factor receptor superfamily, member 17 (Tnfrsf17), mRNA
Sertad3	NM_133210	Mus musculus SERTA domain containing 3 (Sertad3), mRNA
Ppt2	NM_001302395,NM_001302396	Lysosomal thioesterase PPT2 (PPT-2)
Rbm17	NM_152824	Mus musculus RNA binding motif protein 17 (Rbm17), mRNA
H1f10	NM_198622	Mus musculus H1.10 linker histone (H1f10), mRNA
Rabgap1	NM_146121	Mus musculus RAB GTPase activating protein 1 (Rabgap1), transcript variant 1, mRNA

Lrrc26	NM_146117	Mus musculus leucine rich repeat containing 26 (Lrrc26), mRNA
Elmo2	NM_001302754	engulfment and cell motility 2
Fzd1	NM_021457	Mus musculus frizzled class receptor 1 (Fzd1), mRNA
Sprtn	NM_001111141	Mus musculus SprT-like N-terminal domain (Sprtn), mRNA
Ppa2	NM_001293641	Mus musculus pyrophosphatase (inorganic) 2 (Ppa2), transcript variant 2, mRNA.
Armh1	NM_001145637	Mus musculus armadillo-like helical domain containing 1 (Armh1), mRNA
Cracdl	NM_028096	Mus musculus capping protein inhibiting regulator of actin like (Cracdl), mRNA

Trir	NM_026760	Mus musculus telomerase RNA component interacting RNase (Trir), transcript variant 1, mRNA
Olfr1015	NM_146571	olfactory G protein-coupled receptor
Grin2a	NM_008170	Mus musculus glutamate receptor, ionotropic, NMDA2A (epsilon 1) (Grin2a), mRNA
Olfr1355	NM_207571	olfactory G protein-coupled receptor
Smim13	NM_001135577	Mus musculus small integral membrane protein 13 (Smim13), mRNA
Fxyd6	NM_022004	Mus musculus FXYD domain-containing ion transport regulator 6 (Fxyd6), mRNA

Lst1	NM_010734	Mus musculus leukocyte specific transcript 1 (Lst1), mRNA
Pcyox1	NM_025823	Mus musculus prenylcysteine oxidase 1 (Pcyox1), transcript variant 1, mRNA
Olfr281	NM_146280	olfactory G protein-coupled receptor
Ceacam14	NM_025957	Mus musculus CEA cell adhesion molecule 14 (Ceacam14), mRNA
Dhx30	NM_001252682	Mus musculus DEAH (Asp-Glu-Ala-His) box polypeptide 30 (Dhx30), transcript variant 1, mRNA
Sipa1l3	NM_001081028	Mus musculus signal-induced proliferation-associated 1 like 3 (Sipa1l3), mRNA
Tmem201	NM_001284273	Mus musculus transmembrane protein 201

		(Tmem201), transcript variant 4, mRNA
Ndfip1	NM_022996	Mus musculus Nedd4 family interacting protein 1 (Ndfip1), transcript variant 2, mRNA
Dhx30	NM_001252682	Mus musculus DEAH (Asp-Glu-Ala-His) box polypeptide 30 (Dhx30),
Olfr884	NM_001011798	olfactory G protein-coupled receptor
Dennd3	NM_001081066,	DENN/MADD domain containing 3 (Dennd3), transcript variant 1,
Defb43	NM_001039121	defensin beta 43 (Defb43)
Stard13	NM_001163493	StAR-related lipid transfer (START) domain containing 13 (Stard13), transcript variant 1

Rbmxl1	NM_009033,	RNA binding motif protein, X-linked like-1 (Rbmxl1), transcript variant 1
Tas2r126	NM_207028	taste receptor, type 2, member 126 (Tas2r126)
Vmn1r93	NM_207547,	vomeronasal 1 receptor 93 (Vmn1r93)
Plekhg1	NM_001159942	pleckstrin homology domain containing, family G (with RhoGef domain) member 1 (Plekhg1)
Olfr1427	NM_146679	olfactory receptor 1427 (Olfr1427)
Med24	NM_011869	mediator complex subunit 24 (Med24)
Vmn1r203	NM_134236	vomeronasal 1 receptor 203 (Vmn1r203)
Gng7	NM_010319	guanine nucleotide binding protein (G protein), gamma 7 (Gng7)

Cops7b	NM_172974	COP9 signalosome subunit 7B (Cops7b)
Rpp40	NM_145938	ribonuclease P 40 subunit (Rpp40)
Teddm1a	NM_178244	transmembrane epididymal protein 1A (Teddm1a)
Traf6	NM_009424	TNF receptor-associated factor 6 (Traf6)
Rsl1	NM_001013769	regulator of sex limited protein 1 (Rsl1)
Gpat2	NM_001081089	glycerol-3-phosphate acyltransferase 2, mitochondrial (Gpat2), mRNA
Sh3bp2	NM_011893,	SH3-domain binding protein 2 (Sh3bp2)
Zfp458	NM_001001152	zinc finger protein 458 (Zfp458)
Shtn1	NM_001114312	shootin 1 (Shtn1)

Unc5d	NM_153135	unc-5 netrin receptor D (Unc5d)
Btg3	NM_009770	BTG anti-proliferation factor 3 (Btg3)
Syce1l	NM_001048145,	synaptonemal complex central element protein 1 like (Syce1l)
Pik3cg	NM_001146200,	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (Pik3cg),

Table 2. Complete list of potential genetic interactors of BRCA2 identified by CRISPR-activation screening. List of all 189 genes confirmed by Crispra screening.

Primer name	Primer Sequence
hUBL5 Forward	GCAACACGGATGATACCA
hUBL5 Reverse	GGATTCTCATCTATTGAT
hWAPL Forward	CACCAGTCAGGCCTTAG
hWAPL Reverse	CCATAGTATCCTGTATGG
GAPDH Forward	TGCCCCCATGTTTGTGATG
GAPDH Reverse	TGTGGTCATGAGCCCTTCC

Table 3. List of forward and reverse primers used in the present study.