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Addressing The Issue Of Missing Heritability: The Importance Of Apoptosis In Hereditary Breast And Ovarian Cancer And Functional Assessment Of Tp53i3-S252*

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ADDRESSING THE ISSUE OF MISSING HERITABILITY: THE IMPORTANCE OF APOPTOSIS IN HEREDITARY BREAST AND OVARIAN CANCER AND FUNCTIONAL ASSESSMENT OF TP53I3-S252*

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DEDICATION

I dedicate this work to my parents, my siblings and my nieces and nephew. All have been a positive force and motivation in becoming the scientist I am today.

ACKNOWLEDGMENTS

There are many people who deserve recognition for helping me complete my doctoral work. One of the most challenging parts of my time as a graduate student finding the right lab. I was very lucky to be accepted into the lab headed by Dr. Michael Tainsky. He has been an exceptional mentor and I truly appreciate his patience and kindness. For every challenge I have faced during my graduate education, he knows exactly what to say and without fail always pointed me in the right direction. The positive work environment that fostered for the lab is why so many students enjoy their time under his guidance. I cannot thank him enough for all the support he has provided me in the past five years.

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LIST OF ABBREVIATIONS

1,2-NQ	1,2-napthoquinone
ACMG	American College of Medical Genetics
BART	BRCAnalysis rearrangement test
BSA	Bovine Serum Albumin
BWA	Burrows-Wheeler Aligner
COSMIC	Catalog of Somatic Mutations in Cancer
DM	Damaging Mutation
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic acid
DSB	Double Stranded Break
EDTA	Etylenediaminetetraacetic acid
EOC	Epithelial ovarian cancer
FACS	Fluorescence-activated cell sorting
GATK	Genome analysis toolkit
GC	Guanine-cytosine
GFP	Green fluorescent protein
GWAS	Genome wide association studies
HBOC	Hereditary breast and ovarian cancer
HR	Homologous Recombination
HRR	Homologous recombination repair
ICL	Interstrand cross links

IR	Ionizing Radiation
KCIGR	Karmanos Cancer Institute Genetic Registry
MAF	Minor Allelle Frequency
MDR	Medium chain dehydrogenases/reductases
MICR	Microscopy, Imaging & Cytometry Resources
MMC	Mitomycin C
NCCN	National Comprehensive Cancer Network
NCI	National Cancer Institute
NGS	Next generation sequencing
NHEJ	Non-Homologous End Joining
NSCLC	Non-small-cell lung carcinoma
OVCA	Ovarian cancer
PARP	Poly (ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
QOR	Quinone oxidoreductase
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive Oxygen Species
RPA	Replication protein
SDS	Sodium Dodecyl Sulfate
SNP	Singe nucleotide polymorphism
TAE	Tris-acetate-EDTA
TBST	Tris-Buffered Saline, 0.1% TWEEN

TP53I3	Tumor Protein 53 Inducible Protein 3
UV	Ultra-Violet
VCF	Variant call format
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGS	Whole genome sequencing

CHAPTER 1 - INTRODUCTION

1.1 Ovarian cancer and the issue of missing heritability

Ovarian cancer (OVCA) is the eleventh most common cancer amongst women and the fifth leading cause of cancer related deaths, with a five year survival rate of less than 50%(“Ovarian Cancer - Cancer Stat Facts,” 2018). According to the National Cancer Institute (NCI), since 1992 there has been a progressive decrease in the number of new cases of ovarian cancer, so prevention efforts have made an impact. However, this trend does not stand as strongly for the number of deaths due to OVCA. OVCA is a rare form of cancer and the number of deaths from this disease has not changed in recent years. It is still ranked among the top 10 cancers in lethality attributed to its poor survival. The five year survival from 1992 – 2009 was 49.2% and remains almost the same in 2019.

The different types of cancers of ovarian cancer are referred to by the cell type from they are derived from epithelial, germ cell and stromal. In addition, the subtype classification, pathologic grade, histology, are factors in prognosis and treatment (Torre et al., 2018). Epithelial ovarian cancer (EOC) can be classified histologically as serous, mucinous or clear cell. Additionally, EOC can be categorized as type I or II. Type I is considered to be a low-grade carcinoma with a higher survival rate and more associated with somatic mutations. In comparison, Type II EOC is more aggressive with a lower survival due to the spreading of the cancer cells beyond the ovaries, often with late-stage diagnosis (Torre et al., 2018). Less aggressive ovarian malignancies include non-epithelial ovarian

cancers encompassing germ cell, stromal tumors, small cell carcinoma and ovarian sarcoma.

Approximately 25% of all EOC cases are considered to be hereditary but this figure is most likely an underestimation due to missing heritability (Bodmer & Tomlinson, 2010; Manolio et al., 2009). *BRCA1 and BRCA2* are tumor suppressor genes and account for 5 – 10% of all OVCA cases (Ramus & Gayther, 2009). Hereditary EOC is part of the hereditary breast and ovarian cancer syndrome, which has an autosomal dominant inheritance pattern. Patients with a genetic predisposition of EOC are characterized by one or more of the following: family history of ovarian and/or breast cancer, Ashkenazi Jewish heritage, early age of onset, presence of BRCA1/2 mutations, and mutations in other DNA repair genes or mutated mismatch repair genes associated with Lynch syndrome (Saslow et al., 2012). Current EOC patients that fit one or more of these classifications are recommended to undergo genetic testing of buccal or blood DNA. The panel for risk assessment of HBOC consists of 25 genes involved in DNA repair, cell cycle regulation, cell adhesion, RAS signaling, and enzymatic activity. The panel is a comprehensive testing tool for cancers including the breast, ovarian and uterine (Figure 1). However, panel testing is limited to the assessment for mutations already implemented in disease risk. This process does not allow for the discovery of novel risk mutations in panel genes or in genes that are part of the same pathway or have a similar function. Whole genome or exome sequencing (WES) of patient blood and/or tumor DNA is required to identify germline variants that are not among these panel genes. Using the genome analysis toolkit (GATK), a

pipeline optimized for accuracy and performance of next generation sequencing (NGS) analysis, variants of interest are identified based on the American College of Medical Genetics (ACMG) guidelines. While the progression of panel testing for genetic heritability of rare diseases and disorders has grown in the past decade, determining the genetic risk of disease is still complex.

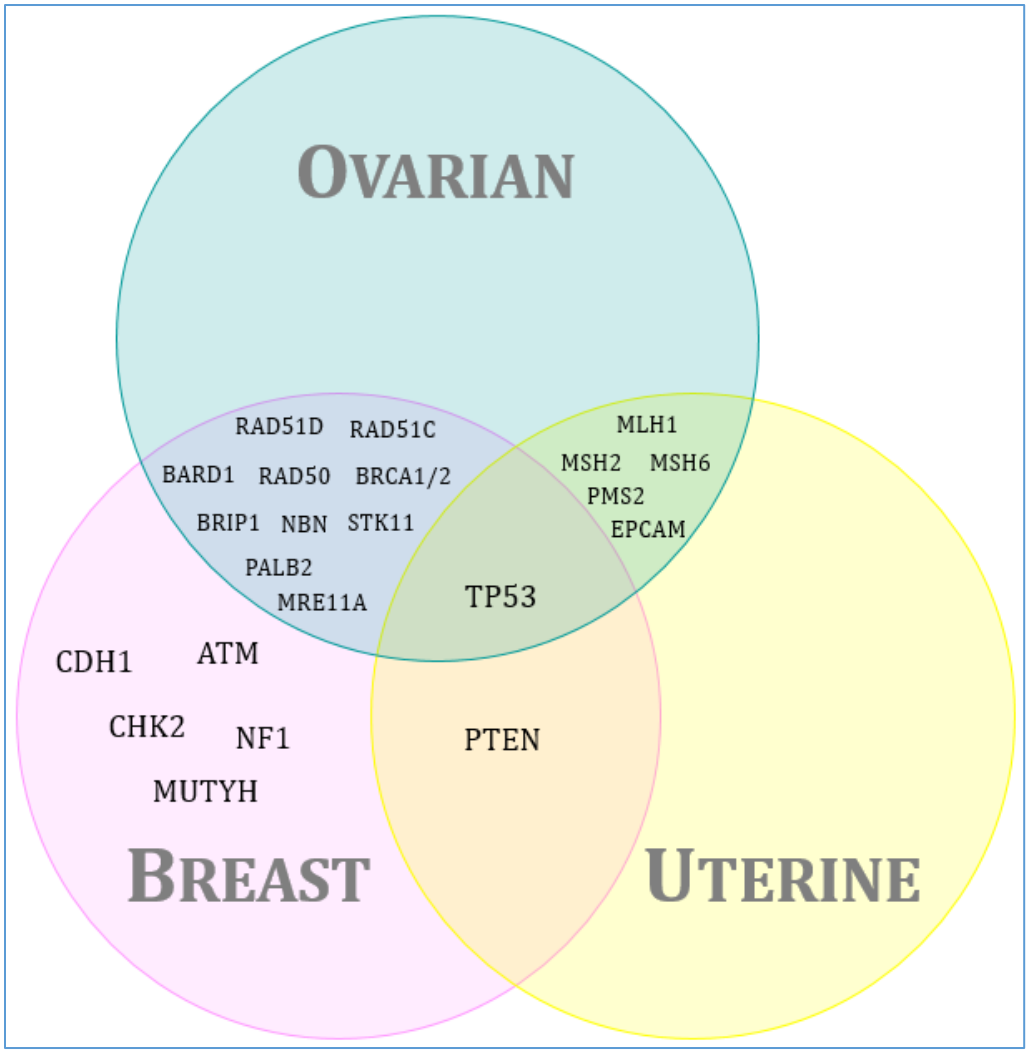

Figure 1: Risk genes associated with ovarian, breast and uterine carcinomas

modified from Ambry Genetics

EOC individuals who have been diagnosed or are suspected to be at risk of the disease are recommended to undergo testing of their germline DNA for known pathogenic mutations in 25 ovarian and breast cancer associated genes (Figure 1). Germline DNA is sequenced using next generation sequencing (NGS) technology and the raw data is processed and formatted to a variant call format (VCF) text file that stores gene sequences. In the case of germline variants, criteria to identify mutations of interest include low minor allele frequency, type of mutation, location of mutation, segregation data, evidence of cancer risk association in publically available databases that report on the relationships of human variation and phenotypes, genotype to phenotype literary evidence, algorithms that predict the impact of the variant, and conservation scores. All variants of interest are confirmed by Sanger sequencing to ensure that false positives are not reported as disease-causing. Clinical geneticists use the molecular genetic profiles to highlight the most significant findings to the patient including important variants found in the associated gene(s), the evidence used to interpret variant, and relevance of the findings to both the patient and family members.

1.2 Variant Classification

Guidelines have been created by the American College of Medical Genetics (ACMG) to determine variant classification (Richards et al., 2015a). Variants can be classified into five groups; pathogenic, likely pathogenic, unknown significance, benign or likely benign. Pathogenic or likely pathogenic variants are sub-classified as very strong, strong, moderate or supporting based on evidence for the particular mutation (Table 1) (Richards et al., 2015a). Benign variants can also be sub-

classified as stand-alone, strong or supporting (Richards et al., 2015b). There are several different types of data that determines if a variant is pathogenic or benign. Population, computational, functional, segregation, de novo, allelic, other databases, population data, computational and functional data determines a mutations impact (Table 1) (Richards et al., 2015b). The purpose of benign sub-classification is to ensure that the variant does not impact protein function and overall patient risk. Variants of unknown significance (VUS) are classified as such if there is conflicting evidence of the mutation being pathogenic or benign. The mutation may be novel, private or there is opposing evidence of its impact on protein function (Amendola et al., 2016; Richards et al., 2015a).

Classification	Benign		Pathogenic			
Sub-Classification	Strong	Supporting	Supporting	Moderate	Strong	Very Strong
Population data	X			X	X	
Computational and predictive data		X	X	X	X	X
Functional data	X		X		X	
Segregation data	X		X	X	X	
De novo data					X	
Allelic data		X				
Other databases		X	X			
Other data		X	X			

Table 1: American College of Medical Genetics guidelines for variant classification (modified).

1.3 Variants of unknown significance impacts missing heritability

A primary contributor to the issue of missing heritability is variants of unknown significance (VUS) (Bodmer & Tomlinson, 2010). The mutations effect on protein function and the patient is not known (Richards et al., 2015b). Often, patients are made aware of a VUS after genetic testing. But they cannot be counseled due to the lack of information about the impact of the mutation on protein function (Richards et al., 2015b). Essentially there is a gap of knowledge that does not allow clinicians to counsel patients on their true genetic risk. An important step in evaluating the significance of a genetic lesion is to use a combination of computational and laboratory techniques. It has been proposed that instead of high throughput sequencing of a set of panel genes, whole genome (WGS) or whole exome sequencing (WES) is a more powerful method of assessing patients who are suspected to have a hereditary risk of cancer (Chaudhry, Stafford, Tainsky, & Levin, 2017). Clinicians can gain a better understanding of the genetic profile of patients, identify novel risk loci outside of the standard genetic panels and have the ability to re-visit the data (Chaudhry et al., 2017). The guidelines for variant assessment is constantly being updated by both National Comprehensive Cancer Network (NCCN) (Pilarski et al., 2018) and ACMG (Richards et al., 2015b). Therefore, reassessing WES/WGS patient data will ease the financial and resource burden of resequencing and data processing.

1.4 The limitations of genome wide association (GWAS) studies

A large portion of individual differences in disease susceptibility is due to genetic factors. Identifying and characterizing novel variants gives a personalized

approach to the prevention, diagnosis, and treatment of disease (Manolio et al., 2009). A previous source of promise was conducting GWAS to identify pathogenic mutations from thousands of affected and/or unaffected individuals. Due to the rarity of OVCA, the frequency of the germline variants in the general population is quite low, but most allele thresholds for GWAS studies at least 5% of the population (Auer & Lettre, 2015). GWAS explains a small fraction of missing heritability because of the inability to identify causal variants and genes in complex traits (Tam et al., 2019). Also, GWAS studies have high false positives and low replication, so this methodology has fallen short in identifying novel risk loci associated with rare diseases such as OVCA (Auer & Lettre, 2015; Tam et al., 2019).

1.5 Identifying novel risk loci TP53I3-S252* in HBOC patients

To address the issue of missing heritability in HBOC, WES is conducted on a cohort of 48 Caucasian women diagnosed with high-grade ovarian cancer. These woman have a personal history of breast cancer or a family history of breast and/or ovarian cancer (Stafford et al., 2017). During the time of sample retrieval, the guidelines for genetic testing for HBOC was limited to assessing for pathogenic mutations in *BRCA1* and *BRCA2*. Thus, these women are an ideal cohort for up to date genetic panel testing to identify clinically actionable mutations. The cohort can also highlight the importance of identifying novel risk mutations in non-panel/candidate genes. After WES of the germline DNA, *in silico* single nucleotide (SNP) assessment is conducted to filter down to clinically actionable or candidates variants for functional assessment. There are 5 clinically actionable mutations in panel genes and 11 additional truncations in non-panel genes involved in DNA

repair and cell cycle regulation (Lopes, Chaudhry, Lopes, Levin, & Tainsky, 2019; Stafford et al., 2017).

Of particular interest is the pre-mature stop gain mutation in Tumor Protein p53 Inducible Protein 3 (*TP53I3*). The nonsense mutations is found in two of the 48 patients, OCJ19 and OCG14. The *TP53I3* gene is unique because it is a quinone oxidoreductase (Porté et al., 2009), involved in the DNA damage response (Contente, Dittmer, Koch, Roth, & Dobbelstein, 2002; B. Li et al., 2013), and p53-mediated apoptosis (Lee et al., 2010; Polyak, Xia, Zweier, Kinzler, & Vogelstein, 1997). Identifying the truncation in *TP53I3* resulted in the expansion of *in silico* SNP assessment to include genes that are part of the conserved programmed cell death pathway way, apoptosis.

1.6 TP53I3 function in apoptosis and oxidative stress

1.6.1 Functional overview

TP53I3, formally known as *PIG3*, is located on chromosome region 2p23.3 (Figure 2). It was originally discovered as a downstream transcriptional target of p53 prior to the understanding of its role in apoptosis (Polyak et al., 1997). Its coding region consists of 5 exons and there are two full-length mRNA variants with different 5'UTR regions. At the N-terminus, there exists a nuclear localization sequence (Lee et al., 2010). The C-terminus is homologous with quinone oxidoreductases (QOR) (Porté et al., 2009). Alternative pre-mRNA splicing events can cause the skipping of exon 4 and result in the splice variant PIG3AS (Nicholls et. al., 2004). TP53I3 is found in most vertebrates except rodents, but it is present in rabbits (Polyak et al., 1997). The gene sequence is a homolog for the plant

gene TED2 which is a plant *NADPH* oxidoreductase and involved in the formation of plant meristems by apoptosis (Polyak et al., 1997). In mammals, the *TP53I3* sequence is most similar to NADPH-quinone oxidoreductase, *ζ-crystallin*, a potent generator of reactive oxygen species (ROS) (Porté et al., 2009). Therefore, TP53I3 is a QOR which is part of the medium chain reductases (MDR) superfamily. The coding sequence contains a conserved binding motif for medium chain dehydrogenases/reductases (MDR). There are also 13 residues in the amino acid sequence of TP53I3 that $NADP^+$ can bind to, 7 of which are conserved (Porté et al., 2009). TP53I3 has also been associated with DNA damage response by effecting phosphorylation of *CHK2* and *γH2AX* (Lee et al., 2010).

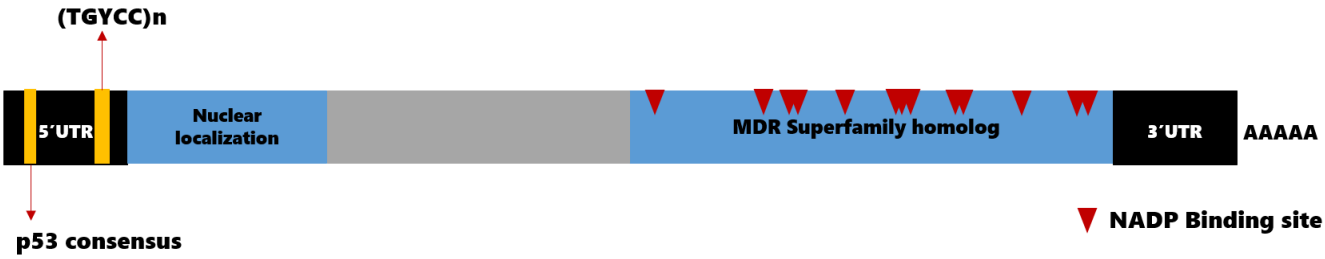

Figure 2*: TP53I3* gene structure and domains

TP53I3 is located on chromosome 2p23.3 and is 1653 nucleotides in length. There are two p53 binding sites at the promoter region including the polymorphic microsatellite $(TGYCC)_n$. The nuclear localization site is close to the N-terminus and the homologous MDR superfamily sequence is near the C-terminus. There are 13 $NADP^+$ binding sites, all within the sequence that shares homology with the medium chain reductase (MDR) family.

1.6.2 Three-dimensional structure of TP53I3 proposed in 2009

In 2009 the crystallographic structure of TP53I3 was determined in the presence of $NADP^+$ to determine the protein's enzymatic function (Porté et al., 2009). The following will explain the protein structure and important domains. Two constructs were crystallized and had identical subunits making the TP53I3 a stable homodimer. The protein structure contains two important domains, a catalytic domain (Met1 – Ala119 and Leu265 – Gln332) and a cofactor-binding domain (Ala120 – Ser264). In-between these two domains is a deep cleft for the $NADP^+$ molecule to bind to. There are 13 amino acid residues that can bind to $NADP^+$, 7 of which are conserved in the TP53I3 sequence. One of the binding sites for $NADP^+$ resides in the conserved binding motif (A/G)XXSXXG and can be found in many other quinone oxidoreductases (Edwards et al., 1996). A missense change from a serine to a valine (TP53I3-S151V), results in enzymatic inactivation due to steric hindrance and preventing $NADP^+$ from binding. NADPH binding to TP53I3 was determined by the quenching of protein fluorescence, the 2' phosphate group binds to Gly173, Lys173, and Tyr192. Many NADPH dependent MDRs are characterized by having a glycine at the C-terminal end of the nucleotide binding domain. The corresponding residue in TP53I3 would be the conserved Gly173. This suggests that TP53I3 is an NADPH dependent enzyme.

The active binding site is formed by amino acids Asn40, Ala42, Met45, Tyr51, Leu51, Leu63, Glu123, Thr127, Leu63, Glu123, Thr127, Leu240, Leu255, Phe256, and Leu265. TP53I3 NADPH-dependent reductase activity was tested with known QOR and ζ-crystallin substrates. The protein exhibited strong

enzymatic activity with 1,2-naphthoquinone (1,2-NQ), an ortho-quinone. Kinetic analysis was conducted with wildtype TP53I3 or TP53I3-S151V in the presence of the 1,2-NQ substrate (Porté et al., 2009). The V_{max} determines the rate of reaction when the substrates saturate the enzyme and is dependent on the affinity of the substrate to bind to the enzyme. To determine if a substrate has a high binding affinity with an enzyme, the Michaelis constant is measured (K_m) (Johnson & Goody, 2011). The K_m measures the concentration of the substrate which permits the enzyme to achieve half V_{max}. The second order rate constant K_{cat}/K_m is the catalytic efficiency of the enzyme (Johnson & Goody, 2011). The K_{cat}/K_m for TP53I3-S151V in the presence of 1,2-NQ was much higher than wildtype TP53I3 indicating the mutant is enzymatically inactive (Porté et al., 2009).

The structure of 1,2-NQ substrate fits into the active binding site of TP53I3 in the appropriate orientation. The production of ROS was detected in the presence or absence of 1,2-NQ substrate, TP53I3 enzyme and/or cofactor NADPH. There was a significant increase in ROS production after a complete reaction consisting of the enzyme, substrate, and cofactor, compared to when there was an absence of one of the components. Intracellular ROS was measured with the 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) in HCT-116 with unmodified TP53I3, overexpressed TP53I3, TP53I3-S151V or phorbol 12-myristate 13-acetate (PMA; positive control). The TP53I3-S151V mutant resulted in a significant decrease in intercellular ROS production compared to cells with overexpression TP53I3 or PMA positive control. So a disruption in the active site

can affect binding affinity for ortho-quinone substrates, causing a hindrance in ROS production.

1.6.3 Transcriptional Regulation of *TP53I3*

There are two *p53* binding sites in the promoter region of *TP53I3* (Figure 2). The first is 308 nucleotides upstream of the transcription start site and is the classic 20 base pair p53 binding sequence. The second preferential binding site, is the polymorphic penta-nucleotide microsatellite sequence $(TGYCC)_n$ positioned between 412 and 517 nucleotides downstream of the transcription start site (Contente et al., 2002). The microsatellite is considered to be the first of its kind to functionally interact with a transcription factor (Contente et al., 2002). Variations of these repeat sequences have been associated with many different cancers including squamous cell carcinoma of the head and neck (Guan et al., 2013), myeloid leukemia (Nomdedéu et al., 2004), breast carcinoma (Gorgoulis et al., 2004), lung carcinoma (Gorgoulis et al., 2004) and invasive bladder cancer (Ito et al., 2006). There were four different motif sizes found in a population of healthy individuals, 10, 15, 16 or 17 with a frequency of 5.1%, 63%, 21.4%, and 11.5% respectively. The greater the number of TGYCC repeats, the stronger the interaction with *p53* (Contente et al., 2002). Transcriptional activation of *TP53I3* can also be regulated by *p63* and *p73* through the penta-nucleotide microsatellite region and mutated *p53* also interacts with the motif but not as strongly (Contente et al., 2002).

TP53I3 is also transcriptionally regulated by alternative splicing, resulting in the skipping of the fourth exon in the pre-mRNA. As a result, there is co-expression

of the splice variant (PIG3AS) with the full-length variant (Nicholls et al., 2004). The splice variant is made up of 248 amino acids lacking most of the 5'UTR region. Unlike the full-length variants, the PIG3AS C-terminal domain is not homologous with QOR (Kotsinas et al., 2012). Under normal conditions, there is a preference for the expression of the full-length variant. However, under ultra-violet irradiation, there is a preference for expression of PIG3AS which has a short life span and is considered non-functional (Nicholls et al., 2004). Therefore, it is possible that defects in the C-terminus of the full-length variant will, at the very least, affect ROS production and cellular apoptosis.

1.6.4 Apoptosis

To fully appreciate the role of *TP53I3* in apoptosis, a basic understanding of the mechanism is necessary. Apoptosis is an essential mechanism that initiates the programming of cell death and the maintenance of tissue homeostasis. There are many forms of programmed cell death, and apoptosis is thought to be of particular importance and distinction (Elmore, 2007). Apoptosis is a defense mechanism when cells are stressed by DNA damage, external toxins, and reaction to the immune system (Norbury & Hickson, 2001). In mouse models, it has been demonstrated that apoptosis and necrosis can occur independently, sequentially or simultaneously (Zeiss, 2003). To differentiate between the two processes, morphological differences can be assessed. Necrosis is characterized by cell swelling and dissolution of the nucleus. On the other hand during apoptosis, cells shrink, cytosol calcium increases, and the nucleus becomes dense and compact and eventually undergoes fragmentation (Elmore, 2007). Apoptosis is a key

regulator of tumorigenesis and treatment response (Fulda & Debatin, 2006). Drugs or irradiation used to target cancer cells can damage the DNA and lead to p53-mediated apoptosis (Elmore, 2007).

1.6.5 Intrinsic apoptosis

There are two main apoptotic pathways, extrinsic/death receptor and intrinsic/mitochondrial. Damage to the cell's DNA elicits apoptosis primarily through the intrinsic pathway (Figure 3). The intrinsic or mitochondrial pathway, is of particular interest because it involves upstream transcriptional activation of p53-mediated apoptosis cells. Recall, *TP53I3* is transcriptionally activated by p53 binding to the polymorphic pentanucleotide repetitive motif $(TGYCC)_n$ to initiate apoptosis. Components involved in the apoptosis process are conserved proteins and physical association with the mitochondria. In terms of the intrinsic pathway, when pro-apoptotic signals occur, disruption in the mitochondrial membrane potential causes the release of cytochrome c into the cytoplasm (Elmore, 2007). Cytochrome c pairs with apoptotic protease activating factor 1 (APAF1) and inactive caspase-9, forming the apoptosome (Elmore, 2007). The apoptosome hydrolyzes adenosine triphosphate (ATP) to cleave and activate. Activated caspase-9 cleaves and activates caspases- 3, 6 and 7 followed by cell apoptosis (Norbury & Hickson, 2001). Cleavage and activation of caspase-3 is the hallmark of apoptosis because it promotes DNA fragmentation and cell death (Cotter, 2009). Permeabilization of the mitochondria membrane also releases Smac into the cytosol which promotes apoptosis by blocking inhibitors of apoptosis proteins (IAPs) (Hongmei, 2012). The B-cell lymphoma (Bcl-2) protein family are the main

regulators of intrinsic apoptosis. The Bcl-2 family activates pro-apoptotic or inhibits anti-apoptotic genes. Some of the best characterized pro-apoptotic proteins are BID, BAD, BIM, BMF, Puma and NOXA contain a Bcl-2 homology 3 domain (BH3) (Kluck et al., 1999). Anti-apoptotic proteins BCL-2, BCL-XL, and MCL-1 have multiple BH3 binding domains and inhibit cytochrome c release (Schuler & Green, 2001).

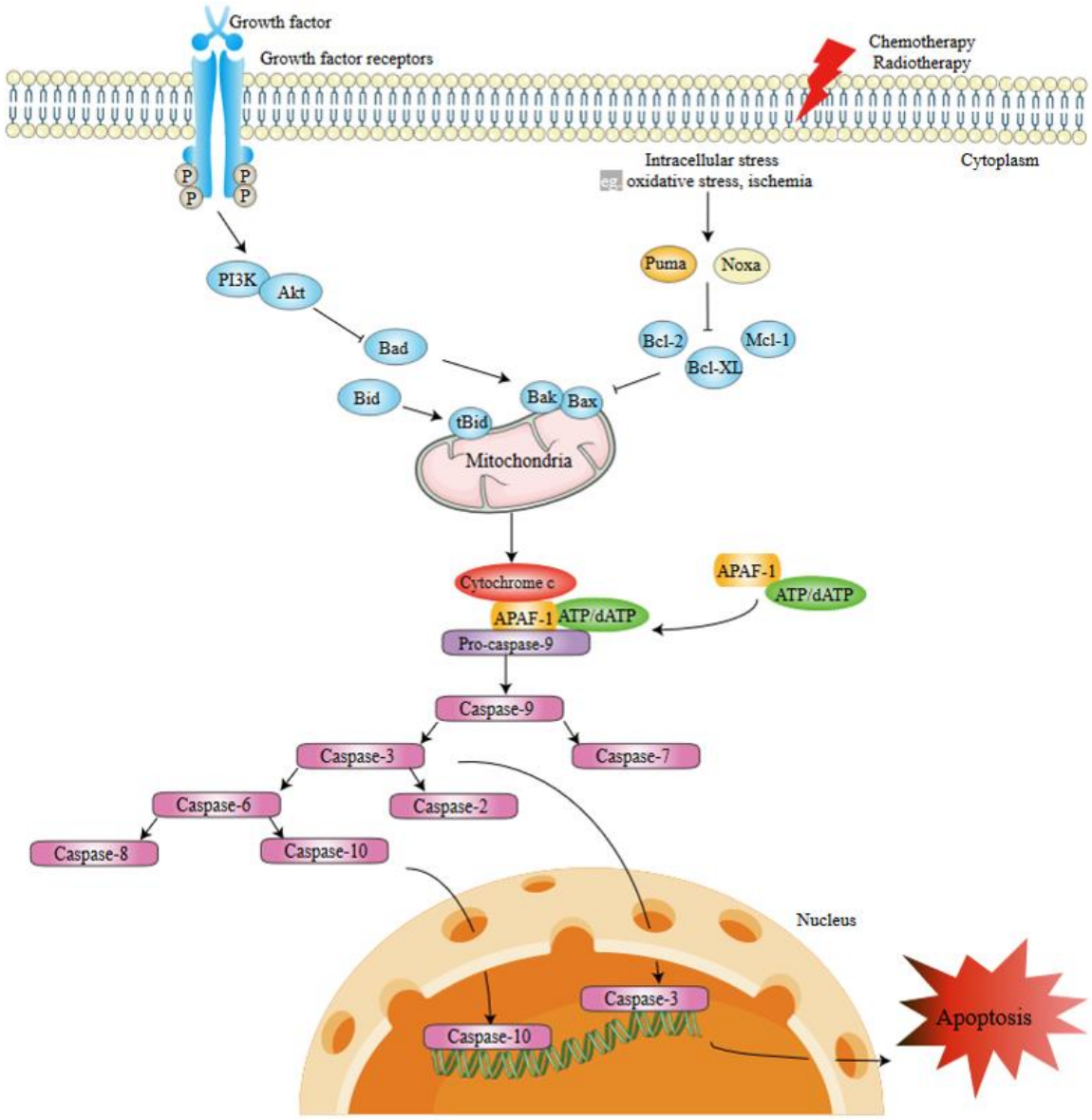

Figure 3: Intrinsic apoptosis pathway

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1.6.6 *p53*-mediated apoptosis

Tumor protein *p53* regulates the expression of many genes involved in a variety of cellular mechanisms including apoptosis, growth arrest, and senescence. The protein consists of four conserved domains, N-terminus, sequence-specific DNA binding, tetramization domain, and C-terminus (Pavletich, Chambers, & Pabo, 1993). When cells are stressed, p53 is stabilized and accumulates in the nucleus. Phosphorylation of p53 is mediated by cellular kinases including check point kinase 1 (*CHK1)* and check point kinase 2 (*CHK2*) as well as ataxia-telangiectasia mutated (*ATM*) (Kuribayashi et al., 2011; Schuler & Green, 2001). Activated p53 initiates the expression of genes leading to programmed cell death (Shen & White, 2001). To promote apoptosis, p53 transcriptionally activates a specific set of genes known as p53-proapoptotic genes including *TP53I3* and *TP53AIP1* as well as *BID, PUMA, NOXA, BAD, BAX, CASP6*, and *APAF1* (Kuribayashi et al., 2011). Depending on which residue is phosphorylated or acetylated in p53, certain pro-apoptotic genes are selectively expressed. Phosphorylation of serine 15 and 20 (Amano et al., 2009) or acetylation at 320 and 373 results in the transcriptional activation of *TP53I3* (Yanagihara et al., 1991). A subset of *p53* targeted apoptosis genes also function as ROS producers, including *TP53I3*. While *p53* is often mutated or not functional in tumors, family members *p63* and *p73* are known to compensate for its loss (Napoli & Flores, 2013).

1.6.7 Quinone reduction reaction for ROS production and downstream apoptosis

Low levels of ROS are important for normal cell cycle progression, proliferation, differentiation, migration, death (Covarrubias et a., 2008), immune response and redox reaction regulation (Schieber & Chandel, 2014). Excessive levels of cellular ROS are detrimental to cellular integrity and can trigger oxidative stress. Increased production of ROS has been associated with cancer, metabolic and neurodegenerative diseases (Kehrer & Klotz, 2015). Production of ROS involves endogenous or exogenous factors. Endogenous superoxide ROS production occurs due to leaks in the mitochondrial electron transport chain, specifically from complex I and II (Dickinson & Chang, 2011). Superoxides are also produced by NADPH oxidases, xanthine oxidases, and cytochrome P450 reductases (Bae, Oh, Rhee, & Yoo, 2011). The TP53I3 protein is a quinone oxidoreductase due to sequence with similarity with the QOR family and reactivity with quinone substrates for ROS production (Porté et al., 2009). Under normal conditions, TP53I3 is localized in the cytosol, a feature shared with other QOR (Flatt et al., 2000).

Oxidative stress is an imbalance between pro-oxidative and anti-oxidative states that leads to an increase in ROS (Schieber & Chandel, 2014). Oxidative stress is commonly associated with causing cellular damages to age-related processes such as cancer (Klaunig & Kamendulis, 2004). The mechanism can be initiated through the redox reaction cycle that involves many oxidoreductases (Oppermann, 2007). HBOC associated gene, *BRCA1* and *ATM* are involved in the

redox reaction by regulating ROS production (Gorrini, Harris, & Mak, 2013; Srinivas, Tan, Vellayappan, & Jeyasekharan, 2019). During respiration, about 5% of molecular oxygen is converted to ROS. Major ROS molecules include superoxide (O^-), H_2O_2, and hydroxyl radical (OH) (Pelicano, Carney, & Huang, 2004). Production of ROS changes the cellular redox state and effects the modification of nucleic acid, proteins, and lipids which are important processes for cancer progression. The redox cycling of quinones is initiated by oxidoreductases, included NADPH-dependent quinone reduction and the understudied class of QOR belonging to the MDR superfamily (Oppermann, 2007). Quinone compounds are reduced to unstable intermediates semiquinone, by one electron, or to hydroquinone by two electrons. Reduction to hydroquinone also requires the presence of quinone reducing agents such as NADPH-oxidoreductase (Figure 3) (Bolton & Dunlap, 2017; Oppermann, 2007; Porté et al., 2009). The reduction of oxygen generates superoxide ROS which is dismutated by superoxide dismutase, generating H_2O_2. The compound is then reduced to another form of ROS, hydroxyl radicals, in the presence of a metal ion (Bolton & Dunlap, 2017). Quinones can sustain the production of ROS leading to DNA modifications and affecting cellular response and defense mechanisms such as apoptosis.

Potent levels of ROS increases the amount of intrinsic apoptosis (Redza-Dutordoir & Averill-Bates, 2016). High amounts of ROS can activate p53 or JNK resulting in the activation of Bcl-2 proteins. Oxidation of cardiolipin and the depolarization of the mitochondrial membrane releases cytochrome c into the cytosol (Figure 3) (Redza-Dutordoir & Averill-Bates, 2016). Cytochrome c forms

the apoptosome triggering the downstream caspase cascade activation effect and eventual apoptosis (Cotter, 2009).

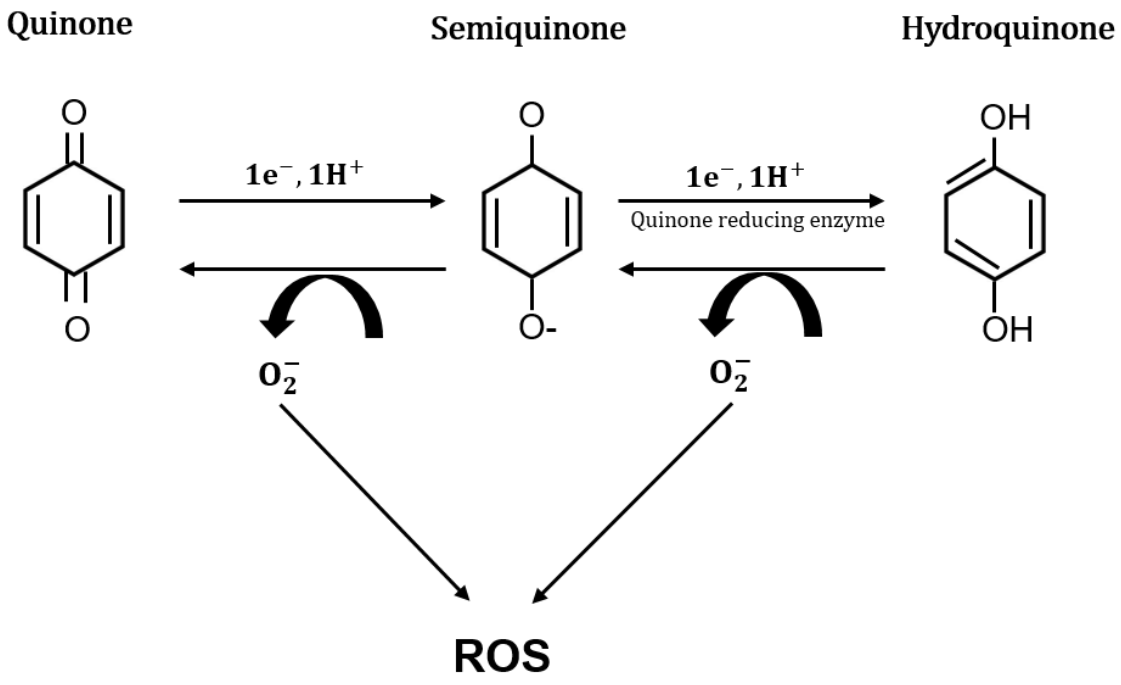

Figure 4: Quinone reduction to reactive oxygen species (ROS)

1.6.8 How TP53I3 connects oxidative stress and DNA Damage Response (DDR)

In order for DDR proteins such as TP53I3 to properly function, recognition of DNA breaks by ATM is required. The protein kinase then activates the p53 antitumor cellular response causing cell cycle arrest, apoptosis or senescence. Also activated by p53 are DNA repair mechanisms that are compromised in malignant cells. Because TP53I3 has a role in two conserved mechanisms, it is thought that loss of function could be toxic to both normal and cancer cells (A Kotsinas et al., 2010).

There is a proposed model for how TP53I3 functions in normal and malignant cells that ties together the effects of oxidative stress and DDR (A Kotsinas et al., 2012). In normal cells under genotoxic stress, DNA damage response (DDR) stabilizes p53 and TP53I3 expression is increased. During low levels of genotoxic stress, DDR TP53I3 triggers DNA repair. When DNA is exposed to genotoxic stress, p53 increases the expression of pro-oxidant TP53I3 even more. This causes lethal levels of ROS production and eventual cellular apoptosis. When malignant cells with wildtype *p53* are under continuous oxidative or genotoxic stress, mutations accumulate. A significant amount of TP53I3 is shuttled to the nucleus to support DDR which adds to the sub-lethal ROS production, maintaining the oxidative stress conditions. The continuous support of DDR by TP53I3 leads to *p53* loss or mutation. However, p63 or p73 can compensate for the loss of p53 to support the positive feedback loop of TP53I3 response to DDR and ROS production.

1.6.9 DNA damage response

DDR involves the mechanisms that sense and signal the presence of DNA (Harper & Elledge, 2007). DNA repair processes include proteins that are highly conserved. Mutations in these proteins can lead to defects in DNA repair and increase predisposition to cancer. Types of DNA damage include bases mismatch, single strand breaks, double strand breaks (DSB), insertions, deletions, bulky DNA lesions, oxidized/deaminated bases, methylated (O^6 or N^7) guanine, pyrimidine dimers, interstrand crosslinks and intrastrand crosslinks (Blanpain, Mohrin, Sotiropoulou, & Passegué, 2011; Pilié, Tang, Mills, & Yap, 2019). Two mechanisms involved in DSB are non-homologous end joining (NHEJ) and homolgous recombination repair (HRR). Many genes that drive HRR are part of the HBOC panel, including *BRCA1, BRCA2, ATM, CHK2, PALB2, RAD50, RAD51D,* and *NBN* (da Cunha Colombo Bonadio, Fogace, Miranda, & Diz, 2018; Liang, Han, Romanienko, & Jasin, 1998).

DSB are made by damaging agents like ionizing radiation, radiomimetic drugs (Limoli, Giedzinski, Bonner, & Cleaver, 2002), replication blocking lesions (Bosco et al., 2004); ROS production (Srinivas et al., 2019) and topoisomerase I and II inhibitors (Degrassi, Fiore, & Palitti, 2004). The inability to repair DSBs can increase cell death or cause chromosomal changes causing genomic instability and the production of cancer cells (Shrivastav, De Haro, & Nickoloff, 2008). The presence of DNA double stranded breaks (DSBs) in eukaryotic cells can be repaired by two main mechanisms, HRR or NHEJ. Homologous recombination repair (HRR) is considered to be a more “error-free” mechanism because there is

less chance of spontaneous mutation formation compared to NHEJ repair (Liang et al., 1998). The determination for which mechanism should be used depends on how the double-stranded break (DSB) was created. For example, if replication fork stalling is recognized by the Fanconi Anemia complex, and will eventually lead to the signaling of *BRCA1* for repair by HRR (Goldstein & Kastan, 2015). On the other hand, DSBs formed by IR can be repaired by HRR or NHEJ either pathways. HRR is a highly conserved mechanism due to the exchange of genetic information between allelic sequences (Liang et al., 1998; San Filippo, Sung, & Klein, 2008; Sung & Klein, 2006). HRR is vital for DNA repair, replication, meiotic chromosome segregation, and telomere maintenance. The HRR mechanism involves the broken ends of the DNA to use the homologous sequence as am repair template, from the sister chromatid or foreign DNA at the S and G2 cell cycle phase.

When a DSB is detected, ATM phosphorylates H2AX histone family member X (H2AX). The DNA damage checkpoint protein 1 (MDC1) binds to the phosphorylated γH2AX and accumulates at sites of DNA damage. DSB repair proteins MRE11, RAD50 and NBS1 form the MRN complex which localizes to the DSB for stabilization and prevention of chromosome breaks. The 5'exonuclease activity of C-terminal binding protein-interacting protein (CtIP) creates single-stranded overhangs and the replication protein (RPA) binds to the 3' single-stranded overhangs (Symington, 2014). RPA is replaced by RAD51, breast cancer 1 (BRCA1) and 2 (BRCA2) proteins to create filaments on the DNA. A homologous sequence from the sister chromatids or foreign DNA is identified by the 3' overhang of Rad51 (Symington, 2014; Tang et al., 2019). Proliferating cell nuclear antigen

(PCNA) produces the deleted DNA fragment which was once broken. Lastly, a Holliday junction is made after the formation of the new DNA fragment and the original DNA sequence is restored (Figure 5).

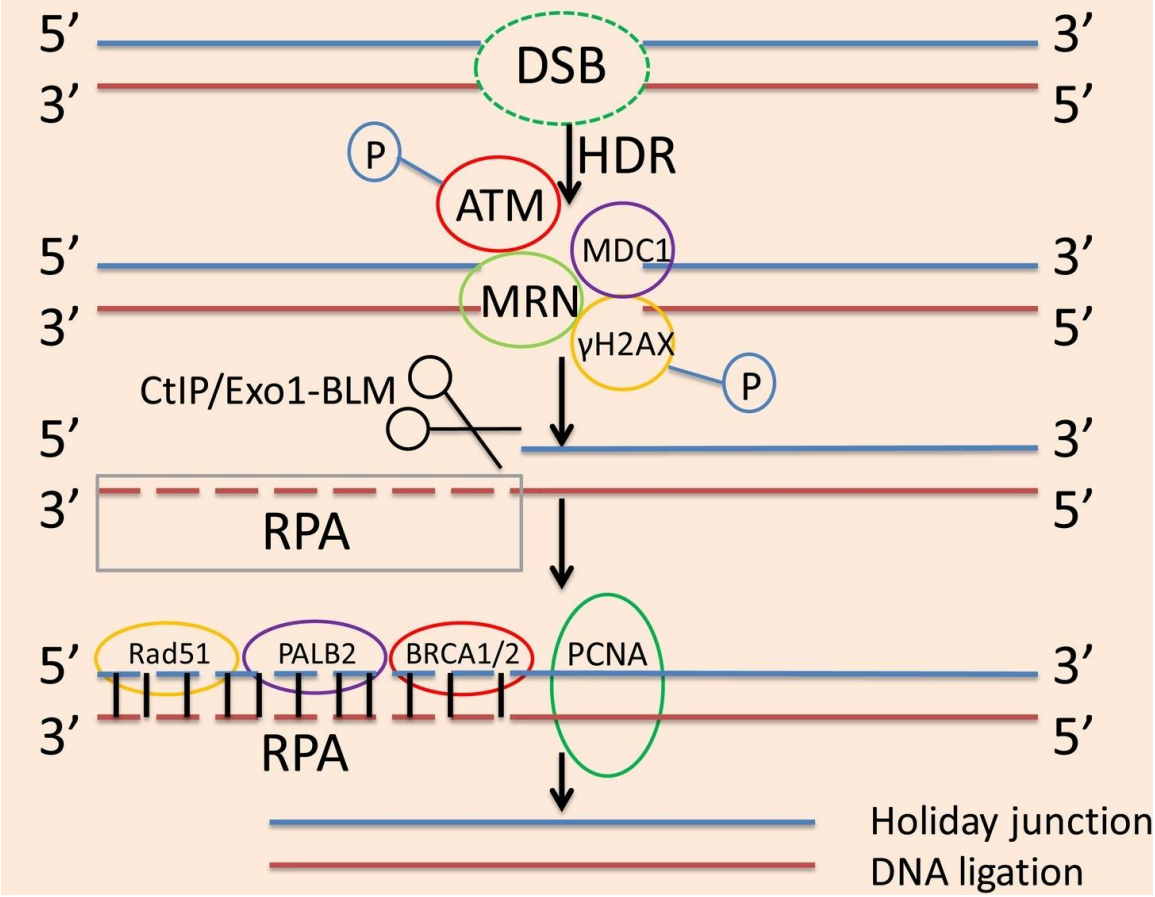

Figure 5: Homologous Recombination Repair (HRR) mechanism

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Mutations in genes involved in DBS repair can be more detrimental than exogenous factors, whether they occurred in the germline or somatically. Approximately 40-50% of all ovarian cancers exhibit a deficiency in homologous recombination repair mechanisms (Elvin et al., 2017). Germline mutations in *BRCA1* and *BRCA2* are key in disrupting HRR (da Cunha Colombo Bonadio et al., 2018). Breast and ovarian cancer were initially associated with HRR impairment due to mutations in *BRCA1* and *BRCA2* (da Cunha Colombo Bonadio et al., 2018; Miki et al., 1994; Wooster et al., 1995). Other HRR genes associated with cancer risk include *RAD51, CtIP, RAD51B, RECQL4, BLM, WEN* and *NBS1* (Helleday, 2010). The expression of *RAD51* is correlated with increased responsiveness to topoisomerase II inhibitor etoposide (Helleday, 2010). Platinum-based therapies are another standard of care with HBOC patients because of mutations in HRR or Fanconi Anemia genes, and are very responsive to treatment (Whitby, 2010). Targeted therapy regimens incorporating the use of PARP inhibitors are also beneficial for patients who carry germline mutations in either *BRCA1* or *BRCA2* (Coleman et al., 2015).

The role of TP53I3 in the DNA repair mechanism is not well defined. However, our recent work determined that it is involved in HRR (Lopes et al., 2019). The knockdown of TP53I3 in HeLa-DRGFP cells resulted in a significant decrease in HRR induction (Lopes et al., 2019). Approximately 30% of TP53I3 is localized in the nucleus and the other 70% in the cytosol (Lee et al., 2010). Knockdown of TP53I3 in U2OS and HeLa cells negatively affects the intra-S phase and G2/M DNA damage checkpoints (Athanassios Kotsinas et al., 2012; Lee et

al., 2010). Additionally, TP53I3 depletion increased cell sensitivity to UV and radiomimetic drugs. Under normal conditions when DDR signaling occurs TP53I3 co-localizes with p-H2AX and 53BP1. When expression of TP53I3 is lost in cells, in the presence of genotoxic stress, there is significant reduction in CHK1, CHK2 and γH2AX (Lee et al., 2010). Breast cancer patients with high expression of TP53I3 and BRCA1 have a significantly higher overall survival. BRCA1 is also thought to regulate TP53I3 in a p53-dependent manner (Zhang et al., 2015). In HCT116 cells, overexpression of BRCA1 increased expression of TP53I3 and p53. In p53-null HCT116 cells, overexpression of BRCA1 did not induce TP53I3 expression (Zhang et al., 2015). Added to the fact that two of our HBOC patients carry a TP53I3 germline truncation (Chaudhry et al., 2017; Lopes et al., 2019; Stafford et al., 2017), it would be beneficial to determine the effect of that mutation on repair of DSB with HRR for potential targeted therapy options.

1.6 Personalized therapy in clinical cancer care

There are two major issues with chemotherapy, drug resistance and relapse of the patient after remission (Leary, Heerboth, Lapinska, & Sarkar, 2018). One solution to mitigate both problems is personalized cancer care based on a patient's molecular genetic profile. Additionally, targeted therapy can result in improved patient outcome. Cancer cells are most sensitive to drugs that do not allow for repair of DNA breaks so that they can eventually die due to the potent amount of damage. In many cancers, including breast and ovarian, one of the standard therapy options is platinum-based agents (Martin, Hamilton, & Schilder, 2008;

Pennington et al., 2014; Reed, 1998). However, platinum-based therapies can often lead to resistance or reoccurrence (Moiseyenko et al., 2014).

Another chemotherapy drug given to HBOC patients is the topoisomerase II inhibitor etoposide, which targets HRR-deficient cells and is involved in ROS production. Relapsed EOC patients are often given a treatment regimen that incorporates the usage of etoposide (Konstantinopoulos, Ceccaldi, Shapiro, & D'Andrea, 2015). Etoposide treatment of EOC with *BRCA1/2* mutations have a higher response, longer time to resistance and better overall survival (Safra et al., 2011). Radiomimetic drug bleomycin inhibits DNA synthesis, B-cell, T-cell and macrophage proliferation (Muller, Yamazaki, Breter, & Zahn, 1972). Bleomycin also reacts with oxygen to form superoxide and hydroxide ROS (Wallach-Dayan et al., 2006). It is often used in combination with etoposide and/or cisplatin. Mitomycin C (MMC) is a chemotherapy drug that alkylates DNA to inhibit synthesis and forms interstrand cross-links like the platinum based drug cisplatin. It is a treatment option for anal, bladder, breast, cervical, colorectal, head and neck and non-small cell lung carcinomas. Ovarian cancer patients with germline *BRCA1/2* mutations have had a complete response, partial response or disease stabilization to MMC (Moiseyenko et al., 2014).

1.6.1 Cancer therapies targeting intrinsic apoptosis

Most of the cancer therapies generate pro-death signals that initiate apoptosis of tumor cells. Apoptosis is no longer reversible once the outer membrane of the mitochondria is permeabilized (Elmore, 2007). Defects in the intrinsic apoptosis pathway affects tumor cells responding to chemotherapy and

can result in resistance. Discovering new therapies that target genes involved in intrinsic mitochondrial apoptosis would be revolutionary in mitigating chemotherapy resistance. *TP53I3* is a *p53* regulated pro-apoptotic gene and part of a larger group of genes involved in regulating mitochondrial membrane potential. Additionally, its role in reacting as an enzyme in the presence of other quinone substrate contributes to ROS production under normal and stressed cellular conditions (Athanassios Kotsinas et al., 2012). Thus, with what is known about chemotherapy resistance, mutated TP53I3 could result in chemotherapy resistance and become the bases for creating new therapies to target intrinsic apoptosis.

1.7 Functional assessment of TP53I3-S252* to address missing heritability

The issue of missing heritability creates a knowledge gap in determining and understanding an individual's true genetic risk of HBOC. A streamlined method of identifying novel risk loci is used in this study to exemplify the advantages of using NGS data analysis and wet lab techniques in combination. I conducted *in silico* assessment of 48 Caucasian non-Finnish women diagnosed with ovarian cancer and a personal or family history of epithelial cancers. WES analysis identified 13 truncations in apoptosis genes, including a rare pre-mature stop-gain mutation TP53I3-S252* (Stafford et al., 2017) in two patients. The truncation in TP53I3 will be functionally assessed *in vitro* to determine its impact on cancer related pathways DNA repair and apoptosis, as well as determining sensitivity or resistance to chemotherapy. The nonsense mutation is upstream of three residues important in maintaining the binding affinity for QOR substrates such as $NADP^+$.

(Porté et al., 2009). When a QOR binds to the enzyme at the active site, ROS is produced in order for damaged cells to undergo apoptosis. Therefore, the TP53I3-S252* truncation could prevent the substrates from binding to the active site and prevent ROS production and reduce apoptotic events.

CHAPTER 2 – METHODS

1.1 Acquiring Samples and Determining Tumor Histology

Patient samples were acquired through the Karmanos Cancer Institute Genetic Registry (KCIGR). An IRB was approved for bio-specimens from females with a personal or family history of breast and/or ovarian cancer. From 1999-2013 over 800 DNA samples were collected at which time HBOC genetic screening only involved *BRCA1/2* risk assessment using BRCAPRO and Myriad II. BRCAPRO is a Bayesian model that determines the probabilities that a patient's *BRCA1* or *BRCA2* mutation accounts for the pattern of breast and ovarian cancer in first- and second-degree relatives (Parmigiani, Berry, & Aguilar, 1998). Myriad II identifies putative *BRCA1/2* mutation carriers based on patient ethnic ancestry (Ashkenazi Jewish or non-Ashkenazi Jewish), breast cancer age of onset (age ≤50 years), and the presence of ovarian cancer in the patient or first- or second-degree relatives (Frank, 1999).

Of the 800 DNA samples, 89 were from high-risk Caucasian women with a personal history of OVCA. Participants were *BRCA1/2* mutation carriers or *BRCA1/2* wildtype after full gene sequencing, BART (BRCAnalysis rearrangement test) or testing for the three common Ashkenazi Jewish mutations. Participants who tested positive for pathogenic *BRCA1/2* germline mutations were excluded from our study sample, resulting in a final count of 48 Caucasian women with one mother-daughter pairing. All subjects gave informed consent, allowing for the collection of blood samples and access to medical records. The protocol

(HIC#024199MP2F(5R)) was approved following a Full Board Review by the Human Investigation Committee at Wayne State University, Detroit, Michigan.

Classification of tumor histology included 26 serous, 5 endometrioid carcinoma, 4 adenocarcinoma, 1 mucinous, 1 clear cell and 9 undefined/unknown. Tumor grades included 6 moderately differentiated, 24 poorly differentiated and 1 well differentiated. Primary diagnosis was determined to be ovarian cancer for 43 patients with a secondary diagnosis of breast cancer (n = 6), colon cancer (n = 2), uterine cancer (n = 1) or melanoma (n = 1). The other 5 patients had a secondary diagnosis of OVCA, with primary being breast cancer (n = 4) or cervical cancer (n = 1) (Table 2).

Histology	**N**	**%**
Serous	26	54
Endometrioid	5	10
Mixed	4	8
Adenocarcinoma, NOS	2	4
Clear Cell	1	2
Mucinous	1	2
Unknown	9	19
Stage	**N**	**%**
I	8	17
II	5	10
III	23	48
IV	3	6
Unknown	9	19
Grade	**N**	**%**
Grade 1	1	**2**
Grade 2	6	13
Grade 3	31	50
Unknown	17	35
Personal and Family History	**N**	**%**
Personal BC/OVCA diagnosis <50 years of age	15	31
Personal second primary cancer diagnosis	12	25
Personal/family history BC	31	65
Family history of OVCA	14	29
Family history of epithelial cancer	47	98

Table 2: Tumor Histology and prevalence of ovarian cancer (OVCA)and breast (BC) in the patient cohort

1.2 Whole exome sequencing and candidate gene analysis

DNA from peripheral blood samples was isolated by the Karmanos Applied Genomics Technology Center, Detroit, MI using Qiagen QIAamp DNA mini kit and WES was performed using Illumina Nextera Rapid Capture Kit. Samples were processed as followed:

1. Samples were demultiplexed with Illumina CASAVE 1.8.2.
2. Read quality assessment was conducted with with FastQC (Andrews, 2010).
3. Alignment to the human reference genome (hg19) (Lander et al., 2001) using Burrows Wheeler Aligner (BWA) (H. Li & Durbin, 2009).
4. For the removal of PCR duplicates, samtools was utilized (H. Li et al., 2009).
5. Local realignment, Qscore recalibration, variant calling, and filtering was performed using the GATK Unified Genotyper (DePristo et al., 2011).
6. Subsequent filters were implemented to remove SNPs of low quality, read count or confidence:
    a. SNP mapping quality = 0 for four or more alignments and the number of alignments that mapped ambiguously were in more than 1/10 of all alignments.
    b. SNP reads less than 5 reads.
    c. SNP quality is less than 50
    d. QD score (variant confidence) is less than 1.5
7. Variant Call Format (VCF version 4.1) files were created with Genome Trax BIOBASE biological databases analysis software.

8. Illumina BaseSpace VariantStudio application v2.2.4 and iVariantGuide were used for variant annotated and predicted variant effects was determined with snpEff (Cingolani et al., 2012).

Our focus was to identify clinically actionable and novel loci in the 25 genes currently on the HBOC genetic testing panels by Ambry OvaNext and Myriad MyRisk. Also included were non-panel genes important to DNA damage response, cell cycle regulation or apoptosis and genes disease causing mutations associated with OVCA designated by HGMD (Table 3). To determine the impact of a variant on cancer risk, various clinically curated databases and bioinformatics tools were used including ClinVar (Landrum et al., 2018), HGMD (Stenson et al., 2017), COSMIC (Forbes et al., 2008), dbSNP (Sherry et al., 2001), gnomAD (Karczewski et al., 2019), SIFT (Ng & Henikoff, 2003), and PolyPhen (Adzhubei, Jordan, & Sunyaev, 2013). To focus on the most interesting variants very conservative filters were applied:

1. Only exonic SNPs
2. Moderate to high impact on protein function (frameshift, nonsense, and missense)
3. A minor allele frequency of the mutations is less than 0.02 rare in the European, non-Finnish population
4. Predicted to be damaging by predictive algorithms SIFT and/or PolyPhen,

Variants that fit these criteria were confirmed by forward and reverse strand Sanger sequence (Table 3).

Ovarian cancer panel gene

ATM	*CDH1*	*MSH2*	*NF1*	*RAD50*	*SMARCA*	*TP53*
BARD1	*EPCAM*	*MSH6*	*PALB2*	*RAD51C*	*4*	
BRCA1	*MLH1*	*MUTYH*	*PMS2*	*RAD51D*	*STK11*	
BRIP1	*MRE11A*	*NBN*	*PTEN*			

DNA repair, apoptosis and cell cycle regulating genes

APEX1	*PCNA*	*XRCC1*	*CDC16*	*KNTC1*	*ARL11*	*ITK*
APEX2	*PMS1*	*XRCC2*	*CDC2*	*KPNA2*	*ATAD5*	*KI*
BLM	*POLB*	*XRCC3*	*CDC20*	*MAD2L1*	*ATF1*	*KMT5A*
DDB1	*POLD1*	*XRCC4*	*CDC34*	*MAD2L2*	*BPIFC*	*KRAS*
DDB2	*POLD2*	*XRCC5*	*CDK2*	*MCM2*	*CAV1*	*LIN28B*
ERCC1	*POLD3*	*XRCC6*	*CDK5R1*	*MCM3*	*CLTC*	*LPAR6*
ERCC2	*POLD4*	*ZFP276*	*CDK5RA*	*MCM4*	*COL18A*	*LPL*
ERCC3	*POLE*	*FANCM*	*P1*	*MCM5*	*1*	*MIR191*
ERCC4	*POLE2*	*HMMR*	*CDK6*	*MKI67*	*CREBBP*	*MIR423*
ERCC5	*POLE3*	*POLK*	*CDK7*	*MNAT1*	*CYP17A*	*MTR*
ERCC6	*RAD1*	*POLQ*	*CDK8*	*RAD9A*	*1*	*MYH9*
ERCC8	*RAD17*	*REC8*	*CDKN1A*	*RB1*	*CYP1A1*	*NCKIPS*
EXO1	*RAD51*	*ABL1*	*CDKN1B*	*RBBP8*	*DCC*	*D*
FANCA	*RAD52*	*ANAPC2*	*CDKN2A*	*RBL1*	*DCP1B*	*NEKB1*
FANCC	*RECQL*	*ANAPC4*	*CDKN2B*	*RBL2*	*DROSHA*	*NOTCH*
FANCE	*RECQL4*	*ATR*	*CDKN3*	*SERTAD*	*E2F2*	*P14ARF*
FANCF	*RECQL5*	*BAX*	*CHEK1*	*1*	*EPHX1*	*PCM*
FANCG	*RFC1*	*BCCIP*	*CKS1B*	*SKP2*	*FMR1*	*PGR*
FEN1	*RFC2*	*BCL2*	*CKS2*	*SKP2.*	*FRG1*	*PPMID*
LIG1	*RFC3*	*BIRC5*	*CUL1*	*SUMO1*	*FSHR*	*PSMC3I*
LIG3	*RFC4*	*CCNB1*	*CUL2*	*TFDP1*	*GSTM1*	*P*
LIG4	*RFC5*	*CCNB2*	*CUL3*	*TFDP2*	*GSTO2*	*RNF213*
MBD4	*RPA1*	*CCNC*	*DDX11*	*UBA1*	*GSTP1*	*SLC4A7*
MGMT	*RPA2*	*CCND1*	*DIRAS3*	*TP53I3*	*GSTT1*	*SLX4*
MLH3	*RPA3*	*CCND2*	*DNM2*	*TP53BP1*	*HFE*	*SPINK1*
MPG	*SMUG1*	*CCNE1*	*E2F4*	*TP53BP2*	*HIP1*	*TERT*
MSH3	*TDG*	*CCNF*	*GADD45*	*ACACA*	*HRAS*	*TNFRSF*
MSH5	*UNG*	*CCNG1*	*A*	*ADH1B*	*IGF2*	*13B*
NEIL1	*UNG2*	*CCNG2*	*GTF2H1*	*AGER*	*IL1A*	*UGT2A3*
NTHL1	*WRN*	*CCNH*	*GTSE1*	*AGO2*	*IL23R*	*WAS*
OGG1	*XPA*	*CCNT1*	*HERC5*	*APOBEC*	*ILI6*	
PARP1	*XPC*	*CCNT2*	*HUS1*	*3B*	*ILIR1*	

Table 3: List of DNA repair, cell cycle regulation and apoptosis genes investigated for the variant assessment of HBOC patients (Stafford et al., 2017).

1.3 Confirming variants of interest

Validation of SNPs involves PCR amplification and Sanger sequencing. Primers targeted the genomic DNA in the patient carrying the SNP were created using Primer3Plus and Thermo Fisher Primer Designer Tool application. Primer constructs were obtained from Sigma-Aldrich. Forward and reverse primers were between 200 – 100 base pairs away from the SNP, had a GC content of approximately 50% and annealing temperature between 50ºC and 52 ºC (Table 4).

1.3.1 PCR amplification

Primers were re-suspended in sterile water for a stock concentration of 100 µM and a working stock is diluted to 10 µM. PCR amplification was conducted using the QIAGEN Fast Cycling PCR Kit (203743).

One reaction 20 µL consists of:

1. 10 µL Qiagen Fast Cycling PCR Master Mix
2. 2 µL CoralLoad Dye
3. 2 µL forward primer (10 µM)
4. 2 µL reverse primer (10 µM)
5. 2 µL sterile H_2O
6. 2 µL of 25 ng/uL patient DNA/positive control/ negative control. The Positive control consists of normal fibroblast genomic DNA and the negative control was water.

PCR amplification protocol requires denaturing, annealing and elongation,

1. DNA heated at 95°C for 5 minutes for original denaturing.

2. PCR Cycle begins with denaturing for 5 seconds at 96°C.
3. Annealing between 50 °C and 62 °C depending on primer specification.
4. Elongation for 15 seconds at 68°C for 15 seconds PCR Cycle ends.
5. Repeat steps 2 to 4 for 36 cycles.
6. Final elongation at 72 °C for 2 minutes.

1.3.2 Agarose gel electrophoresis

DNA amplification occurred was confirmed using gel electrophoresis. DNA bands are separated by size, the smaller the fragment size the lower it migrates down the agarose gel. Since DNA is negatively charged, when applying electric current the fragments will move toward the positive electrode. The CoralLoad binds to the DNA and emits red fluorescence. A 1% agarose gel made with 1X TAE buffer was used (Thermo Fisher 16500100). Prior to casting the gel, propidium iodide was added to allow for the visualization of the product. The gels are submerged in 1 x TAE buffer (40 mM Tris base, 2 mM EDTA, 20 mM Acetic Acid, pH 8.5). Approximately, 2 – 5 µL of the PCR product was loaded along with 2 µL of a 100 base pair DNA ladder (Invitrogen™ 100 bp DNA ladder, 15628019). The gel was run at 100 volts for 60 minutes and visualized under the Odyssey LI-COR scanner.

1.3.3 Sanger sequence confirmation

After confirming that the target region had been properly amplified with gel electrophoresis, the amplified genomic DNA from the PCR products were purified using the QIAGEN QIAquick PCR Purification Kit (28106).The concentration of the purified genomic DNA was quantified using the Nanodrop 2000. The sequencing

reactions were assembled and sent to Genewiz. One reaction consists of 5 µL of forward or reverse primer, 2 µL of 25 ng/ µL amplified genomic DNA and 8 of µL sterile water.

Gene	SNP	AA Change	Forward	Reverse
PIK3C2G	rs61757718	*1446S	CAGAGCTCCAAGGACATGTC	CTGCTTTTAACTGTAGGCACAC
TP53I3	rs145078765	S252*	TCTGAAATCGGGTTCCCTCT	AGGCCTCATAAATGGTGAACTT
TP53AIP1	rs141395772	Q22fs	GCAAAAGACCGTCTCGGTTTTC	CCTAACAACAAATGAGGAGAAGCCA
TP53AIP1	rs140191758	S32*	GCAAAAGACCGTCTCGGTTTTC	CCTAACAACAAATGAGGAGAAGCCA
BCLAF1	rs61731960	E403*	GTTTGACTTCAGGACGGTGA	AGGATCAGAGAAAGGGAGGG
BCLAF1	rs140096922	H847fs	TGTAAAACGACGGCCAGT	CAGGAAACAGCTATGACC
PPP1R15A	rs139708522	E160*	GAGAAACACTGGGGCTGAAA	TGTGTGCCTTTTCCTCCTTC
DOCK1	rs768625958	D248fs	AACAGACAAGCCAAGTTTGC	GGCAGGCACCATTCTAAATG
NLRP1	rs771551366	R138fs	CCCTATCCTTCCTCTGCTGT	GTTGGCCCAATAAAGCACAG
PTH	N/A	K85fs	TGTATTGTTGCCCTACACTGT	TACCTGCAAAAGACATGGCT
ANGPTL4	rs747940485	G275fs	ATGGCTCAGTGGACTTCAAC	GCATGTAAGGAAGAGGTGGG
NOD2	N/A	W289*	TCAGTCTCGCTTCCTCAGTA	TGCAGAAGGTTGAAGAGCAG
GZMM	rs200398398	Q161*	TAGCTGGACGGGAAAGTGAA	GCTAAACCTGTCTGAGCCTC

Table 4: PCR primer sequences for SNP confirmation found apoptosis genes.

1.4 Cell lines and Cell culture

A variety of different cell lines were used to find the best *in vitro* model for DNA damage and cell death pathways. Epithelial ovarian cancer cells SKOV-3 are derived from a 64-year-old Caucasian female with ovarian serous cystadenocarcinoma (Fogh, Fogh, & Orfeo, 1977). SKOV-3-DRGFP cells were gifted by Dr. Z Ping Lin from Yale University School of Medicine (Lin, Ratner, Whicker, Lee, & Sartorelli, 2014). However, SKOV-3 cells are p53 null and the population only exhibited a 2% induction of the DNA repair mechanism. Sub-cloning was attempted on the SKOV-3-DRGFP cells, but the HRR induction rate did not improve significantly. High grade ovarian serous adenocarcinoma OVCAR8 cells were also evaluated for functional assessment of DNA repair and cell death. OVCAR-8-DRGFP was provided by Dr. Larry Karnitz from the Mayo Clinic. However, OVCAR8 is also p53 null and induction of HRR was not successful. Therefore, the SKOV4-DRGFP and OVCAR8-DRGFP cells were not used for *in vitro* assessment of DNA repair and cell death.

HeLa cells are immortalized and derived from cervical cancer cells from a 31-year-old African American woman. HeLa cells are well characterized and have been used across a variety of research topics in the medical fields. HeLa-DR-GFP cells were provided by Dr. Jeffery Parvin from Ohio State University. The induction of HRR after DNA DSB in HeLa-DRGFP ranged from 10% – 20% of the cells within the population.

All cells types were maintained in Dulbecco's Modified Eagle Medium (DMEM; 31600-034), low glucose, pyruvate with 10% fetal bovine serum (Hyclone,

SH30071.03IR), 1% penicillin-streptomycin (Gibco™, 15070063), and 0.5 - 1.5 µg/mL puromycin for selection of pDR-GFP (InvivoGen, ant-pr-1).

1.5 Transfection reagent, siRNA and antibodies

The focus of this work was to determine the effect of candidate apoptosis genes, *TP53I3*, and the associated nonsense mutation that two of the HBOC patients carried on cellular function using transient transfection. Transient transfection employs exogenous nucleic acid for a limited period of time followed by functional assays. For protein knockdown, high quality and pure siRNAs were used targeting the 3'UTR not contained in the plasmid expression vectors. Exogenous TP53I3 wildtype, TP53I3-S252* mutant and empty vector DNA plasmids were delivered to the cells by transient transfection. All transfections are conducted using jetPRIME Transfection Reagent (Polyplus, 114-15). Information about plasmid DNA, siRNA protein knockdown, and primary and secondary antibodies for protein quantification can be found in Table 5. Based on knockdown efficiency, the concentration of siRNA used for all proteins was 110 picomole/well in a 24 well plate and scaled up to the appropriate cell culture plate when needed.

Product	Manufacturer	Catalog No.	Concentration/Dilution
Hs_ TP53I3_2 FlexiTube siRNA 20 nmol	Qiagen	SI00069636	110 pmol in a 24 well plate
Hs_BRCA1_13 FlexiTube siRNA 20 nmol	Qiagen	SI02654575	110 pmol in 24 well plate
P53AIP1 siRNA (h) 10µM	Santa Cruz	SC-37459	110 pmol in 24 well plate
Negative Control siRNA 20 nmol	Qiagen	1027310	110 pmol in 24 well plate
PIG3 Antibody- mouse (A-5)	Santa Cruz	SC-166664	1:1000 overnight incubation
BRCA1 Antibody- mouse (D-9)	Santa Cruz	SC-6954	1:200 overnight incubation
P53AIP1- rabbit Antibody	Invitrogen	PA5-20355	1:200 overnight incubation
Beta-actin- rabbit AC-74 Antibody	Sigma-Aldrich	A53160-100UL	1:10,000 overnight incubation
Donkey anti-Rabbit IgG (H+L) Alexa Fluor® 790	Thermo Fisher	A11374	1:10,000; 1 hour incubation
Donkey anti- Rabbit IgG (H+L) Alexa Fluor® 680	Thermo Fisher	A10043	1:10,000; 1 hour incubation
Donkey anti-Mouse IgG (H+L) Alexa Fluor® 790	Thermo Fisher	A11371	1:10,000; 1 hour incubation
Donkey anti-Mouse IgG (H+L) Alexa Fluor® 680	abcam	ab175774	1:10,000; 1 hour incubation
PIG3 cDNA Clone, Human, C-OFPSpark® tag	Sino Biological	HG15531-ACR	N/A

Table 5: Materials for knockdown, westerns, and plasmid constructs.

1.6 Cell Lysate Preparation and Western blots

Identification of specific protein expression from a mixture of proteins can be done using the western blotting technique (Mahmood & Yang, 2012). For the preparation of protein mixtures, lysed cells and proteins are solubilized using ice-cold RIPA (Radio Immunoprecipitation Assay Buffer) with Halt™ protease (Thermo Fisher, 87786) and phosphatase inhibitor cocktails (Thermo Fisher, 78420). Cells are scraped and shaken for 15 minutes at 4ºC. To ensure that the DNA was sheared, lysates are passed through a 21-gauge and incubated on ice for 30 minutes. Cell lysate are centrifuged for 10,000xg for 10 minutes at 4ºC. The total cell lysate supernatant was transferred to a new 0.6 mL centrifuge tube. For protein concentration, the DC™ Protein Assay was used and consisted of protein assay reagent A (Biorad, 5000113), protein assay reagent B (5000114) and protein assay reagent S (Biorad, 5000115). Protein concentration was quantified using the BioTek Synergy H1 Hybrid Multi-Mode Reader. Prior to loading the gel the protein mixture was reduced and denatured using the LI-COR loading buffer and 2-mercaptoethanol (BME).

The three main parts of western blot methodology involves separating proteins by size, transferring proteins to a membrane and targeting protein for visualization with primary and secondary antibodies (Mahmood & Yang, 2012). For separation of proteins, a polyacrylamide sodium dodecyl sulfate (SDS) gel was used, the size of the protein determines the thickness of the gel. For TP53I3, TP53AIP1 and β-actin 12% separating gel was optimal and 6% - 7.5% for BRCA1 visualization. Cell lysates (50 – 150 µg) are prepared with a 4x Li-CORE protein

sample buffer (928-40004) and BME before running each gel. The samples and 5 µL of the protein standard marker (Biorad, 1610374) were loaded. The gel was run at 150 – 180 volts until the dye ran off the gel. The proteins are then transferred to a 0.45 µM or 0.22 µM nitrocellulose membrane (VWR) at 250 mA for one hour on ice (TP53I3, TP53AIP1, and β-actin) or 30 V for 18 hours at 4ºC (BRCA1).

Membranes were removed from the transfer apparatus and set to dry for 30 minutes. Next, the blot was blocked with 5% bovine serum albumin (BSA) in 1x Tris Buffered Saline with 0.1% Tween 20 (TBST) for 60 minutes at room temperature. The membranes were incubated overnight with the appropriate primary antibody diluted in 5% BSA in 1x TBST. The membrane was washed with 1x TBST for 10 minutes three times. After one hour incubation with the secondary in the dark at room temperature, membranes were washed with 1 x TBST for 3 minutes three times (Table 5). Visualization and quantification of protein are determined by the LI-COR Odyssey® CLx Imaging System.

1.7 *TP53I3* gene editing and transfection

TP53I3 expression vector, pCMV3-C-OFPSpark-TP53I3, was acquired from Sino Biological (HG15531-ACR). The plasmid contains an open reading frame for the full coding sequence of *TP53I3* followed by an orange fluorescent protein (OFP) marker at the C-terminus. Using the wildtype plasmid, site-directed mutagenesis was conducted using the Q5-Site-Directed Mutagenesis Kit from New England BioLabs Inc. (E0554S). To emulate the mutation change in two of our 48 ovarian cancer patients the kit was used to create the rs145078765 (c. 755C>G, S252*) truncation. The primers for the TP53I3-S252* mutant were

forward 5'-CCCCTGTTTTAAAAAGCTACTTTTTAAG-3' and reverse 5'-CCCATTGATGTCACCTCC-3'. Purified DNA of the selected mutant and wildtype clones was conducted using the Qiagen QIAamp DNA mini kit. PCR primers were created to sequence the open reading frame of the plasmid in order to capture the entire coding region of TP53I3 in both the wildtype mutant plasmid (Figure 6). In a 60 mm culture plate, a 3 ng/µL concentration of unmodified transfection was sufficiently expressed pCMV3-C-OFPSpark-TP53I3 and similar to endogenous to TP53I3. The pCMV3-C-OFPSpark-TP53I3 will be addressed as TP53I3-WT and the mutant plasmid will be TP53I3-S252*. Due to the orange fluorescent protein tag on the C-terminus of the wildtype construct, the size of the exogenous TP53I3-WT is 64 kDa. Mutated TP53I3-S252* mutation is 28 kDa and endogenous TP53I3 is 36 kDa. Assessment of the mutant was conducted using a transient transfection methodology, where endogenous TP53I3 was depleted with siRNA and the TP53I3-S252* plasmid or the TP53I3-WT plasmid or pCMV3 empty vector was incorporated into the HeLa-DR-GFP cells. The TP53I3 siRNA targeted a region at the C-terminus, downstream of the truncation (Figure 7).

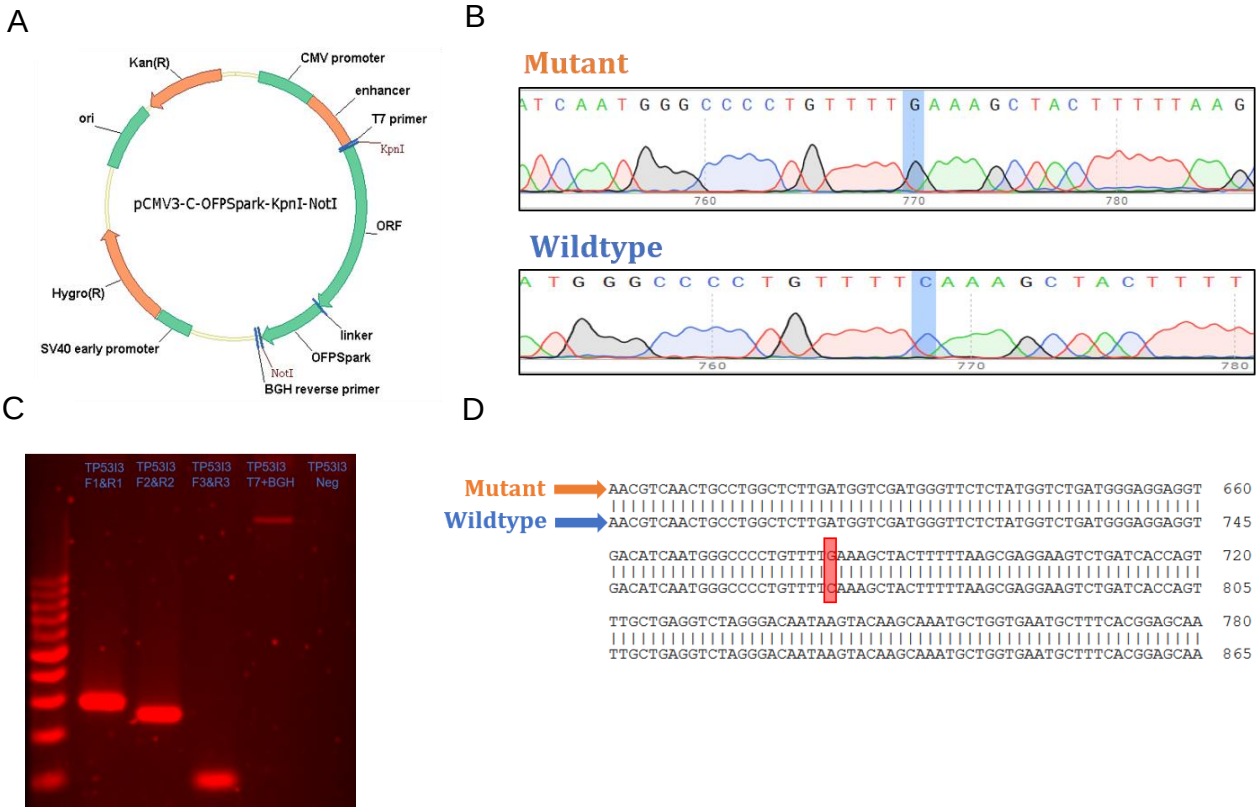


Figure 6: *TP53I3* site-directed mutagenesis for S252* mutant.

The pCMV3-C-OFPSpark plasmid (A) carries the full coding sequence of TP53I3 and confirmed with PCR, gel electrophoresis and Sanger sequencing (C). Using the NEB Q5® Site-Directed Mutagenesis kit, the cytosine at position 755 of the DNA sequence was modified to guanine resulting in a pre-mature stop codon exhibited in the OVCA cohort (B). The mutant plasmid DNA was compared to unmodified wildtype to insure that no off-target effects occurred (D).

>NM_147184.3 Homo sapiens tumor protein p53 inducible protein 3 (TP53I3), transcript variant 2, mRNA

AACGGCTCCTTTCTCTTCTCTTAGCAGCACCCAGCTTGCCCACCCATGCTCAAGATGGG
CGGGATGCCAGCCTGTTACATAAATGTGCCAAAAGCCTGGCCATGCCTGGAAAATGGAC
CAATCCGCCCGCCAAGAGGTTGGGTCTCGTTCCCTAGAGAGAAGGAAGTTTCCTCTCCT
TGAAGTGAGAGCTAGAATCGCACTTTCTGTCAAGCTGAGAGAAAGACTCTTTTCCAGAG
GCTAAAAGGACAAGAAAATCTGATTTGCTTGCTTCTAACTTTGCGTTTTAAAGGGGGAA
GGAGGAAAGGAAAGAGGGGGAGGGTGGTTCTGCTTAGCCCCACCCCTCCGGCTACCCCA
GGTCCAGCCGTCCATTCCGGTGGAGGCAGAGGCAGTCCTGGGGCTCTGGGGCTCGGGCT
TTGTCACCGGGACCCGCAGGAGCCAGAACCACTCGGCGCCGCCTGGTGCATGGGAGGGG
AGCCGGGCCAGGAACAAT**ATGTTAGCCGTGCACTTTGACAAGCCGGGAGGACCGGAAAA**
CCTCTACGTGAAGGAGGTGGCCAAGCCGAGCCCGGGGGAGGGTGAAGTCCTCCTGAAGG
TGGCGGCCAGCGCCCTGAACCGGGCGGACTTAATGCAGAGACAAGGCCAGTATGACCCA
CCTCCAGGAGCCAGCAACATTTTGGGACTTGAGGCATCTGGACATGTGGCAGAGCTGGG
GCCTGGCTGCCAGGGACACTGGAAGATCGGGGACACAGCCATGGCTCTGCTCCCCGGTG
GGGGCCAGGCTCAGTACGTCACTGTCCCCGAAGGGCTCCTCATGCCTATCCCAGAGGGA
TTGACCCTGACCCAGGCTGCAGCCATCCCAGAGGCCTGGCTCACCGCCTTCCAGCTGTT
ACATCTTGTGGGAAATGTTCAGGCTGGAGACTATGTGCTAATCCATGCAGGACTGAGTG
GTGTGGGCACAGCTGCTATCCAACTCACCCGGATGGCTGGAGCTATTCCTCTGGTCACA
GCTGGCTCCCAGAAGAAGCTTCAAATGGCAGAAAAGCTTGGAGCAGCTGCTGGATTCAA
TTACAAAAAAGAGGATTTCTCTGAAGCAACGCTGAAATTCACCAAAGGTGCTGGAGTTA
ATCTTATTCTAGACTGCATAGGCGGATCCTACTGGGAGAAGAACGTCAACTGCCTGGCT
CTTGATGGTCGATGGGTTCTCTATGGTCTGATGGGAGGAGGTGACATCAATGGGCCCCT

GTTTTCG**AAAGCTACTTTTTAAGCGAGGAAGTCTGATCACCAGTTTGCTGAGGTCTAG**
GGACAATAAGTACAAGCAAATGCTGGTGAATGCTTTCACGGAGCAAATTCTGCCTCACT
TCTCCACGGAGGGCCCCCAACGTCTGCTGCCGGTTCTGGACAGAATCTACCCAGTGACC
GAAATCCAGGAGGCCCATAAGTACATGGAGGCCAACAAGAACATAGGCAAGATCGTCCT
GGAACTGCCCCAGTGAAGGAGGATGGGGCAGGACAGGACGCGGCCACCCCAGGCCTTTC
CAGAGCAAACCTGGAGAAGATTCACAATAGACAGGCCAAGAAACCCGGTGCTTCCTCCA
GAGCCGTTTAAAGCTGATATGAGGAAATAAAGAGTGAACTGGAAAAAAAAAA

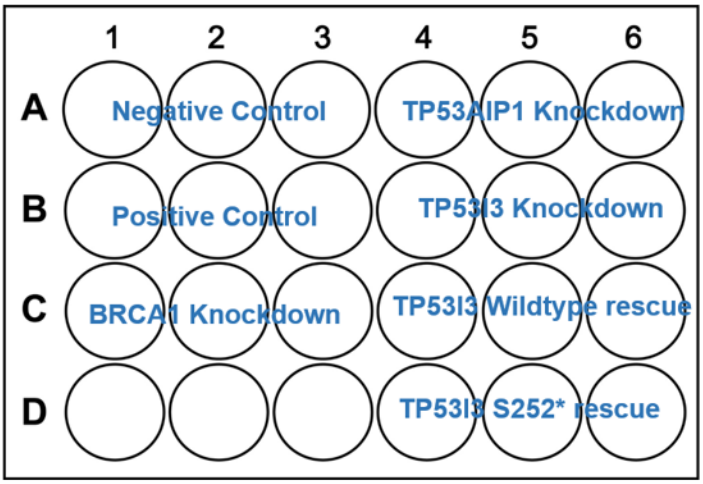
siRNA target sequence Mutation **Coding region**

Figure 7: *TP53I3* transcript sequence and targeted regions

1.8 Homologous Recombination Repair (HRR) Assay

Hela-DR-GFP cells provided by Dr. Jeffery Parvin from Ohio State University School of Medicine are used for *in vitro* assessment of HRR. Within the genome, the cells contain the DR-GFP plasmid which consists of two copies of green fluorescent protein (GFP) and transfection of I-*Sce1* endonuclease causes a DSB in the GFP allele 1 containing the I-*Sce1* recognition site. The truncated GFP allele 2 serves as a donor of DNA repair, resulting in the activation of the repaired GFP allele 1. Therefore, GFP expression is a proxy for HRR occurring in the cells (Pierce, Johnson, Thompson, & Jasin, 1999a). To maintain the HeLa-DR-GFP selection, cells were cultured with 1.5 µg/mL Puromycin (Figure 8). Quantification of GFP expression after transfection was determined using the BD FACSCanto II at the Wayne State University Microscopy, Imaging & Cytometry Resources (MICR) core. To establish how proteins of interest affect the induction of HRR, siRNAs are used (Figure 8). As a negative control, empty vector pCMV3 was used. HeLa-DR-GFP cells were seeded 24 hours prior to transfection. The transfection complex consists of pCMV3 empty vector or TP53I3-S252* or wildtype TP53I3 with pCBASceI, siRNA and jetprime®PRIME reagent diluted into jetPRIME® Buffer. All conditions were conducted in triplicate for each experiment and raw values were normalized to the positive control.

Day 1: Seed 35,000 HeLa-DRGFP CL4A8 cells.
For protein knockdown efficiency seed 200,000 cells in 6 well plates.



Day 2: Transfection
1 RxN ➔ 1.5 µg pCBASce1/pCMV3 + 0.875 ng pCMV3/pCMV3-OFPSpark-TP53I3-WT/pCMV3-OFPSpark-TP53I3-ΔS252 + 150 µL jetPRIME buffer + 3 µL jetPRIME Reagent.

DNA	siRNA	jetPRIME Buffer	jetPRIME Reagent
4.5 µg pCMV3	300 pmole Scramble	150 µL	9 µL
4.5 µg pCBASce1 + 2.625 ng pCMV3	300 pmole Scramble	150 µL	9 µL
4.5 µg pCBASce1 + 2.625 ng pCMV3	300 pmole siBRCA1	150 µL	9 µL
4.5 µg pCBASce1 + 2.625 ng pCMV3	300 pmole siTP53AIP1	150 µL	9 µL
4.5 µg pCBASce1 + 2.625 ng pCMV3	300 pmole TP53I3	150 µL	9 µL
4.5 µg pCBASce1 + 2.625 ng pCMV3-OFPSpark-TP53I3-WT	300 pmole TP53I3	150 µL	9 µL
4.5 µg pCBASce1 + 2.625 ng pCMV3-OFPSpark-TP53I3-ΔS252	300 pmole TP53I3	150 µL	9 µL

Day 5: Collect cells and quantify with BDFACSCantoII.
Lyse 6 well plates to quantify knockdown efficiency.

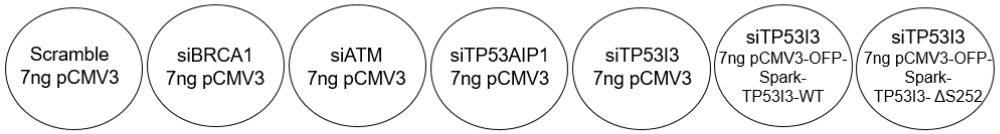
Figure 8: Detailed protocol for HRR assay.

The HRR assay was conducted in HeLa-DRGFP cells. Knockdown and rescue conditioned cells for protein quantification are conducted in parallel.

1.9 Colony Survival Assay

The clonogenic assay can be used to determine a single cell's ability to survive and grow into a colony (Franken, Rodermond, Stap, Haveman, & van Bree, 2006). The *in vitro* cell assay can be used to ascertain the cell's reproductive death after being conditioned with various cytotoxic agents (Franken et al., 2006). To determine how TP53I3 knockdown, TP53I3-S252* and TP53AIP1 knockdown cells respond to chemotherapy agents the colony survival assay was utilized (Figure 9). For each condition, 300 cells are seeded in triplicate. Conditioned cells were treated with the IC50 of bleomycin (1.5 µM), mitomycin C (100 nM), etoposide (4 µM) or hydrogen peroxide (H_2O_2, 125 µM). To prevent the rapid degradation of H_2O_2, it was diluted in phenol red-free and sodium bicarbonate free DMEM. Clones were grown for seven days post-treatment then fixed and stained with 0.5% crystal violet (Sigma-Aldrich, C0775). Surviving colonies consisting of 50 or more cells were counted using a light microscope to determine plating efficiency and surviving fraction.

Day 1: Seed 400,000 HeLa cells in 60 mm plate for each protein knockdown condition.



Day 2: Conduct siRNA knockdown with Polyplus transfection jetPRIME.

DNA	siRNA	jetPRIME Buffer	jetPRIME Reagent
7 ng pCMV3	525 pmole Scramble	150 µL	9 µL
7 ng pCMV3	525 pmole Scramble	150 µL	9 µL
7 ng pCMV3	525 pmole siBRCA1	150 µL	9 µL
7 ng pCMV3	525 pmole siATM	150 µL	9 µL
7 ng pCMV3	525 pmole siTP53AIP1	150 µL	9 µL
7 ng pCMV3	525 pmole TP53I3	150 µL	9 µL
47 ng pCMV3-OFPSpark-TP53I3-WT	525 pmole TP53I3	150 µL	9 µL
7 ng pCMV3-OFPSpark-TP53I3-ΔS252	525 pmole TP53I3	150 µL	9 µL

Day 3: Seed 300 HeLa cells for each protein knockdown/rescue condition in triplicates for each drug treatment condition in 6 well plates. Seed left over cells in 60 mm plates for protein knockdown quantification

Day 4: Treat cells with bleomycin, mitomycin C (MMC), etoposide, H_2O_2 or leave untreated. For protein knockdown quantification lyse carry over plates from Day 3.

Knockdown/rescue	Treatment conditions
Scramble + 7ng pCMV3	Untreated, 1.5 µM Bleomycin 3 hours, 100 nm MMC 3 hours, 1 µM Etoposide 3 hours, or 200 µM H_2O_2 3 hours
siBRCA1 + 7 ng pCMV3	
siTP53AIP1 + 7 ng pCMV3	
siTP53I3 + 7 ng pCMV3	
siTP53I3 + 7 ng pCMV3-OFPSpark-TP53I3-WT	
siTP53I3 + 7 ng pCMV3-OFPSpark-TP53I3-ΔS252	

$$Plating\ Efficiency\ (PE) = \frac{number\ of\ colonies\ formed}{number\ of\ colonies\ seeded} \times 100$$

$$Surviving\ Fraction\ (SF) = \frac{number\ of\ colonies\ formed}{number\ of\ colonies\ seeded\ \times PE}$$

Day 11: Fix and stain cells with 0.5% Crystal violet. Count cells and account for plating efficiency (PE) and surviving fraction for treated cells (SF).

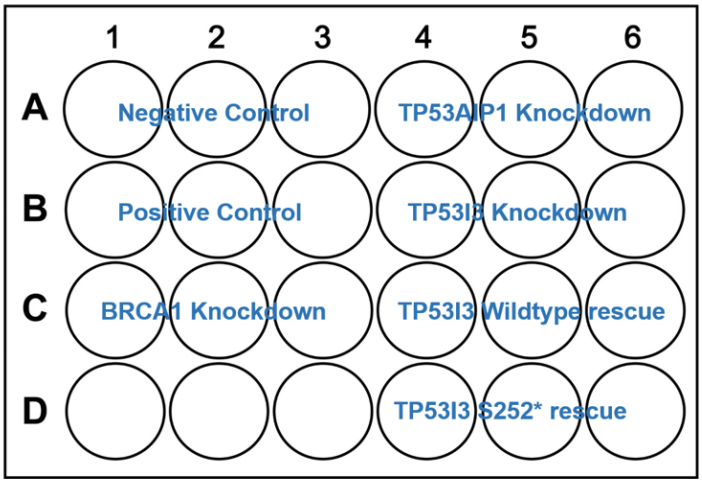
Figure 9: Detailed protocol for clonogenic assay

The clonogenic assay was conducted using HeLa-DRGFP cells. Knockdown and rescue conditioned cells for protein quantification were conducted in parallel.

2.1 Mitosox assay for mitochondrial reactive oxygen species (ROS) production

To detect for production of ROS in the presence of H_2O_2 or etoposide, the Mitosox probe for intra-mitochondrial superoxide ROS was used (Kauffman et al., 2016). Mitosox is a positively charged probe that accumulates and emits red fluorescence (excitation: 510 nm, emission: 580 nm) in the mitochondria to detect for superoxide ROS (Kauffman et al., 2016). For each treatment (H_2O_2 or etoposide), cells were seeded 24 hours prior to transfection with each knockdown condition was conducted in triplicate (Figure 10). An additional plate was used to serve as an untreated control for knockdown conditions in triplicate. In parallel cells were also seeded in 6 well plates for cell lysate and protein quantification. The transfection complex includes TP53I3-252* or TP53I3 wildtype or pCMV3 DNA with siRNA and jetPRIME® reagent. Cells were then treated with etoposide 60 µM or 125 µM of H_2O_2 for four hours. Next, treatment was removed and cells were washed three times with warm PBS (37ºC) and then stained with 5 µM Mitosox for 30 minutes at 37ºC at 5% CO_2 in the dark. Fluorescence intensity was quantified using the BioTek Synergy H1 Hybrid Multi-Mode Reader.

Day 1: Seed 35,000 HeLa-DRGFP CL4A8 cells in two 24 well plate (untreated and H_2O_2 conditions). For protein knockdown efficiency in triplicate seed 200,000 cells in 6 well plates.



Day 2: Conduct siRNA knockdown with Polyplus transfection jetPRIME.

DNA	siRNA	jetPRIME Buffer	jetPRIME Reagent
5.25 ng pCMV3	300 pmole Scramble	150 µL	9 µL
5.25 ng pCMV3	300 pmole Scramble	150 µL	9 µL
5.25 ng pCMV3	300 pmole siBRCA1	150 µL	9 µL
5.25 ng pCMV3	300 pmole siTP53AIP1	150 µL	9 µL
5.25 ng pCMV3	300 pmole TP53I3	150 µL	9 µL
5.25 ng pCMV3-OFPSpark-TP53I3-WT	300 pmole TP53I3	150 µL	9 µL
5.25 ng pCMV3-OFPSpark-TP53I3-ΔS252	300 pmole TP53I3	150 µL	9 µL

Day 4: Treat cells with 200 µM H_2O_2 using phenol red and sodium bicarbonate free DMEM-20%FBS in parallel with untreated conditions for four hours. After four hour remove media and incubate with 5 µM of Mitosox (excitation/emission 510/580) or DCFDA and quantify florescence (excitation/emission 488/515) with the BioTek microplate reader. Lyse 6 well plates for protein quantification.

Figure 10: Detailed protocol for Mitosox staining

The mitosox assay was conducted in HeLa cells. Knockdown and rescue conditioned cells for protein quantification are conducted in parallel. Untreated and treated conditions include, Scramble siRNA + pCMV3 treated, siBRCA1 + pCMV3, siTP53AIP1 + pCMV3, siTP53I3 + pCMV3, siTP53I3 + TP53I3-WT plasmid, siTP53I3 + TP53I3-S252* plasmid.

3.1 Statistical Analysis

The values reported in graphs are the mean±standard error (S.E.) from experiments conducted in triplicate. A standard two-way student t-test was conducted to compare all conditions to the positive control. A value of $p<0.05$ was considered statistically significant.

CHAPTER 3 – RESULTS

1.1 Identifying clinically actionable germline mutations in HBOC patients

From 1999 – 2015, 800 DNA samples of breast and/or ovarian cancer patients were collected. I assessed the WES data of the germline DNA of 48 Caucasian patients suspected to have HBOC but negative for pathogenic mutations in *BRCA1* and *BRCA2*. During the period of time in which the samples were collected, genetic panel testing had not yet been established as a standard of care, as usually only BRCA1 and BRCA2 were analyzed. Therefore, this sample subset was an excellent cohort to explore genetic variation associated with OVCA because they have not undergone up-to-date genetic panel testing. The WES data from these patients can also help identify additional mutations in non-panel genes. Variant caller files (VCF) were created for each patient and further annotated using the Illumina Variant Studio 3.0 application. All mutations are annotated based on ACMG and NCCN guidelines. Variants were filtered to only include those in the coding region, with a minor allele frequency of at most 2%. I focused on truncation or missense annotations as well as pathogenic or likely pathogenic in ClinVar, damaging or deleterious in Polyphen or SIFT, high or moderate in SnpEff, type and definition in COSMIC, germline in TCGA or damaging in HGMD. Five unrelated patients carry a clinically actionable mutation (Table 6), including a premature stop-gain or frameshift in *FANCM, RAD51D* or *ATM* (Stafford et al., 2017). All five truncations were cross-referenced with the HGMD database and the *FANCM* R1931* (rs144567652, MAF = 0.000946) and *RAD51D* R206* (rs38790683, MAF = 0.0001) nonsense mutations are reported as damaging

mutations (DM). Patient OCJ19 carries the *FANCM* rs144567652 nonsense mutation, and consistent with our study it was associated with an increased the risk of triple-negative breast cancer (Figlioli et al., 2019; Peterlongo et al., 2015) and hereditary ovarian cancer (Dicks et al., 2017). Additionally, patient OCJ19 carries a truncating mutation in TP53I3-S252*, an oxidoreductase involved in the process of apoptosis.

Clinically actionable variants					
Gene	**dbSNP ID**	**Mutation**	**MAF**	**OBS**	**HGMD**
FANCM	rs144567652	R1931*	0.000946	1	DM
RAD51D	rs587781756	Q171*	N/A	1	N/A
RAD51D	rs387906843	R206*	0.0001	1	DM
ATM	N/A	CATCTG>C	N/A	1	N/A
ATM	N/A	G>GA	N/A	1	N/A

Variants of interest in candidate genes				
Genes	**dbSNP ID**	**Mutation**	**MAF**	**Observed**
TP53I3	**rs145078765**	**S252***	**0.001648**	**2**
FANCM	**rs144567652**	**R1931***	**0.000946**	**1**
HMMR	rs146791423	E352*	0.003498	1
POLQ	rs148626322	Q2513*	0.000285	1
RAD52	rs4987207	Y415*	0.0128	1
RAD52	rs4987208	S346*	0.01969	1
REC8	N/A	W365*	N/A	1
CHEK1	N/A	G>GA Exon7	N/A	1
MCM4	N/A	GGC>G Exon12	N/A	1
POLK	N/A	GA>G Exon10	N/A	1
RAD1	N/A	CT>C Exon 6	N/A	1

Table 6: Clinically actionable and novel risk loci in DNA repair genes (Lopes et al., 2019; Stafford et al., 2017)

From left to right is gene name, SNP ID from dbSNP, the mutation was either nonsense (*) or frameshift, gnomAD non-Finnish population minor allele frequency (MAF), and the number of individuals in the HBOC cohort carrying the mutation.

1.1.1 Novel risk loci in DNA repair, cell cycle regulating and apoptosis candidate genes

To address the issue of missing heritability in HBOC, we conducted targeted screening to identify high impact variants in genes not currently on the genetic panels. Genetic aberrations disrupting DNA repair, cell cycle regulating and apoptosis can result in tumorigenesis. Therefore, variants of interest were narrowed down to those found in KEGG annotated as DNA repair, cell cycle regulating and/or apoptosis genes (Table 5). There are eleven high impact truncations in DNA repair and cell cycle regulating non-panel or "candidate" genes (Stafford et al., 2017). Of particular interest was the TP53I3 S252* rs145078765 stop-gain mutation caused by the point mutation of a cytosine at position 755 to guanine in the gene's coding sequence (Figure 6). This nonsense mutation was present in two unrelated patients, OCG14 and OCJ19. There are multiple incidences of epithelial cancers in the family history for both patients including ovarian, breast, prostate, pancreatic, stomach and melanoma (Figure 11A and B). Patient OCJ19 also carries the *FANCM* R1931* (rs144567652) truncation (Stafford et al., 2017). Her family history includes two members previously diagnosed with pancreatic cancer or multiple myeloma (Figure 11A). HBOC syndrome is known to increase the risk of prostate, pancreatic, male breast and melanoma in gene variant carriers (Solomon, Das, Brand, & Whitcomb, 2012). Patient OCG14 has siblings diagnosed with ovarian, breast, stomach, and eye cancer (Figure 11B). Approximately 1-3% of stomach cancer patients have an inherited cancer predisposition syndrome, including HBOC (Petrovchich & Ford, 2016). There are

also some family members with unknown cancer diagnoses, an issue that is often found in self-reported pedigrees.

Gene	Consequence	Amino Acid	Exon	SNP ID	MAF	OBS
PIK3C2G	STOP LOST	*1446S	32/32	rs61757718	0.017	2
TP53I3	STOP GAIN	S252*	4/5	rs145078765	0.0016	2
TP53AIP1	FRAMESHIFT	Q22fs	3/4	rs141395772	0.007	1
TP53AIP1	STOP GAIN	S32*	3/4	rs140191758	0.0009	1
BCLAF1	STOP GAIN	E403*	5/13	rs61731960	0.007	1
BCLAF1	FRAMESHIFT	H847fs	2/13	rs140096922	0.0003	3
PPP1R15A	STOP GAIN	E160*	2/3	rs139708522	0.006	1
DOCK1	FRAMESHIFT	D248fs	8/52	rs768625958	N/A	1
NLRP1	FRAMESHIFT	R138fs	2/17	rs771551366	0.00007	1
PTH	STOP GAIN	K85fs	1/3	N/A	N/A	1
ANGPTL4	FRAMESHIFT	G275fs	6/7	rs747940485	0.0002	1
NOD2	STOP GAIN	W289*	4/12	N/A	N/A	1
GZMM	STOP GAIN	Q161*	4/5	rs200398398	0.014	1

Table 7: Candidate risk mutations in apoptosis genes

From left to right; Gene name, Consequence = modification due to the mutation, Amino acid = translated amino acid change due to nonsense (*) or frameshift (fs) mutation, Exon = location of truncation, SNP ID = dbSNP ID, MAF = gnomAD non-Finnish population minor allele frequency and OBS = number of patients in the HBOC cohort carrying the truncation.

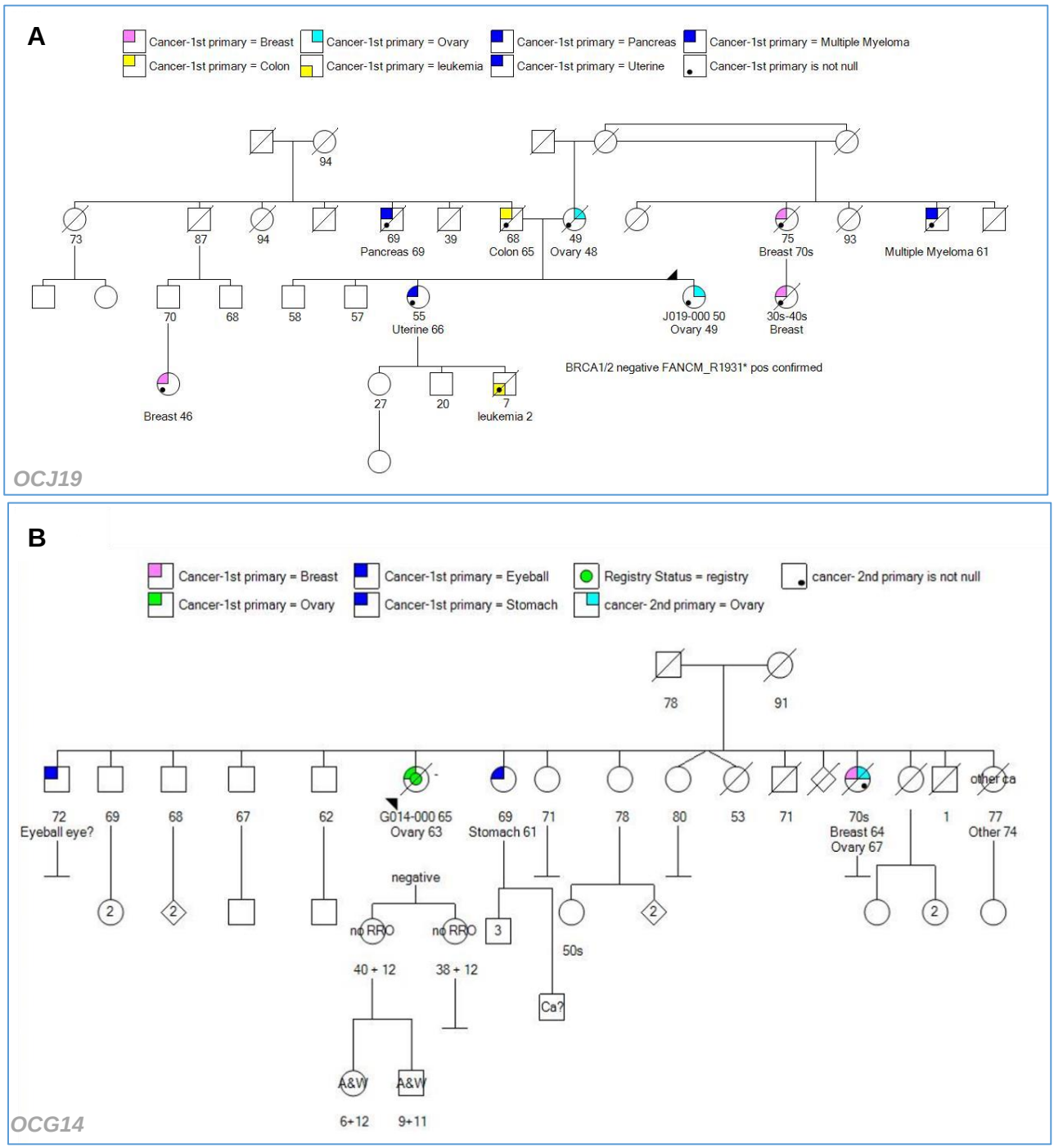


Figure 11*:* Patient pedigree for carriers of TP53I3-S252*

Patient OCJ19 (A) pro-band (arrow) carries TP53I3-S252* and pathogenic variant FANCM-R1931* and has a family history of BC and OVCA. (B) Patient OCG14 has a family history of BC and OVCA, no RRO = no intervention, A&W = alive and well.

As stated previously, the individuals in the HBOC cohort also carry several truncations in apoptosis genes. Several of the truncations in apoptosis genes were present in more than one of the 48 HBOC patients (Table 7). Of particular interest are 12 truncating mutations in 10 genes, including *TP53I3*, a TP53-regulated gene. *TP53I3* is involved in both DNA damage response and p53-mediate apoptosis. To date, there has been no germline variant in *TP53I3* associated with cancer. However, there have been several studies indicating that the gene affects the progression of a variety of cancers including outcome of breast (Zhang et al., 2015), NSCLC (M. Li et al., n.d.), colon (Park et al., 2017) and papillary thyroid (XU et al., 2015). TP53I3 overexpression significantly results in an increase in breast cancer survival (Zhang et al., 2015). Loss of TP53I3 expression promotes NSCLC, colon and papillary thyroid cancer (M. Li et al., n.d.; Park et al., 2017; XU et al., 2015).

Tumor Protein P53 Regulated Apoptosis Inducing Protein 1 (*TP53AIP1*) is a mitochondrial protein involved in p53-mediated apoptosis. TP53AIP1 induces the release of cytochrome *c* from the mitochondria and interacts with BCL-2, affecting TP53AIP1-mediated apoptosis through regulation of the mitochondrial membrane potential (Matsuda et al., 2002; Oda et al., 2000). Reduced expression of TP53AIP1 has been associated with increased progression of non-small cell lung cancer (Fang et al., 2019). Two unrelated patients carry either the Q22fs (rs141395772, MAF=0.007) truncation or the TP53AIP1 S32* (rs140191758, MAF=0.0009) variants in *TP53AIP1*. These two high impact mutations have been identified in cutaneous melanoma (Benfodda et al., 2018). There is conflicting data

as to whether the two truncations are associated with prostate cancer risk (Luedeke et al., 2012a; Wang et al., 2006).

Three unrelated patients carry the same nonsense mutation in BCL2 associated transcription factor 1 (*BCLAF1*) (rs61731960, E403* MAF=0.007) and another patient has a frameshift mutation (rs140096922, H847fs MAF=0.0003) in this gene. *BCLAF1* is a tumor suppressor that interacts with anti-apoptotic members of the BCL-2 family (Cuconati & White, 2002). The BCLAF1- E403* nonsense mutation found in our study was previously identified in four unrelated individuals of a larger population study of germline and somatic variants in ovarian cancer patients (Kanchi et al., 2014). An *in vitro* study found that colon cancer cells deficient in *BCLAF1* have decreased cell growth and colony formation. Colon cancer cells expressing BCLAF1 were injected into nude mice. Knockdown of BCLAF1 resulted in a decrease in tumor incidences and tumor formation (Zhou et al., 2014).

Another mutation over-represented in the cohort was the stop lost in Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2 Gamma (*PIK3C2G*), which belongs to the phosphoinositide 3-kinase (PI3K) family. The gene has a been associated with poor colorectal cancer patient outcome (A. Li et al., 2015) and promotion of type 2 diabetes mellitus (Daimon et al., 2008). In the case of colorectal cancer, low copy number variants in *PIK3C2G* resulted in an increased risk of reoccurrence and poor survival (A. Li et al., 2015). There are five SNPs in *PIK3C2G* significantly associated with HbA1c and/or insulin levels

(Daimon et al., 2008). Diabetes negatively affects the overall survival of ovarian cancer patients (Shah et al., 2014).

1.2 Functional Assessment of TP53I3- S252*

1.2.1 Loss of TP53I3 or TP53I3-s252* significantly decreases homologous recombination repair (HRR)

Many of the genes currently on the HBOC testing panel are involved in DNA repair mechanisms. Ovarian and breast cancer panel genes such as *ATM, BRCA1, BRCA2, CHEK2, PALB2, RAD51D, and RAD50* have important roles in the HRR mechanism. For repair of DSB with HRR, the ATM protein detects the break and phosphorylates numerous proteins including Chk2 and BRCA1 (Maréchal & Zou, 2013). A key regulator of ATM activation is the MRN complex which consisting of Mre11, Rad50 and Nbs1 (Maréchal & Zou, 2013; Symington, 2014). Rad51 interacts with BRCA1 and BRCA2 as well as PALB2 to replace RPA and form filaments on the DNA. To determine how *TP53I3* effects HRR, I used HeLa-DR-GFP cells. The pDR-GFP plasmid contains two inactive green fluorescent protein (GFP) allele, one has the SCE-1 endonuclease recognition site and the other is truncated. Upon transfecting HeLa-DR-GFP cells with pCBASce1, a DSB occurs and the truncated allele serves as a template for HRR of the lesion and activation of GFP (Pierce, Johnson, Thompson, & Jasin, 1999b). This assay was employed to determine whether loss of TP53AIP1, TP53I3, and TP53I3-S252* mutation effects HRR (Figure 12). Because BRCA1 is well established to have a primary role in the HRR mechanism, it is expected that knockdown of the endogenous protein will result in a significant reduction in HRR. Therefore, the

BRCA1 siRNA knockdown condition was used as a concurrent control for the transient co-transfections of the siRNA-test gene and the pCBASCE1 plasmid. Overall, knockdown of TP53I3 significantly reduced by an average of 20% (p-value ≤ 0.05) HRR. Attempting to rescue wildtype TP53I3 after knockdown was successful, similar experiments with TP53I3-S252* also succeeds, but to a lesser extent, indicating that the presence of the truncation negatively impacts its role in HRR (p-value ≤ 0.05). Although the impact of *TP53I3* (p-value ≤ 0.05) on HRR was not as significant as panel gene *BRCA1* (p-value ≤0.001), it has a similar effect to other panel genes like *CHEK2* and *ATM* (Lopes et al., 2019). The depletion of TP53AIP1 with siRNA did not result in a significant reduction in HRR. This is consistent with the primary function of the protein in p53-mediated apoptosis and maintaining the mitochondrial membrane potential (Oda et al., 2000).

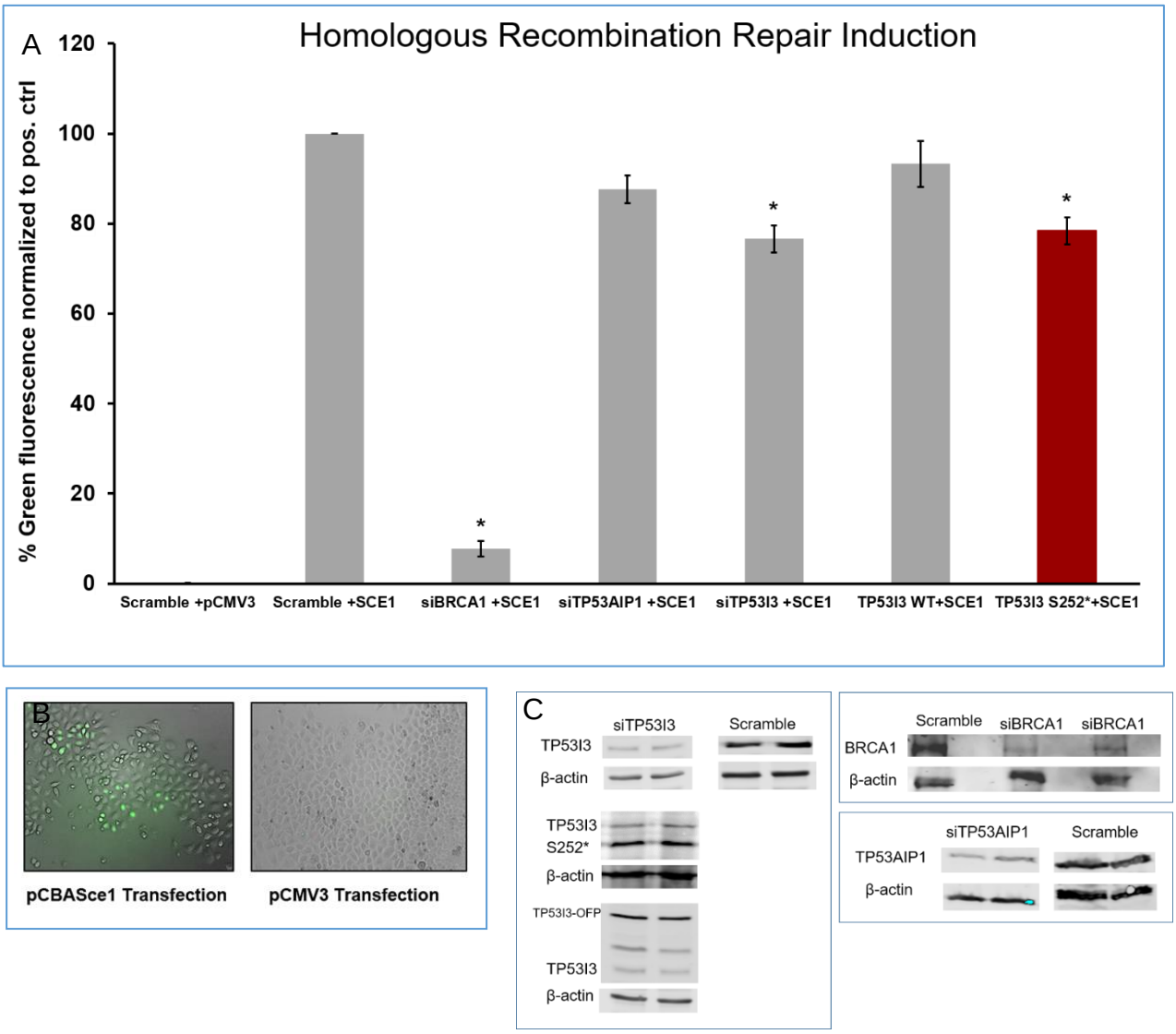


Figure 12: TP53I3-S252* significantly defects Homologous Recombination Repair

HeLa-DRGFP cells contain the p-DRGFP plasmid which has two inactive GFP alleles. The SCE-1 endonuclease recognition side is located in the first allele and the second allele is truncated. Introduction of pcBASce-1 to the cells causes a double stranded break in the first GFP allele and the second GFP allele acts a template for HRR of the lesion. Active GFP is a proxy for HRR having occurred in the cell. (A) Effect of siRNA knockdown of BRCA1, TP53AIP1, TP53I3 or TP53I3

with wildtype (TP53I3 WT) or mutant plasmid (TP53I3 S252*). (B) Fluorescent imaging of HeLa-DRGFP cells repairing DSB (after pCBASce1 transfection) with HRR compared to empty vector pCMV3. (C) Western blots for knockdown of TP53I3, TP53I3 rescue with wildtype, TP53I3 rescue with mutant, BRCA1 and TP53AIP1.

1.2.2 TP53I3 deficient cells are sensitive to mitomycin C, bleomycin and etoposide

Chemotherapy agents often given to HBOC patients were used, including mitomycin c (MMC), bleomycin and etoposide. To determine how TP53I3 and TP53I3-S252* affect drug response and cell death, the clonogenic assay was employed. Because *BRCA1* is a known HBOC panel gene, knockdown of the protein with siRNA in HeLa-DRGFP cells was used to ensure siRNA transfection could affect a drug response on cell survival. I determined the IC50 dosages to be 100 nM for mitomycin C (MMC), 1.5 µM for bleomycin and 4 µM for etoposide, administered for four hours. In the absence of these cytotoxic agents knockdown of BRCA1 and TP53I3 exhibited, respectively, 20% or 18% loss in the number of viable clones (p-value ≤ 0.05) (Figure 13).

As expected BRCA1-deficient HeLa cells exhibited significant additional sensitivity to MMC (p-value ≤ 0.001), bleomycin (p-value ≤ 0.001), and etoposide (p-value ≤ 0.001). In the case of etoposide, there is an almost complete absence of viable clones. Following a similar trend, loss of TP53I3 resulted in the cells being significantly more sensitive (p-value ≤ 0.05) to all three drug treatments (Figure 14). Approximately 60% of the cells survived after TP53I3 knockdown, compared to the 80% in the scramble control (p-value ≤ 0.05). About 80% of cells survived after knockdown of endogenous TP53I3 and rescue with the wildtype plasmid. Similar to the scramble control. Knockdown of TP53I3 with siRNA and attempted rescue with the TP53I3-S252* mutant plasmid resulted in a slight increase in surviving cells, 82%, compared to the scramble control.

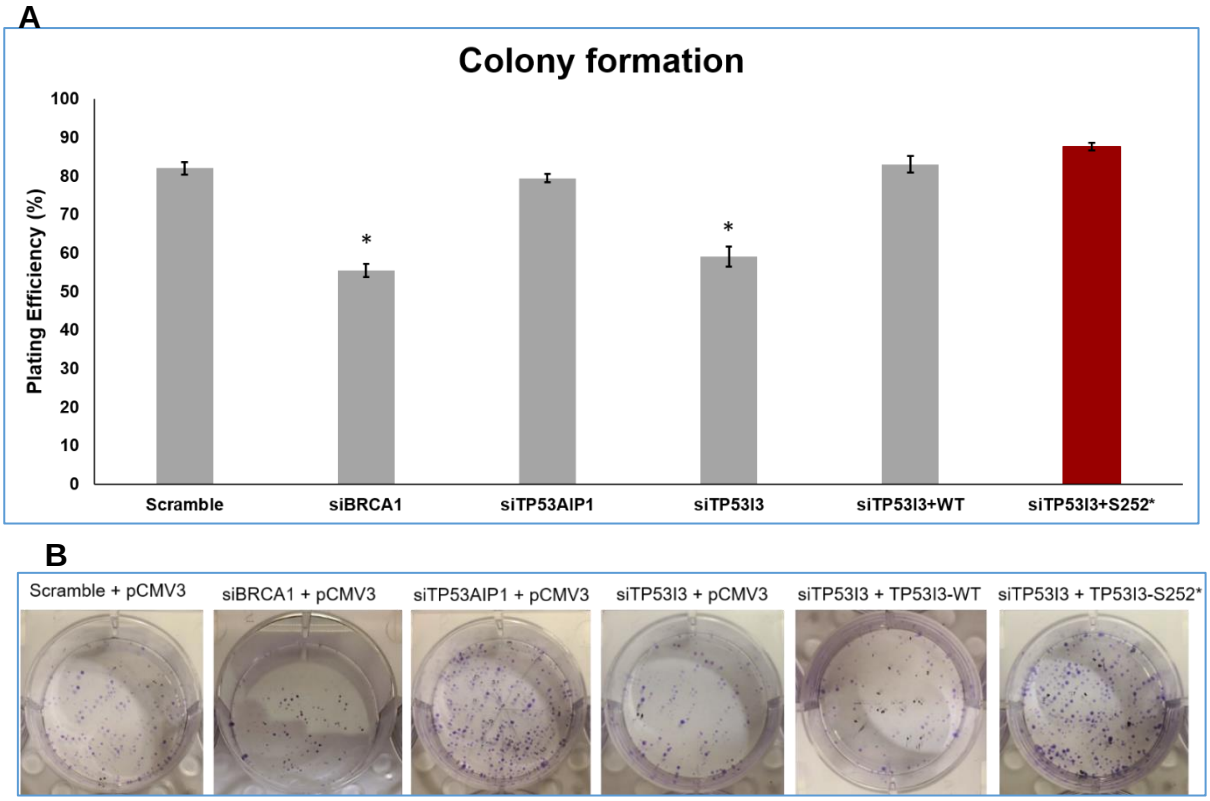


Figure 13: TP53I3-S252* and TP53AIP1 reduces cell death

(A) Plating efficiency after knockdown of proteins and introduction of empty vector (pCMV3), wildtype TP53I3 (TP53I3-WT), or S252* mutant (TP53I3-S252*). (B) Representative qualitative images of each conditioned cell type.

$$SF = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} \times PE}$$

***p-value ≤ 0.001
**p-value≤0.01
*p-value ≤ 0.05

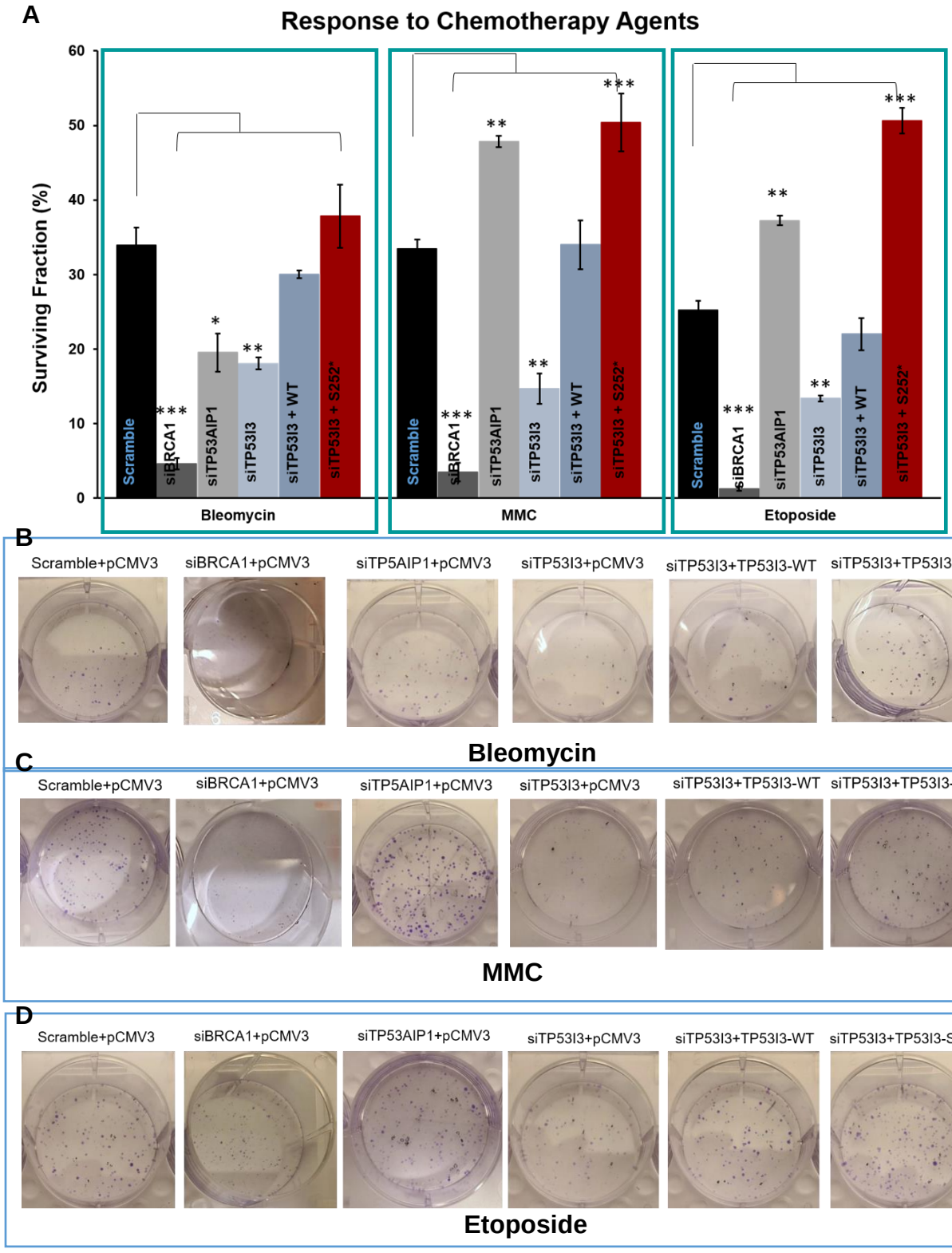


Figure 14: TP53I3-S252* resistant to OVCA chemotherapy

(A) Surviving fraction after treatment with bleomycin, MMC or etoposide incorporates plating efficiency. Representative images of surviving clones after knockdown of protein of interest and introduction of empty vector (pCMV3), wildtype TP53I3 (TP53I3-WT) or TP53I3 S252* mutant (TP53I3-S252*) after bleomycin (B), MMC (C) or etoposide (C) treatment.

1.2.3 TP53I3 S252* resistant to mitomycin C, bleomycin and etoposide

The depletion of endogenous TP53I3 and rescue with exogenous TP53I3-S252* causes in a slight increase in the number of surviving clones compared to the scrambled control (Figure 13). There was a significant increase in the number of surviving clones after treatment with MMC (p-value ≤ 0.001) or etoposide (p-value ≤ 0.001), displaying the resistance of the mutation to DNA damaging agents (Figure 14A, C and D). This phenomenon could be explained by the mutant cells multiplying and becoming more resistant to chemotherapy, gene amplification, disruption of drug transportation across the cell wall, an alternative method of DNA break repair or inactivation of the drug in the presence of the mutant. After bleomycin treatment, an increase in the number of clones is observed but not significantly.

1.2.4 TP53AIP1 response to mitomycin C, bleomycin and etoposide

The knockdown of TP53AIP1 did not significantly change the number of surviving clones compared to scramble control (p-value = 0.595, Figure 13). Similar to TP53I3-S252* cells, depleted TP53AIP1 HeLa-DRGFP cells were resistant to mitomycin C (p-value ≤ 0.01) or etoposide treatment (p-value ≤ 0.01) (Figure 14A, C and D). An opposite response was observed after bleomycin treatment, with a significant decrease in surviving clones (p-value ≤ 0.05) (Figure 14A and B). Bleomycin is a radiomimetic drug that inhibits the synthesis of DNA, indicating that a selective sensitivity to this drug by TP53AIP1 could provide a targeted therapy option.

1.2.5 TP53I3-S252* increases cell viability in the presence of oxidative stress

TP53I3-S252* may contribute to the resistance of chemotherapy often given to HBOC patients. To further explore this finding, the clonogenic assay was also utilized to determine how the mutation could affect cell death, due to TP53I3's role in ROS production and p53-mediated apoptosis. Hydrogen peroxide (H_2O_2) has a well-defined role in apoptosis induction and ROS production by increasing the formation of superoxide and hydroxyl radicals. Knockdown of TP53I3 in HeLa cells followed by a four-hour H_2O_2 treatment (125 µM), did not change colony formation compared to scramble control (Figure 15). However, in the presence of TP53I3-S252* after endogenous TP53I3 knockdown and exposure to H_2O_2, there was a significant increase in colony formation (p-value ≤ 0.001). This suggests the mutant disrupts a conversed region of the protein which, under wildtype conditions, is involved in activating cell death. The mutant is adjacent to conserved residues that make up the active binding site that interacts with QOR substrates for the formation of ROS and resulting in eventual apoptosis (Porté et al., 2009). Under conditions of TP53AIP1 knockdown and subsequent treatment, there was no change in the number of surviving clones compared to the scramble control.

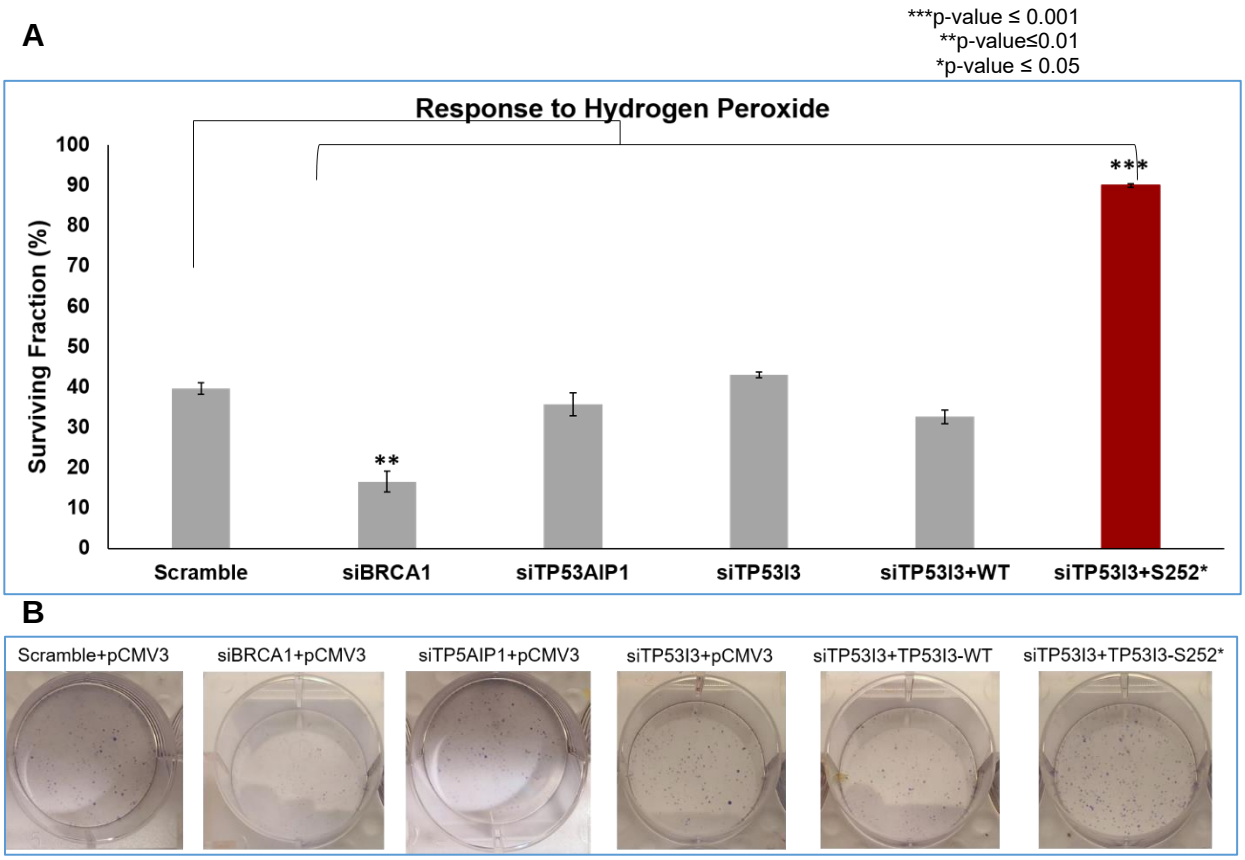


Figure 15: TP53I3 - S252* response to oxidative stress

(A) Surviving fraction after treatment with H_2O_2 expressed in terms of plating efficiency. (B) Representative qualitative images of each knockdown condition with empty vector (pCMV3), wildtype TP53I3 (TP53I3-WT) or S252* mutant (TP53I3-S252*).

1.2.6 TP53I3-S252* decreases ROS production in the presence of H_2O_2 or etoposide

The unique response of TP53I3-S252* in the clonogenic assay begs the question of what mechanism is preventing cell death. *TP53I3* has been described as a member of the quinone oxidoreductase gene family that can catalyze the formation of superoxide and hydroxyl ROS. The MitoSox probe allows for the quantification of the induction of ROS bodies in the mitochondria. H_2O_2 and etoposide had the most prominent effect on cell proliferation in the presence of TP53I3-S252*. Also, both superoxide producing H_2O_2 and topoisomerase II inhibitor etoposide have a well-defined role in ROS production (Wu & Yotnda, 2011). The response to etoposide was of particular interest because of it is frequently employed in the treatment of a many cancers, including those seen in HBOC high-risk subjects. HeLa cells depleted of TP53I3 or TP53AIP1 significantly increased the production of ROS after exposure to H_2O_2 (p-value ≤ 0.05, Figure 16). In contrast, there was a decrease in ROS production in the presence of TP53I3-S252* and treatment with H_2O_2 (p-value ≤ 0.05) or etoposide (p-value ≤ 0.05, Figure 16). This is likely due to the fact that the truncation interrupts 3 downstream nucleotides that are conserved and part of the active binding site that is needed form ROS in the presence of ortho-quinone (Porté et al., 2009)

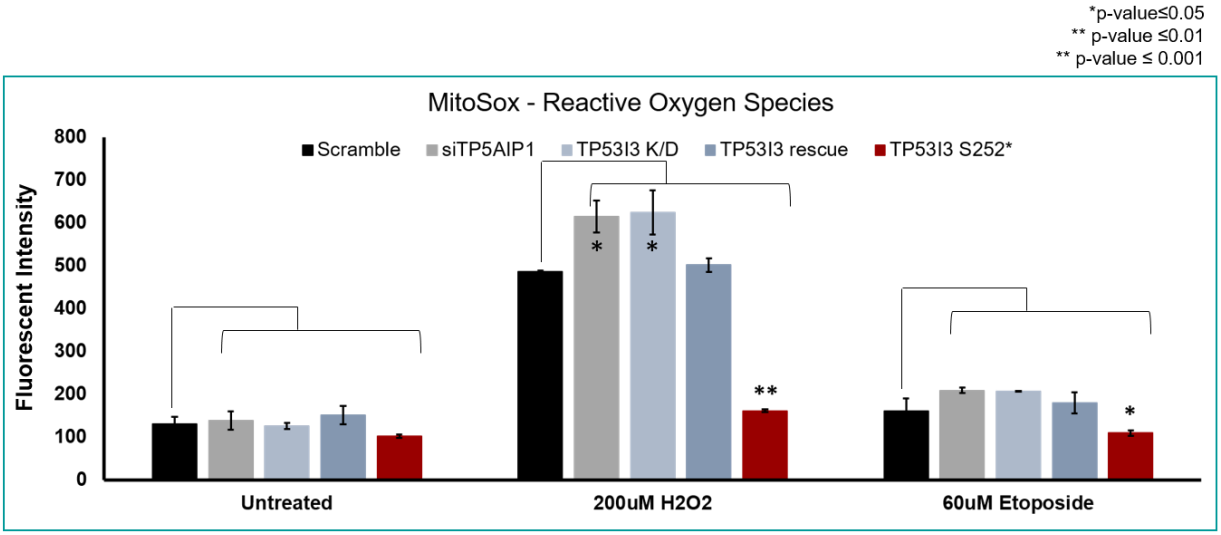


Figure 16: TP53I3-S252* disrupts mitochondrial ROS production

Proteins of interest were knockdown in HeLa cells (without DR-GFP) and/or rescued with TP53I3 wildtype or TP53I3-S252* followed by treatment with H_2O_2 or etoposide.

CHAPTER 4 – DISCUSSION

1.1 Whole exome sequencing is a more powerful tool for genetic risk assessment than a traditional candidate gene approach

Whole exome sequencing provides an enriched data set to identify disease risk loci. In the past decade, there has been an influx of larger and more inclusive gene panels, but the issue of low diagnostic yield and missing heritability remains (Chaudhry et al., 2017). As genetic panels are being continuously updated and with a rapid decrease in the cost of WES/WGS it would be beneficial to take a NGS approach to identify an individual's true genetic risk. This study has been able to demonstrate the benefits of utilizing WES data of high-risk hereditary breast and ovarian cancer patients. Variant assessment of WES resulted in the identification of clinically actionable mutations and post hoc assessment of candidate risk loci in DNA repair and cell cycle regulation. In addition, the findings in this thesis implicate apoptosis genes and the apoptosis pathway as a whole to be important in addressing the issue of missing heritability in HBOC.

Traditionally, patients undergo genetic panel testing involving high throughput sequencing of target genes. When using WES all genes on a panel test can be assessed and the data can be mined any time in the future when additional novel risk loci are identified. We found five clinically actionable genetic variants (Lopes et al., 2019; Stafford et al., 2017) in our cohort of 48 HBOC patients. An exciting finding among them was the FANCM R1931*(rs144567652) nonsense mutation. The *FANCM* gene is not on any cancer genetic panel, but there is a consensus amongst clinicians and researchers that it soon will be. *FANCM* is

specifically involved in the recognition of interstrand cross-links (ICL) and recruits *BRCA1* for downstream DNA repair by HRR (Whitby, 2010; Xue, Sung, & Zhao, 2015). Additionally, consistent with our findings (Chaudhry et al., 2017; Lopes et al., 2019; Stafford et al., 2017) the rs144567652 variant was found to increase the risk of triple-negative breast cancer (Figlioli et al., 2019; Peterlongo et al., 2015) and hereditary ovarian cancer (Dicks et al., 2017).

1.2 The necessity to assess cell death pathway genes for genetic risk assessment of cancer

From our OVCA cohort, there was an enrichment of rare, high impact mutations in apoptosis genes. Many of these truncated genes already have an association with a variety of cancers. Two unrelated OVCA patients carry either the rs141395772 or rs140191758 truncation in *TP53AIP1*. Both these SNPs have been previously associated with melanoma (Benfodda et al., 2018) and could be considered VUSs due to opposing reports on the effect of the mutations on prostate cancer (Luedeke et al., 2012b). There was an overrepresentation of truncations in *BCL2* in our cohort, with four of the patients carrying rs140096922 or rs61731960. Given *BCL2's* prominent role in the regulation of apoptosis and mitochondrial membrane potential, defects in its protein function can have detrimental defects to a variety of programmed cell death and DNA repair mechanisms. A total of 18 patients carried a truncated apoptosis gene, as well as the patients carrying 11 high impact DNA repair truncations. Apoptosis is a tightly controlled and conserved mechanism, and therefore programmed cell death pathways should not be overlooked when examining hereditary cancer risk.

1.3 TP53I3 has an important role in DNA damage and apoptosis

Two of the HBOC patients carry a rare, premature stop gain mutations S252* in *TP53I3*. Based on overexpression, knockdown or allele frequency,this gene has been associated with squamous cell carcinoma of the breast (Gorgoulis et al., 2004), squamous cell carcinoma of the head and neck (Guan et al., 2013), myeloid leukemia (Nomdedéu et al., 2004), lung (Gorgoulis et al., 2004) and invasive bladder (Ito et al., 2006). However, there has yet to be an example of germline mutational changes in *TP53I3* associated with disease risk in cancer patients. The gene is important to mechanisms associated with maintaining the integrity of DNA and cellular homeostasis. The mechanisms include *TP53I3* being transcriptionally regulated by *TP53* (A Kotsinas et al., 2010) for response to DNA damage (Lee et al., 2010) and apoptosis (Flatt et al., 2000). *TP53I3* is a quinone oxidoreductase involved in redox reaction to continuously produce ROS, vital to maintaining cellular homeostasis (Bolton & Dunlap, 2017; Oppermann, 2007; Porté et al., 2009). Therefore, mutations genes involved in the pathways can have detrimental consequences for cells to properly function (Jeggo, Pearl, & Carr, 2016). *TP53I3* is considered to be an upstream regulator of the DDR pathway, which, unlike*TP53*, are rarely altered. So the potential of a positive feedback loop of *TP53I3* being p53 transcriptionally activated and its role DDR will likely not be compromised because mutated p53 is enough to effect repair and cell death mechanisms. In short, loss of TP53I3 would have a negative impact on both normal and cancer cells (Athanassios Kotsinas et al., 2012). Therefore, mutations in this gene should be significant, whether they are truncations or missense mutations.

The TP53I3-S252* mutation is in the fourth exon and in its presence, the cells did not rescue DDR, demonstrated a decrease in ROS production, and a resistance to cell death in the presence of cytotoxic agents. The PIG3AS is a splice variant for *TP53I3* in which exon 4 is spliced out, resulting in an inactive protein. A functional consequence of when the splice variant is preferentially translated is the disruption of ROS formation. This is due to the absence of the C-terminus sequence, which is homologous with a QOR a subclass of the MDR superfamily (Nicholls et al., 2004). Additionally, the missense mutation of serine at position 151 to valine in the protein disrupts a conserved binding motif for $NADP^+$ (A/G)XXSXXG (Edwards et al., 1996). This makes the protein enzymatically inactive because of steric hindrance and not allowing $NADP^+$ to bind. In the presence of substrate quinone 1,2-NQ and cofactor NADPH, there was a decrease in binding affinity to the enzyme resulting in a loss of ROS production (Porté et al., 2009). The presence of the TP53I3-S252* mutation at the fourth exon resulted in a truncation due to a nonsense mutation. There are two possible explanations as to why the mutant exhibits opposing phenotypes of ROS production and cell death when comparing scramble siRNA to TP53I3 siRNA knockdown. Either there was a loss of the enzymatic ability to the TP53I3 due to the truncation or the mutation has a dominant negative effect that changes ROS production and cell death.

The location of the nonsense mutation is in position 252 of the TP53I3 protein sequence, where serine is altered to a premature stop codon. At the very least, this nonsense mutation found in *TP53I3* shortens the protein and it is nonfunctional. Due to the position of the truncation, this could be an issue for the

enzymatic ability of the protein. Recall that the residues involved in the active site include Leu255, Phe256, and Leu265 (Porté et al., 2009). *TP53I3* is disrupted by a nonsense mutation found in two of the OVCA patients which prevents the translation of the mRNA sequence after position 252. This means that three of the 15 residues necessary for the enzymatic activity of *TP53I3* are not present. This likely affects the active site conformation, preventing quinone substrates like 1,2-NQ from binding efficiently, if at all. Also, the ability to reduce free molecular oxygen to produce ROS species in the presence of a cofactor is severely diminished. This is supported by the fact that TP53I3-S252* cells had a significant decrease in mitochondrial ROS production in the form of H_2O_2, which can be reduced to hydroxyl radicals (Figure 16). Furthermore, I observed that in the presence of H_2O_2 the TP53I3-S252* cells experienced less cell death than wildtype TP53I3 (Figure 15). After the administration of chemotherapy drugs bleomycin, MMC or etoposide there was also a resistance to cell death in the TP53I3-S252* mutant cells compared to the scramble control (Figure 14). In the case of MMC and etoposide, there was a significant increase in the number of surviving cells in the presence of the nonsense mutation in TP53I3. To maintain normal cellular homeostasis, high levels of cellular ROS should lead to the activation of programmed cell death such as apoptosis. The inability to regulate apoptosis can result in the accumulation of old and damaged cells, which will could lead to tumorigenesis.

An alternative hypothesis could be that a dominant negative phenotype is observed in the presence of the TP53I3-S252* mutant. A dominant negative

phenotype is when the mutant outcompetes the wildtype. The dominant negative observed by the mutant in the presence of MMC, etoposide or H_2O_2 could be due to the disruption in the area of the protein responsible for oxidoreductase of superoxide ROS (Figure 14 and 15). This is further suggested by the reduction of mitochondrial superoxide ROS after H_2O_2 treatment in the presence of TP53I3-S252* (Figure 16). A particular surprising observation is an increase in ROS production after TP53I3 knockdown. The TP53I3 siRNA targets the 3'UTR, which is outside of the region that is homologous with the MDR superfamily. Since siRNA knockdown is not a complete knockout of protein function and there is no disruption of the residues involved in the conformation of the active site, the TP53I3 siRNA would therefore not hinder the effects of cells undergoing ROS production (Figure 16) and eventual cell death (Figure 14 and 15). The opposing effects on cell death and oxidative stress when comparing TP53I3 knockdown cells to TP53I3-S252* cells depletion of TP53I3 and TP53I3-S252* suggests a dominant negative pattern. Tumor suppressor genes p53 (Willis, Jung, Wakefield, & Chen, 2004) and *BRCA1* (Thangaraju, Kaufmann, & Couch, 2000) and *ATM* (Chenevix-Trench, 2002) genes are known to have dominant-negative mutations that result in carcinomas. However, due to the fact that TP53I3-S252* mutation is positioned adjacent to three amino acid residues necessary for the integrity and confirmation of the active binding site of quinone substrates, it is more likely that mutation is affecting enzymatic activity of the protein.

Chapter 5 – LIMITATIONS AND FUTURE DIRECTIONS

This study extended the scope of our previously published work to examine apoptosis associated genes role in cancer risk. A larger set of missense mutations in apoptosis associated genes were also identified and passed the computational filters applied including low MAF, high read quality, annotated as deleterious or possibly deleterious by Polyphen, Sift or SnpEff and found in clinical databases ClinVar, ACMG, COSMIC, TCGA, SNPEff. This is a dataset that can answer questions about polygenetic effect where there are multiple medium or low impact mutations observed in cancer patients. Additionally, pathway analysis of the HBOC patients against a normal population from a database such as 1000genome or TCGA would also shed light on other mechanisms that are often overlooked.

While the second portion of this project focused on the functional assessment of TP53I3-S252* mutation, there were several other high impact mutations found in apoptosis genes. Some of the mutations were found in multiple patients like those in TP53AIP1, BCLAF1, and PIK3C2G. These genes are involved in programmed cell death. To determine the effect of the truncations found in the OVCA patients, similar experiments to those used for TP53I3-S252* can be used. This would include, the clonogenic assay using DNA damaging and apoptosis cytotoxic agents, the Annexin V assay, and mitochondrial membrane potential-dependent assay.

To further support the hypothesis that TP53I3-S252* is enzymatically inactive, kinetic analysis of the mutant in the presence of a QOR like 1,2-NQ with

cofactor NADPH would be required. Comparing the effect of the truncation at amino acid to the known inactivating TP53I3-S151V would also be beneficial and to determine if the deficiency in substrate binding is similar. Testing to see how both intercellular and mitochondrial ROS is affected by both mutants will also help assess the severity of the nonsense truncation on oxidative stress response and downstream apoptosis. To directly determine the effect of TP53I3-S252* on apoptosis, the Annexin V assay can be utilized in the presence of similar cytotoxic drugs used in this study. It would be important to include a drug that is considered a positive control for apoptosis, such as camptothecin (CPT). CPT leads disrupts mitochondrial membrane potential, resulting in the release of cytochrome c release, caspase-3 activation, and ROS formation. TP53I3-S252* and TP53I3-S151V expressing TP53I3 knockout cell lines would also be advantageous for the above mentioned experiments to determine the heterozygote and homozygote effects of both mutants.

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ABSTRACT

ADDRESSING THE ISSUE OF MISSING HERITABILITY: THE IMPORTANCE OF APOPTOSIS IN HEREDITARY BREAST AND OVARIAN CANCER AND FUNCTIONAL ASSESSMENT OF TP53I3-S252*

by

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A quarter of all cases of ovarian cancer (OVCA) cases are associated with inherited risk. However, due to unclassified variants or variants of unknown significance (VUS), much of an individual hereditary risk remains unknown. We have established the importance of whole exome sequencing to answer the question of missing heritability. Five clinically actionable and eleven novel risk loci in the DNA repair and cell cycle regulation pathways were identified by *in silico* SNP assessment of a cohort of women diagnosed with OVCA, wildtype for BRCA1/BRCA2 and suspected to be hereditary due to family history of breast cancer/OVCA. Equally as important was the exploration and discovery of novel risk loci in the apoptosis pathway. A total of thirteen truncating mutations in apoptosis genes were found in over 35% of our patient cohort. The TP53I3-S252* premature stop gain was identified in two unrelated patients, one of whom also carries the clinically actionable truncating variant in FANCM. The proposed function of TP53I3 is its ability to maintain DNA damage response and is

transcriptionally activated by p53 to cause ROS induced apoptosis. It has been hypothesized to be a key gene that connects DNA repair mechanisms with downstream apoptosis as a quinone oxidoreductase. Additionally, two truncations in *TP53AIP1*, two in *BCLAF1* and one in *PIK3C2G* were identified in multiple individuals. This study highlights the importance of the often overlooked pathway of apoptosis. The importance of genetic assessment of the apoptosis pathway was further strengthened back the observation that TP53I3-S252* significantly decreases homologous recombination repair (HRR) and significantly resists response to chemotherapy drugs bleomycin, mitomycin c (MMC) and etoposide. Additionally, in the presence of oxidative stress from hydrogen peroxide and/or etoposide there was a reduction in the formation of reactive oxygen species, which is an important precursor to apoptosis. Lastly, the combination of computational and bench lab techniques allows for a streamlined assessment of an individual's true genetic risk of disease.

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Education

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Publications

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