Identification of the Functional Significance of a Novel Genetic Modifier of p53---Ovca1

A dissertation presented to

the faculty of

the College of Arts and Sciences of Ohio University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy

Shuhua Du

June 2011

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This dissertation titled

Identification of the Functional Significance of a Novel Genetic Modifier of p53---Ovca1

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ABSTRACT

DU, SHUHUA, Ph.D., June 2011, Biological Sciences

Identification of the Functional Significance of a Novel Genetic Modifier of p53---Ovca1

(94 pp.)

Director of Dissertation: Susan C. Evans

Our lab discovered novel genetic modifiers of \( p53 \): a recessive modifier of \( p53 \) (\( mop1 \)) locus from CE mice and a dominant modifier of \( p53 \) (\( mop2 \)) locus from the 129 mice. We identified the tumor suppressor gene \( Ovca1 \) as the modifier gene for \( mop2 \) locus. The polymorphic change from 321P to 321S on OVCA1 increases the expression level of Ovca1, increases the cell proliferation rate, alters the cell cycle, and leads to a different sub-cellular localization pattern of the OVCA1 protein.

In this study to further understand the functional significance of the particular polymorphic change of \( Ovca1 \), and to explore the cellular function of OVCA1, we found that the 321P OVCA1 protein is more stable than the 321S OVCA1 protein, which helps to explain the higher protein level of 321S OVCA1. In 129 kidney cells, OVCA1 (321P) colocalizes uniformly with eEF2 in the cytoplasm; while in CE kidney cells, OVCA1 (321S) has a punctate distribution in the cytoplasm, which does not perfectly colocalize with eEF2. This suggests that the polymorphic change might alter OVCA1’s function in the biosynthetic pathway of diphthamide on eEF2. Bioinformatics studies show that the polymorphic change might alter the secondary structure of the OVCA1 protein, thereby leading to a global tertiary structural change. We also found that OVCA1 is involved in BRCA1 signaling. Overexpression of \( Ovca1 \) leads to an increase in BRCA1 level in
MCF-7 cells. OVCA1 level decreases as an early response to UV stimulus in MCF-7 cells, and this decrease is primarily due to the dramatic decrease of cytosolic OVCA1, while the level of nuclear OVCA1 stays the same after UV treatment. Moreover, OVCA1 is post-translationally modified by phosphorylation in the cytoplasm in MCF-7 cells, and the phosphorylated OVCA1 decreases after UV as well. OVCA1 regulates cell cycle at G1/S checkpoint: overexpression of Ovca1 in A2780 cells leads to an increase in the S phase cell population, which is consistent with the increased pRB level. The effort to discover OVCA1 interacting proteins revealed that OVCA1 function might be associated with the cytoskeleton structure, the function of RNPs, and the apoptosis pathways.

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ACKNOWLEDGEMENT

At the completion of my graduate study, I would like to express my deepest appreciation to the individuals who have helped me with my PhD work and who have always being there to support me throughout this journey.

My advisor Dr. Susan C. Evans is the first and most important person I want to give my gratitude to. She has being a most helpful and patient advisor, from the very beginning of the research project, to all the guidance and discussions as the project goes on, to helping me practicing presentations and carefully and patiently correcting my manuscript and dissertation. She gave me the independence to choose and explore the research project I want to pursue, she gave me great guidance and inspirations as the project went on, and she gave me tremendous support and encouragement throughout my graduate study; to which I am greatly thankful.

I want to thank Dr. Donald Holzschu, Dr. Xiaozhuo Chen, and Dr. Lonnie Welch for being my committee members and bringing up sparkling questions in the committee meetings and the proposal defense. I want to give special thanks to Dr. Donald Holzschu, who has being a very helpful graduate chair, a challenging but inspiring committee member, and a great instructor to work with during my teaching work.

I want to thank my previous and current lab members who have helped me greatly and made my research experience very enjoyable. They are Dr. Yan Liu, Eroica Soans, and Elroy Fernandes. I want to give special thanks to Eroica Soans for her help in editing and formatting my dissertation, for being a wonderful labmate, and for the lots of fun we had together.
I also want to thank our collaborators who are involved in this project. They are Dr. Lonnie Welch and Dr. Dazhang Gu, who have helped with my bioinformatics studies, and Dr. Daniela Volke and Stefan Jezierski, who have helped with the Mass Spectroscopy analysis. I also want to thank Dr. Fabian Benecia for helping me with the FACS analysis.

Last but not least, I would like to give my deep appreciation to Yang Shi, my parents, and families. Thank you for always being there for me through all my ups and downs in these years. You have been my endless source of love and support, to which I am forever thankful!
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CHAPTER 1 INTRODUCTION

1.1 Tumor suppressors, p53 and its mutation

The concept of tumor suppressor genes arose around the late 1980s (Klein, 1987). There have been intensive studies on tumor suppressor genes ever since. The focus of early studies on tumor suppressor genes was on several heavily studied, well characterized tumor suppressors such as p53 and retinoblastoma sensitive protein (RB) (Levine, 1990). While these most well-known tumor suppressors remain under the spotlight of cancer research, there have been many new tumor suppressors or putative tumor suppressor genes that are identified in recent years. To give several examples, RNA binding motif-5 (RBM5) was found to be a putative tumor suppressor gene for lung cancer (Sutherland, Wang, & Robinson, 2010), AMPK functions as a metabolic tumor suppressor gene that controls metabolism and cell growth (Luo, Zang, & Guo, 2010), epithelial membrane protein 3 (EMP3) is emerging as a candidate tumor suppressor gene for solid tumors (Fumoto et al., 2009), and the Inhibitor of Growth (ING) proteins have been identified as biomarkers and new tumor suppressors that play role in tumor initiation and progression (Ythier, Larrieu, Brambilla, Brambilla, & Pedeux, 2008).

P53 is one of the most well studied tumor suppressor genes. It is situated at the center of a network of signaling pathways that are essential for cell growth regulation and apoptosis induced by a wide variety of intrinsic and extrinsic stresses (Vogelstein, Lane, & Levine, 2000; Vousden & Lu, 2002). P53 is highly conserved throughout evolution as a stress response gene (Junntila & Evan, 2009). P53 has been well identified as a tumor suppressor gene with potent transcription ability. As
demonstrated in Figure 1, in response to many regulatory factors after cell stresses, \( p53 \) becomes active and it induces a complex of pathways including cell cycle control, proliferation, DNA damage repair, and apoptosis (Amaral, Xavier, Steer, & Rodrigues, 2010; Lane, 1992). In normal cells, the half-life of \( p53 \) protein is very short, limited to minutes, due to its ubiquitin-dependent degradation by MDM2/HD M2 (Eischen & Lozano, 2009; Kubbutat & Vousden, 1998). But under extrinsic genotoxic stresses such as ultraviolet (UV), ionic radiation (IR) and intrinsic, non-genotoxic stresses like the activation of oncogenes (Debbas & White, 1993; Ruiz et al., 2008), nutrient depletion (Linke, Clarkin, Di Leonardo, Tsou, & Wahl, 1996) and hypoxia (Graeber et al., 1994; Zhao, Chen, & Du, 2009), the half-life of \( p53 \) protein is extended to hours. Different stresses trigger the activation of \( p53 \) to perform in distinct signaling pathways. The cyclin dependent kinase (CDK) inhibitor p21 is a critical target for \( p53 \) in the G1 cell cycle arrest pathway (Garner & Raj, 2008; Harper, Adami, Wei, Keyomarsi, & Elledge, 1993). Proteins GADD45 and 14-3-3σ are important mediators of P53-dependent G2 arrest pathway (el-Deiry, 1998; Zhu et al., 2008). P53-dependent apoptosis pathway includes protein mediators such as Bax and Fas/APO1 (Burns & El-Deiry, 1999). P53 is involved in the DNA damage repair pathway via transcription factor ERCC3 (X. W. Wang et al., 1994). Cytoplasmic \( p53 \) also regulates autophagy when under conditions like hypoxia and nutrient depletion (Tasdemir et al., 2008). P53 can also control tumor growth by inducing potent angiogenesis inhibitors such as thrombospondin 1 (Tsp1), thereby preventing angiogenesis (el-Deiry, 1998). The roles
of p53 in metabolism have gained more and more attention in recent years (Vousden & Ryan, 2009).

p53 is more commonly mutated in human tumors than any other single gene (Greenblatt, Bennett, Hollstein, & Harris, 1994). It is mutated in about 50% of human tumors (Soussi, Legros, Lubin, Ory, & Schlichtholz, 1994), and loss or mutation of p53 is strongly related to an increased risk to cancer (Vousden & Lane, 2007). Most of the mutations in p53 are missense mutations in its DNA binding domain. Mutant p53 can function as a dominant-negative inhibitor of wild-type p53. Although mutant p53 cannot completely abolish wild-type p53 activity, there might still be a small, but nevertheless important, shortcoming in wild-type p53 activity in cells in which both wild-type and mutant proteins are expressed (Vousden & Lane, 2007).

A proper amount of p53 protein is also important in normal embryonic development (Armstrong, Kaufman, Harrison, & Clarke, 1995; Chakraborty, Uechi, Higa, Torihara, & Kenmochi, 2009; Jones, Roe, Donehower, & Bradley, 1995; Sah et al., 1995). It was suggested that p53 has a role in embryogenesis from observations of developmentally regulated expression of p53 (Schmid, Lorenz, Hameister, & Montenarh, 1991).

Nevertheless, it was demonstrated later that p53-null mice are viable, which led to the opinion that p53 is entirely dispensable for normal embryonic development (Donehower et al., 1992). However, further analyses show that the majority of p53-null female mice have severe developmental abnormalities including profound neural-tube defects (Armstrong et al., 1995). Results from more studies show that in addition to impairing normal development, loss of p53 also leads to deficiency in the differentiation processes
and acts in the apoptosis processes in the developing embryo (Almog & Rotter, 1997; Choi & Donehower, 1999). In the last decade or so, it has also been shown that p53 influences embryonic development in the genetic context of deficiency in other genes. Most of these cases involve p53 deficiency rescuing embryonic lethal phenotypes which are caused by deficiency of another growth regulatory or DNA repair-related gene (Jones et al., 1995; Pani, Horal, & Loeken, 2002).

Figure 1. Schematic demonstration of major p53 signaling pathways.
1.2 Li-Fraumeni syndrome

Inherited mutations in p53 have been identified in individuals with Li-Fraumeni syndrome (LFS), a rare autosomal dominant disorder characterized by early onset of a wide variety of tumors in multiple family members (Evans & Lozano, 1997). This familiar cancer disorder was first identified by Dr. Fred Li and Dr. Joseph F. Fraumeni Jr. at the US National Cancer Institute in 1969 (Li & Fraumeni, 1969). The exact prevalence of LFS is unclear, however, according to the Genetics Home Reference, about 400 people from 64 families have this disorder, from one U.S. registry of LFS patients.

LFS is diagnosed if an individual has sarcoma before 45 years of age, who has at least two first-degree relatives who have been diagnosed with any type of cancer before 45 years of age (Evans & Lozano, 1997). The most distinct characteristics of LFS are the young age when patients develop cancer, the fact that the patients usually develop multiple primary tumors, and the high frequency of cancers within the family. Different from other familiar cancer syndromes, a wide spectrum of different cancer types occurs in LFS families. The most common cancers in LFS are sarcomas, breast, leukemia, brain, and adrenocortical carcinoma. Less frequent tumors include melanoma and cancers of the lung, pancreas, cervix, prostate, and ovary (Kleihues, Schauble, zur Hausen, Esteve, & Ohgaki, 1997).

The cause of the predisposition to various cancers in LFS patients remained elusive until 1990, when the link between inherited mutation in p53 and LFS was revealed (Malkin et al., 1990). Since then, germline mutations in p53 have been identified in approximately 70% of LFS families (Frebourg et al., 1995; Varley et al., 1997).
However, some LFS families clearly do not have p53 germline mutations nor is cancer predisposition genetically linked to the p53 locus (Evans et al., 1998). In this study by Evans et al., after using a variety of methods (including a transcriptional activation assay to exclude a mutation within the DNA-binding domain of p53, a single-stranded conformational-polymorphism analysis, and a linkage analysis to exclude potential mutations in the noncoding regions of p53) to accurately determine the p53 status in a large LFS kindred, the presence of a p53 germline mutation was excluded in a classic LFS family. These observations indicate that the alteration of at least one other gene leads to the development of cancers in some of these families. Indeed, individuals in LFS kindreds both with and without inherited p53 mutations develop diverse tumors at various ages. Examination of the clinical histories of patients with germline mutations in p53 show that the age of onset and types of tumor vary within and among families despite the inheritance of an identical mutation (Hwang, Lozano, Amos, & Strong, 2003; Kleihues et al., 1997). This variation further suggests variations in the genetic background, in addition to an inherited mutation in p53, contributing to the cancer phenotype in LFS kindreds. In 1999, CHECK2 was identified as a second gene involved in LFS (Bell et al., 1999). And there is another LFS predisposition locus that was mapped on human chromosome 1q23 (Bachinski et al., 2005).

1.3 Genetic modifiers

It has long been known that traits that are inherited in Mendelian fashion can vary in their phenotypes in subtle ways (Dipple & McCabe, 2000). Alternative alleles,
environmental factors and modifier genes are among the different causes of variable phenotypes for the Mendelian traits. The modification effects occur at different levels: from the transcription regulation of the target gene, to the intermediate phenotypes at the molecular or cellular levels, to end phenotypes at the organ, system or organismal levels (Nadeau, 2001). Genetic modification events are extremely common in humans as well as model organisms. It is actually relatively rare for the same phenotypes to present exactly the same way in all different genetic backgrounds. In most cases that involve genetic modifiers, the genetic basis for modification is totally unknown; in several of these cases, the genetic modifiers have been mapped; only in some cases, the candidate genes for the genetic modifiers are being evaluated; and in very few cases, the modifier genes have been identified and further studied (Nadeau, 2001).

Genetic modifiers are factors that alter the expressivity, penetrance, dominance modification and pleiotropy of other genes (Nadeau, 2001). Based on the nature of the phenotypic effect of the genetic modifiers, they may cause enhanced phenotypes, reduced phenotypes, novel phenotypes or wild-type phenotypes (Nadeau, 2001). Expressivity is defined as the extent to which a particular trait may be enhanced or reduced by a particular genotype. The trait may also be suppressed so that the mutants appear to be normal. Penetrance refers to the frequency of affected individuals out of the carriers of a specific genotype. In humans, the individuals with the genotype that makes him/her susceptible for a disease who nevertheless shows “unaffected” phenotype are evidence of reduced penetrance. If genetic linkage and mapping analysis of these unaffected
individuals suggest a role of another independent gene in the background, then the basis for the reduced penetrance is a modifier gene. With different genetic background, dominance of a specific genotype usually varies; for instance, a trait that is inherited in a dominant manner on one genetic background can be inherited in a recessive or semi-dominant manner on another genetic background. Pleiotropy refers to the various phenotypes resulting from the same single-gene mutation. Modifier genes of pleiotropy can lead to various combinations of traits on different background, and they can also result in novel phenotypes that exist exclusively on a particular genetic background.

One established example is a modifier gene for Cfr gene---cystic fibrosis transmembrane conductance regulator gene, in cystic fibrosis (Rozmahel et al., 1996). This genetic modifier is located on the mouse chromosome 7 with the corresponding location in the human chromosome 19q13.2–q13.4. Cfr-deficient mice that completely lack this modifier gene die soon after birth; mice heterozygous for the modifier survive until weaning; while mice homozygous for the modifier survive for several months longer than mice of the same Cfr genotype that lack the modifier.

1.4 Discovery of novel genetic modifiers of p53 involved in embryonic lethality

Previous studies showed that there are LFS families that do not have p53 germline mutations (Evans et al., 1998). In LFS kindreds both with and without inherited p53 mutations, diverse tumors occur in individuals at various ages. These data suggest that genes other than p53 are involved in the cancer predisposition and disease phenotype in these patients. As an approach to identify risk modifiers of p53 that influence
tumorigenesis in LFS families or perhaps to uncover a novel tumor suppressor pathway involved in the disease, p53-null mice (129/Sv; hereafter 129) were crossed to an inbred strain of mice (CE/J; hereafter CE) that has a low susceptibility to tumors and long tumor latency. Our lab reasoned that they could increase the penetrance and decrease the timing of tumor growth in the CE mice by incorporating the p53 null mutation. Only then could they map the tumor suppressor or modifier locus that appeared to segregate with the cancer phenotype.

The CE inbred strain of mice is susceptible to many of the tumors seen in LFS, such as ovarian and breast carcinomas, adrenocortical carcinomas, and sarcomas (Evans, Liang, Amos, Gu, & Lozano, 2004). Importantly, adrenocortical carcinomas are rare in the general population but frequent in LFS kindreds. All these tumors, however, develop with long latency and low penetrance in LFS.

Evans et al hypothesized that the wide range of tumor types with low incidence and long latency is due to a defect in CE mice in a tumor suppressor gene. In addition, it was hypothesized that in a cross with mice having a p53-null allele, the time until tumor onset would be decreased and the tumor incidence increased in the CE mice. An increase in tumor incidence has been shown in 129 mice that were crossed with mice null for p53. Ordinarily, they have a modest predisposition to testicular tumors (approximately 3%), but in the presence of a p53-null allele, the penetrance of testicular tumors increased to 50% and latency decreased (Donehower et al., 1995). Likewise, Balb/c mice are prone to mammary tumors. When p53 is heterozygous in Balb/c mice, the incidence of mammary tumors increases significantly (Blackburn et al., 2003).
By crossing CE mice with the p53-null 129 mice, it was observed that in the F2 generation a subset of p53 heterozygous and homozygous male and female mice die (Evans et al., 2004). Modeling of the data suggests that a dominant modifier \((mop2)\), in addition to a recessive modifier \((mop1)\), when combined with a p53 null allele, causes embryonic lethality (Evans et al., 2004). This genetic mouse model was used to identify a genetic modifier locus of p53, \(mop1\), on chromosome 11 centromeric to p53 (Evans et al., 2004). In a more recent study, a second locus was identified telomeric to p53 on mouse chromosome 11, \(mop2\), which genetically modifies p53 (Liang et al., 2008). Interestingly, loci telomeric to p53 have higher CE/CE recombination frequency than expected. This suggests that lethality depends on the presence of a 129 allele \((mop2)\).

NCBI, MIT and Jackson Laboratory databases were searched for genes within this region. The \(mop2\) locus contains a number of interesting genes, such as \(Ngfr\), \(Nog\), \(Rara\), and \(Ovca1\). The \(Ovca1\) gene was sequenced because it is related to embryonic development and has been attested to genetically modify cancer progression and embryonic development with p53 (Chen & Behringer, 2004). A proline to serine polymorphism in \(Ovca1\) at codon 321 was present in CE mice but not 129 mice when the sequence was aligned with those of C57BL/6J mice from the NCBI DNA library (Liang et al., 2008). The human counterparts of the \(mops\) may play a role in tumorigenesis and embryogenesis in humans.

1.5 \(Ovca1\) tumor suppressor gene

Ovarian cancer is a leading cause of gynecological malignancy (Chen & Behringer, 2004). \(Ovca1\) (ovarian cancer gene 1) is a tumor suppressor gene which shows
frequent loss of heterozygosity in breast and ovarian carcinomas (Schultz et al., 1996). Overexpression of \textit{Ovca1} induces growth suppression in ovarian cancer cell lines (Bruening et al., 1999). Moreover, loss of \textit{Ovca1} increases tumor incidence in p53 homozygous null mice (Chen & Behringer, 2004). \textit{Ovca1}, which is mapped to a highly conserved region on human chromosome 17p13.3, is reduced in tumors and inhibits cell growth of ovarian cancer cells, and cotransfection of cyclin D1 is able to override \textit{OVCA1}’s suppression of clonal outgrowth (Bruening et al., 1999).

Studies of \textit{Ovca1} mutant mouse embryonic fibroblasts (MEFs) show that \textit{Ovca1} regulates cell proliferation and embryonic development (Chen & Behringer, 2004). Inactivation of \textit{Ovca1} causes developmental abnormalities in the mouse embryos. Cell growth and cell cycle profile are altered in the \textit{Ovca1} mutant MEF cells: \textit{Ovca1}-knockout MEFs exhibit proliferation defects and decreased S phase population related to a reduction of Rb phosphorylation.

\textit{Ovca1} gene is evolutionarily conserved, with homologs existing from archaea to chimpanzee and human (Chen & Behringer, 2005). Mammalian \textit{Ovca1} is the counterpart of the yeast DPH2 gene, which encodes a diphthamide biosynthesis protein involved in diphtheria toxin (DT) resistance pathway. Diphthamide is a rare amino acid derived from a histidine residue within the translation elongation factor 2 (EF-2) through a series of post-translational modifications (Mattheakis, Sor, & Collier, 1993). Although the biochemical function of \textit{OVCA1} protein is not yet fully understood, it is shown that \textit{OVCA1} is a component of the biosynthetic pathway of
diphthamide on EF-2, revealed by a gene trap mutagenesis-based forward genetic approach (Nobukuni, Kohno, & Miyagawa, 2005). Interestingly, eEF1α2 is shown to be a putative oncogene in ovarian cancer (Anand et al., 2002), suggesting there might be correlation between the upregulation in the translational machinery and the cause of oncogenic transformation. Ovca1 expression was identified to be induced by the Brca1 (breast cancer 1, early onset) in breast cancer cell line MCF-7 cells using suppression subtractive hybridization (Atalay, Crook, Ozturk, & Yulug, 2002), suggesting that there are potential interactions between these two tumor suppressors, Brca1 and Ovca1. Four different subcellular localizations of OVCA1 protein with 321P polymorphism have been reported: cytoplasmic diffusion, nuclear and cytoplasmic diffusion, nuclear punctate, and peri-nuclear punctate (Chen & Behringer, 2001). The function related to these different localizations, however, is not yet deciphered. A yeast two-hybrid assay indicates that two highly conserved RNA-binding proteins RBM8A and RMB8B interact with human OVCA1, but the biological significance of this interaction is not yet clear (Salicioni et al., 2000).

1.6 Brca1 (breast cancer 1, early onset)

Breast cancer associated gene 1 (Brca1) is closely linked to familial breast cancers and germline mutations of Brca1 are found to predispose women to both breast cancer and ovarian cancer. Brca1 is also downregulated in sporadic breast cancers (Baldassarre et al., 2003). Although mutations in Brca1 gene are rarely found in sporadic breast carcinomas (Futreal et al., 1994), women who carry Brca1 mutations have 50 – 80%
risk to develop breast cancer and 30 – 40% chance of developing ovarian cancer by the age of 70 (Antoniou et al., 2003).

In mouse studies, disruption of Brca1 gene results in embryonic lethality, which is accompanied by embryonic growth retardation, cell cycle defects and genomic instability (Deng & Scott, 2000). In an animal model in which the Brca1 gene is mutated specifically in mammary epithelia tissues, the tumors develop in mutant glands at a low frequency after a long latency. Importantly, p53 status has shown to have important impact on the Brca1-related tumorigenesis: in Brca1 conditional mutant mice, introduction of a p53-null allele significantly increased mammary gland tumor formation (Xu et al., 1999) and made the mice more sensitive to oxidative stress (Cao et al., 2007).

BRCA1 is a tumor suppressor which plays important roles in DNA damage repair, development and tumorigenesis (Deng & Wang, 2003). However, the roles of BRCA1 in DNA damage repair are the most thoroughly studied (Huen, Sy, & Chen, 2010). Since its being cloned in 1994, BRCA1 is found to be involved in many cellular signaling pathways (Figure 2). Some well established cellular processes that BRCA1 is involved in include DNA repair, cell cycle checkpoint control, ubiquitylation, and chromatin remodeling (Narod & Foulkes, 2004).

Brca1 is involved in homologous recombination, a number of non-homologous end joining (NHEJ) and nucleotide excision repair (NER) DNA repair pathways (Huen et al.; Narod & Foulkes, 2004). In response to DNA double strand breaks, BRCA1
associates with RAD50 (Y. Wang et al., 2000) and RAD51 (Narod & Foulkes, 2004), then recruits a number of other proteins (including ATM which is a kinase that phosphorylates BRCA1 and other associated proteins) to form a large protein complex called BASC (BRCA1-associated genome surveillance complex), which is involved in the repair process by homologous recombination. *P53, RB* (retinoblastoma), and *p21* are involved in the downstream signaling of *Brca1*’s regulation on G1/S phase checkpoint (Figure 2). BRCA1 is not only a member of BASC, but also a member of another protein complex that contains chromatin remodeling factors such as SW1 and SNF (Narod & Foulkes, 2004). It is shown that BRCA1 directly interacts with the SW1-SNF complex and suggested that these proteins might be involved in the chromatin remodeling events that occur surrounding the DNA damage site.

1.7 Possible coordination of the tumor suppressors *Brca1, p53, Ovca1 and Hic1*.

Loss of heterozygosity (LOH) studies have revealed that human chromosome 17 is a hotspot for mutations and chromosomal aberrations in breast (Phelan et al., 1998) and ovarian cancers (N. Phillips, Ziegler, Saha, & Xynos, 1993; Wiper, Zanotti, Kennedy, Belinson, & Casey, 1998). Candidate oncogenes and tumor suppressors located on human chromosome 17 include *p53, Brca1, Her2/Neu, Nf1, Nm23, Hic1*, and two putative tumor suppressor genes *Ovca1* and *Ovca2* (Jensen & Helin, 2004).
Figure 2. Schematic demonstration of major BRCA1 signaling pathways (adapted from Fig. 2, Narod and Foulkes, 2004).

*Ovca1* is localized to human chromosome band 17p13.3 and *p53* is localized at 17p13.1, suggesting a linkage of these two genes and their potential synchronized allelic loss in tumors (Cornelis et al., 1994; N. J. Phillips et al., 1996). Another tumor suppressor *Hic1* (hypermethylated in cancer) is located next to *Ovca1* on 17p13.3, which is a hotspot for deletion in ovarian cancers (Chen & Behringer, 2001). *Ovca1* can modify p53-induced tumor formation: *p53* deficiency can rescue the *Ovca1* mutant MEF proliferation defects.
and partially rescue \textit{Ovca1} mutant embryonic phenotypes (Chen & Behringer, 2004). \textit{P53} activates expression of \textit{Hic-1} (Wales et al., 1995). Several similarities are found between \textit{Ovca1$^{-/-}$} and \textit{Hic1$^{-/-}$} embryos (Chen & Behringer, 2004). The tumor suppressor \textit{Brca1} is also located on human chromosome 17. \textit{Ovca1} is found to be one of the \textit{Brca1}-inducible genes by a study using suppression subtractive hybridization to identify genes induced by \textit{Brca1} in breast cancer cell lines (Atalay et al., 2002). Therefore, there might be coordination of the tumor suppressor loci \textit{Brca1}, \textit{p53}, \textit{Ovca1} and \textit{Hic1} on human chromosome 17 by undefined tumor-suppressive mechanisms during carcinogenesis.

1.8 **Research significance**

Understanding the molecular and cellular mechanisms by which genetic modifiers exert their influence on phenotypes will provide insights into developmental and physiological pathways that are vital for fundamental biological processes, therefore revealing novel targets for therapeutic interventions in human diseases (Nadeau, 2001).

Previous study in our lab using a mouse model identified two novel genetic modifiers of \textit{p53}, \textit{mop1} and \textit{mop2}, which cooperate with \textit{p53} in influencing embryonic development. Of special interest to us is the tumor suppressor gene \textit{Ovca1} within the \textit{mop2} modifier. \textit{Ovca1} acts as a genetic modifier of \textit{p53} and affects embryogenesis and tumorigenesis. The polymorphic change from proline to serine at amino acid 321 is associated with changes in \textit{Ovca1} mRNA and protein expression, protein localization, and cell cycle arrest.
Although previously identified as a putative tumor suppressor gene, the cellular functions of OVCA1 were not clearly understood. My study aimed to identify the functional significance of the particular polymorphic change of the Ovca1 gene, as well as to enhance our understanding of the general molecular and cellular functions of OVCA1. The results of this study shed light on the detailed mechanism of this particular genetic modification, how it affects protein structure, and how it interacts with the genetic modifier mop1, and p53. This study also reveals how Ovca1 and its modification interact with other important cellular proteins and signaling pathways, such as BRCA1 and the DNA damage repair pathway, the eEF2 and the protein synthesis pathway, and the RB and the cell cycle regulation pathway. In this study, we examined and compared characteristics of 321P OVCA1 and 321S OVCA1. We also studied the general functions of the 321S OVCA1 protein, employing standard molecular and cell biology methods as well as bioinformatics approaches.

The knowledge gained from this study and subsequent work about the OVCA1 network and the specific Ovca1 single nucleotide polymorphism (SNP) can contribute to understanding its possible roles in cancer predisposition, tumor spectrum, and penetrance in LFS as well as in sporadic cancers.
CHAPTER 2    MATERIALS AND METHODS

2.1 Cell lines and cell culture

Both CE/J (CE) mice and 129-Trp53\textsuperscript{tm1Tyj} (129) mice were obtained from The Jackson Laboratory. Primary cells from the kidney of CE and 129 mice were cultured in DMEM-RS cell medium (CyClone Pharmaceuticals) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin/Amphotericin B (P/S/A, CyClone Pharmaceuticals). Breast cancer cell line MCF-7 was purchased from ATCC. MCF-7 cells were cultured in DMEM cell medium with 5% Bovine Growth Serum (BGS) and 1% Penn/Strep/Amphotericin. Ovarian cancer cells line A2780 is a gift from Dr. Fabian Benicia (Ohio University). A2780 cells were cultured in DMEM cell medium with 10% FBS and 1% P/S/A. All the cultured cells were incubated at 37°C and 5.0% CO\textsubscript{2}.

2.2 Antibodies and immunoblotting

Rabbit polyclonal OVCA1 antibody (ab40733) and mouse monoclonal BRCA1 antibody (ab16780) were both purchased from Abcam. Mouse monoclonal pRB antibody (32G8) was purchased from Santa Cruz (sc-69790).

Ten percent SDS PAGE gel was used for electrophoresis. Fifty to one hundred micrograms of total protein was used for each sample. After adding 5ul of 4X protein dye to 15ul of each diluted sample, the samples were heated at 95°C for 5 minutes and then cooled before they were loaded on the gel. Gel electrophoresis was performed at 150 V in the stacking gel and 200 V in the resolving gel. The gel was transferred to Immobilon-P PVDF membrane at 0.05A for 1 hour using a semi-dry transfer device. After transfer, the
membrane was blocked in a solution of 5% milk/PBS-T (milk: non-fat dry milk powder; PBS-T: Phosphate Buffered Salien (PBS) plus 0.1% Tween-20) at room temperature for one hour. The primary antibody was diluted in 5% milk/PBS-T to the desired dilution (1:1000 to 1:2000 dilution was used for OVCA1 antibody, 1:100 dilution for BRCA1 antibody, and 1:1000 dilution for pRB antibody) and the membrane was incubated overnight at 4°C. Then the membrane was washed three times for 10 min each with PBS-T, and blocked with 5% milk/PBS-T for 15-30 minutes. The corresponding secondary antibody diluted 1:10,000 in 5% milk/PBS-T was added to the membrane and incubated for 1 hour at room temperature. Then the membrane was washed 4 times with PBS-T, 5 minutes each time. LumiGLO substrate reagent (Cell Signaling) was added to the membrane. After incubation for 5 minutes, the membrane was exposed to an X-ray film for 1 minute.

2.3 Protein stability assay

Cycloheximide (CHX) powder (CalBiochem, Catalog # 80059-086) was dissolved in 95% ethanol to a concentration of 100mg/ml upon arrival. When cultured CE and 129 kidney cells grew to the desired confluence (~ 80% density), 10 ml complete culture medium with 100 ug/ml CHX was added to each plate. Protein lysates were harvested at different time points after adding CHX: 8 hours, 16 hours, 24 hours, 32 hours and 48 hours. A plate with no CHX served as the negative control. Protein lysates were subjected to western blotting with OVCA1 antibody. Densitometry was performed on the film to calculate the relative intensity of each band on the Kodak In-VivoF image station.
2.4 Immunofluorescence

To grow cells, glass coverslips were treated as follows: 1M HCl for 1 hour, rinse in distilled water, 1M NaOH for 1 hour, rinse in distilled water, and then stored in 70% ethanol at room temperature. Cells were cultured on treated cover slips in plates containing complete medium until they reached the desired confluence (~60% density). After any further treatment was applied (e.g. UV treatment as in Section 3.6), the cells were fixed at the desired time point (e.g. 24 hours post-UV as in Section 3.6).

To fix cells, medium was removed from the plates containing coverslips and the plates were washed twice with PBS. Cells were then fixed for 5 minutes by adding cold 95% ethanol/5% acetic acid to the plates. After washing the plates twice with 1xPBS, the cover slips were removed with forceps and placed “cells-up” on a parafilm strip for a third wash with PBS. After aspirating the PBS, the cover slips were blocked in 2% chicken serum/PBS for 30 minutes at room temperature. A drop (~210 ul) of primary antibody with the desired dilution (1:1000 for OVCA1 antibody and 1:500 for eEF-2 antibody) in 2% chicken serum/PBS was put on the parafilm beside each cover slip. Cover slips were turned “cells-down” over the drop of antibody and incubated for 1 hour at room temperature. After two washes with PBS, coverslips were incubated with the secondary antibody (1:500 dilution) for 1 hour in the dark. After two final washes with PBS, the coverslips were mounted “cells-down” on slides using the Vectashield Mounting Medium with DAPI (Vector Laboratories). Cells were observed under an immunofluorescent microscope (Nikon Eclipse E600).
2.5 Bioinformatics studies

I-Mutant v2.0 (http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant2.0/I-Mutant2.0.cgi) is a web-based server for prediction of protein stability changes upon single amino acid mutations. 321P OVCA1 protein sequence was the input, the position of change was at the 321st amino acid, with the new residue Serine. Temperature and pH values were kept as default: 25°C and 7.

JPred software is a protein secondary structure prediction server run by the University of Dundee. It is available at http://www.compbio.dundee.ac.uk/jpred_v2/

Robetta is an *Ab initio* protein 3D structure prediction server, which was accessed at http://robetta.bakerlab.org/. 321P OVCA1 protein sequence in FASTA format was the input. The output structure in Rasmol model form was analyzed with Swiss-PdbViewer Deep View v4.0 downloaded from http://spdbv.vital-it.ch/.

2.6 Plasmids and transfection

*Ovca1* plasmid pcDNA3.0-*Ovca1* (321P) was a generous gift from Dr. Andrew Godwin (Fox Chase Cancer Center, Philadelphia, PA). An empty pcDNA 3.0 plasmid was used in all the transfection experiments as a negative control. Serum resistant ExpressFect transfection reagent was used for transfection. Cells were seeded and grown in normal growth medium to 60% to 70% confluence on 100mm plates. Six micrograms of *Ovca1* plasmid DNA was diluted in 300 ul serum-free and antibiotic-free medium and mixed gently. ExpressFect solution was warmed at 50°C for 3 minutes and mixed gently. Nine microliters to 18 ul warmed transfection solution was diluted in 300 ul serum-free and
antibiotic-free medium. The diluted transfection reagent solution was added into the diluted DNA solution and vortexed before incubation for 20 minutes at room temperature, allowing the formation of polymer/DNA complex. Six hundred microliters of polymer/DNA complex was added into each plate drop by drop while gently swirling the plate. Cells were analyzed 24 hours after transfection.

2.7 UV treatment

Culture medium was removed from the cells before UV treatment. Cells were then exposed to 30 J/m² UV, with the plate lid off in a TL-2000 Ultraviolet Translinker. Culture medium was added to the cells, then plates were returned to the incubator and analyzed after 24 hours.

2.8 Nuclear and cytosolic cellular fraction isolation

Nuclei EZ Prep nuclei isolation kit (Sigma) was used for the nuclear/cytosolic cellular fraction isolation. Briefly, cells (24 hours post-UV or non-UV treatment) were grown to about 90% confluence. After aspirating the medium, the plates were set on ice and washed with 10 ml ice cold PBS. Two milliliters of ice cold Nuclei EZ lysis buffer was added to each plate. Cell lysates were collected by thoroughly scraping each dish and transferring to a 15 ml centrifuge tube. After a brief vortex, the lysates were set on ice for 5 minutes, and then centrifuged at 500xg for 5 minutes at 4°C. The supernatant (cytoplasmic fraction) was carefully removed with a pipette and saved; the pellet (nuclear components) was resuspended and washed with 4ml of ice cold Nuclei EZ lysis buffer. The washed nuclear fraction was centrifuged again at 500xg for 5 minutes at 4°C. The
supernatant was carefully aspirated out and discarded, and the nuclear pellet was set on ice. Each nuclear pellet was resuspended in 200 ul of ice cold Nuclei EZ storage buffer. Both the cytoplasmic and nuclear fractions were immediately stored at -80°C.

2.9 Immunoprecipitation assay for finding OVCA1-interacting proteins

Protein A sepharose beads were used for immunoprecipitation. To prepare the protein A beads, 0.125 g of protein A sepharose was weighed and added to 1ml of protein lysis buffer. After vortexing, the beads were left overnight at 4°C. The beads were spun for 30 seconds at max speed (round per minute, RPM), and the supernatant was discarded. After washing the beads with 500 ul lysis buffer twice, equal volume of lysis buffer was added to the beads and the beads were stored at 4°C. The desired amount of OVCA1 antibody (1 ug of OVCA1 antibody was added to each 500 ug whole cell extract) was added to the resuspended beads in each eppendorf tube for each sample, and incubated overnight at 4°C on the rocker.

To reduce the false positive results and antibody contamination, we used the imidoester crosslinker DMP (dimethyl pimelimidate) to covalently crosslink OVCA1 antibody to the protein A beads. Briefly, after the overnight incubation of protein A beads and OVCA1 antibody, the beads were washed once with the SNNTE buffer (5% sucrose, 500 mM NaCl, 50 mM Tris/HCl, 5 mM EDTA and 1% NP-40). The beads were then washed twice with 10 volumes of 0.1M sodium borate (pH=9). Ten volumes of borate buffer was prepared with 20 mM DMP and stored at 4°C. After washing, the beads were spun down at 4°C, the supernatant was discarded, and beads were resuspended and incubated with
prepared borate/DMP at room temperature for 30 minutes on the rocker. The centrifugation, resuspension and incubation steps were repeated once more. Then the beads were spun down and washed twice with 10 volumes of 50 mM glycine (pH=2.5). After washing 4 times with SNNTE buffer, the beads-antibody-DMP complex was stored at 4°C as a 50% slurry.

Twenty microliters of the beads-antibody-DMP complex was added to each sample (5,400-5,600 ug total protein) and incubated at room temperature on the rocker for 1 hour. Then the samples were spun down for 1 minute at max speed, the supernatant was discarded, and the beads were washed 4 times with SNNTE buffer. Finally, 13ul of lysis buffer and 5ul of 4x protein loading buffer were added to each tube, heated at 95°C for 5 minutes and loaded on a gradient gel. The SDS-PAGE gel was stained by Denville Blue (Denville Scientific). As negative controls, cell lysates without antibody were handled similarly. The desired bands (present in the experimental lanes but absent in the corresponding negative control lanes) were marked and the gels were sent to Dr. Hoffman’s lab (University of Leipzig, Germany) for Mass Spectroscopy analysis.

2.10 Phosphorylation detection assay

Cultured MCF-7 cells were UV treated and total cellular protein was harvested at different time point post-UV: 4 hours, 12 hours, and 24 hours. Lysate from cells without UV treatment was used as the control. ExactCruz C (Santa Cruz) IP matrix was used for immunoprecipitation to prevent contamination with the IP antibody. To form the antibody-IP matrix complex, 50 ul of the suspended IP matrix, 2ug of the Phospho-Tyrosine antibody (PY99 or 2C8 from Santa Cruz), and 500 ul of PBS were added to an
eppendorf tube and incubated at 4°C on a rotator for at least one hour. The matrix was then pelleted via microcentrifugation at max speed for 30 seconds at 4°C. After discarding the supernatant, the pelleted matrix was washed twice with 500 ul PBS. Total cellular protein samples were then added to the pelleted matrix and incubated at 4°C on a rotator overnight. After incubation, IP matrix was pelleted by centrifugation and washed 4 times using SNNTE buffer. Then the samples were separated on a 10% SDS-PAGE and subjected to western blotting using OVCA1 antibody.

2.11 FACS cell cycle analysis

GFP plasmid pEGFP-N1 (4.7 kb) was a generous gift from Dr. Shiyong Wu (Ohio University). Cultured A2780 cells were co-transfected with GFP plasmid and OVCA1 plasmid with a ratio of 1:20 so that any cells that are positive for GFP should also contain Ovca1. Cells co-transfected with GFP plasmid and an empty pcDNA3.0 plasmid were used as the negative control. Twenty-four hours post-transfection, about 2x10^6 cells were harvested and washed with cold PBS. Cells were then resuspended in 200 ul cold PBS and vortexed. To a 15 ml centrifuge tube containing 4 ml of ice cold 70% ethanol, 200 ul cells in PBS was slowly added while constantly vortexing the centrifuge tube for rapid dispersion of the cells. Cells were fixed at -20°C overnight. The next day, cells were pelleted at 1,200 rpm (100g) for 10 minutes at 4°C. After carefully aspirating the supernatant, cells were resuspended in the Propidium Iodide master mix, which contains 100 ul of 100 ug/ml RNase, 200 ul of propidium iodide (50 ug/ml), and 2 ml of PBS. Cells were incubated at 37°C for 30 minutes prior to analysis.
The cells were analyzed by the flow cytometer FACSsort (Becton Dickinson) under the guidance of Dr. Fabian Benecia. In general, after appropriate setting of voltage and threshold using the CellQuest software (Becton Dickinson), we counted 20,000 cells and sorted them in a gated channel. Proper area on the scatter-plot was drawn which corresponds to the cells with green fluorescence. Then, a sorting of the gated cells (cells with GFP) was done based on the fluorescence produced by propidium iodide, which stains the DNA. The flow cytometer calculates the DNA amount in each of the cells with GFP, and groups the cells by their DNA amount into G1, S and G2/M phases. The raw data obtained from the FACSsort were then analyzed by ModFit LT v 3.2 software.
CHAPTER 3     RESULTS

3.1  321P OVCA1 protein has higher stability than 321S OVCA1 protein

Previous studies in our lab show that the specific Ovca1 polymorphism has effects on the expression level of Ovca1, at both the mRNA level and the protein level (Chapter One, Liang et al., 2008). To further understand the cause of the difference in the protein level, we looked at the half-life of the 321P OVCA1 and 321S OVCA1 protein in 129 and CE kidney cells, respectively. The result of this experiment would indicate if the higher level of 321S OVCA1 in CE cells are due to higher stability of 321S OVCA1. If 321S OVCA1 (CE) is more stable than 321P OVCA1 (129), the higher expression level of 321S OVCA1 could be the result of the longer OVCA1 protein half-life; if 321P OVCA1 (129) is more stable than 321S OVCA1 (CE), the higher expression level of 321S OVCA1 could only be explained by the higher CE Ovca1 expression at the mRNA level.

Cycloheximide (CHX) is a universal inhibitor of cellular protein synthesis. After applying cycloheximide to cultured cells, the synthesis of new cellular proteins is stopped, while the already-existing cellular proteins undergo their degradation processes. Therefore, the level of a particular cellular protein would be expected to decrease over time after the application of cycloheximide; the rate of decrease corresponds to the half-life of the particular protein. In our experiment to compare the stability of 321P OVCA1 (129) and 321S OVCA1 (CE), we applied the same concentration of cycloheximide to the cultured CE kidney and 129 kidney cells. After different time points, cycloheximide was removed from the medium, and total cellular proteins were harvested from the cells.
Western blotting experiments were carried out to detect the levels of OVCA1 in each sample.

Since there is no previous record about the half-life of OVCA1 protein, we used different time points in sequential experiments to define the half-life of the protein. In the first experiment, the decrease of OVCA1 proteins starts to become obvious when reaching 24 hours post-CHX treatment (Figure 3). Then, after extending the time points to 48 hours, we saw significant difference in the half-life of 321P OVCA1 (129) and 321S OVCA1 (CE) (Figure 4). From the results of these two stability assays using different time points, it is obvious that 321P OVCA1 (129) is more stable than 321S OVCA1 (CE). Protein densitometry was used to quantify the decreasing protein expression of 321P and 321S OVCA1 over time and is represented by the line graphs in Figure 5.

![Image of Western blot results](image-url)

Figure 3. OVCA1 stability assay. OVCA1 protein level in CE and 129 kidney cells at different time points (8h, 16h and 24h) after CHX treatment. 0 h is without CHX treatment. B-actin was used as a loading control.
Figure 4. OVCA1 stability assay. OVCA1 protein level in CE and 129 kidney cells at different time points (24h, 32h and 48h) after CHX treatment. 0 h is without CHX treatment. B-actin was used as a loading control.

Figure 5. Relative OVCA1 protein level in CE and 129 cells at time points (24h, 32h, and 48h) post-CHX treatment.
To explore the difference in 321S and 321P OVCA1 stability, we also conducted bioinformatics predictions using I-Mutant v2.0 (http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant2.0/I-Mutant2.0.cgi), which predicts protein stability change upon a single point mutation. The *in vitro* experimental data about the change of OVCA1 protein stability are consistent with the *in silico* bioinformatics predictions. I-Mutant v2.0 prediction results are displayed in Table 1.

When substituting Pro with a Ser at the 321st amino acid position, the protein stability free energy change (DDG), which is the unfolding Gibbs free energy value of the 321S OVCA1 protein minus the unfolding Gibbs free energy value of the 321P OVCA1 in Kcal/mol, is negative (DDG <0) (http://gpcr2.biocomp.unibo.it/~emidio/I-Mutant2.0/I-Mutant2.0_Details.html). This suggests that under physiological conditions, change from 321P OVCA1 to 321S OVCA1 decreases the stability of the OVCA1 protein. Thus, 321P OVCA1 (129) might be more stable than 321S OVCA1 (CE).

### 3.2 OVCA1’s colocalization with eEF2 in CE and 129 kidney cells

Earlier studies of *Ovca1* show that the expression of OVCA1 is reduced in tumors and exogenous expression of OVCA1 inhibits the growth of ovarian cancer cells (Bruening et al., 1999). *Ovca1* gene knock-out mice show that *Ovca1* regulates embryonic development, cell proliferation, and tumorigenesis (Chen & Behringer, 2004). However, the biochemical mechanism by which *Ovca1* carries out its cellular function is not fully understood. A study using gene trap mutagenesis-based forward genetic approach reveals
that OVCA1 is a component of the biosynthetic pathway of diphthamide, a unique amino acid on eukaryotic elongation factor 2 (eEF2) (Nobukuni et al., 2005). This suggests that the biochemical functions of OVCA1 might take place on the protein level, and the tumor suppressing functions of Ovca1 might be associated with protein synthesis.

Table 1

*Prediction of protein stability changes upon point mutations (by I-Mutant v2.0)*

<table>
<thead>
<tr>
<th>Position</th>
<th>WT</th>
<th>NEW</th>
<th>DDG</th>
<th>Stability</th>
<th>RI</th>
<th>pH</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>321</td>
<td>P</td>
<td>S</td>
<td>-1.01</td>
<td>Decrease</td>
<td>5</td>
<td>7.0</td>
<td>25</td>
</tr>
</tbody>
</table>

Note:  
WT: Amino acid in Wild-Type Protein  
NEW: New Amino acid after Mutation  
DDG: DG (New Protein)-DG (WildType) in Kcal/mol  
DDG<0: Decrease Stability  
DDG>0: Increase Stability  
RI: Reliability Index  
T: Temperature in Celsius degrees  
pH: -log[H+]
Chen *et al* shows four distinct sub-cellular localization patterns of OVCA1 in COS-7 cells: diffuse cytoplasmic staining, both cytoplasmic and nuclear staining, nuclear punctate staining, and perinuclear staining (Chen & Behringer, 2001). Our previous study demonstrates that 321P OVCA1 is diffused within the cytoplasm in various tissues from 129 mice, whereas 321S OVCA1 has a punctate staining pattern in different tissues from CE mice (Liang et al., 2008). Therefore, the different sub-cellular localizations of OVCA1 might be associated with different functions of OVCA1 in both transcription and translation (Chen & Behringer, 2005). Furthermore, the 321P/S polymorphism in OVCA1 protein might alter its interaction with other OVCA1 associated proteins thereby affecting its localization.

To better understand the interaction between OVCA1 and eEF2, and also to gain insights as how the 321(326 in human) Pro/Ser polymorphism of OVCA1 might affect OVCA1’s involvement in the eEF2 pathway, we determined whether the two proteins colocalized in the cell. After fixation of cultured kidney cells from CE and 129 mice, primary antibodies for OVCA1 and eEF2 were added, then two secondary antibodies which were tagged with FITC and TexasRed respectively, were used. The merged images show that OVCA1 and eEF2 partially colocalize in the cells (Figure 6).

In 129 kidney cells, OVCA1 (321P) co localizes uniformly with eEF2 in the cytoplasm but there is also distribution of OVCA1 in the nucleus. In CE kidney cells, OVCA1 (321S) has a punctate distribution, predominantly in the cytoplasm, which does not perfectly colocalize with eEF2. This difference suggests that the 321P/S polymorphism in
OVCA1 might alter OVCA1’s function in the biosynthetic pathway of diphthamide on eEF2, leading to different colocalization patterns.

![Subcellular colocalization of eEF2 and OVCA1 proteins in CE and 129 cells.](image)

Figure 6. Subcellular colocalization of eEF2 and OVCA1 proteins in CE and 129 cells. eEF2 is stained green and OVCA1 is stained in red. In the merged images, yellow stains show the cellular colocalization of eEF2 and OVCA1. Arrows show areas where OVCA1 and EF2 do not colocalize in these CE cells.

3.3 Bioinformatics studies on OVCA1’s secondary and tertiary structures

Since we found several functional differences between 321P OVCA1 and 321S OVCA1, we hypothesized that the single nucleotide polymorphism (SNP) of Ovca1 is critical and might alter the function of the protein via the alteration of its structure. We employed bioinformatics approaches to understand the influence of the OVCA1 polymorphism
(321P/S) on both the secondary and tertiary structures of this protein. Since proline and serine are two amino acids with distinct properties, this switch might induce secondary structure changes, and furthermore, alter the global 3-D structure of this protein. Since protein functions are closely related to its structure, it is not surprising that one amino acid substitution might result in dramatic functional changes.

We used the JPred software tool (http://www.compbio.dundee.ac.uk/jpred_v2/) for the prediction of the OVCA1 protein secondary structures. The prediction result is listed in Table 2. Each letter represents the secondary structure status of the specific amino acid at that position (“H” stands for “helix”, “C” stands for “random coil”, and “E” stands for “strand”). For example, the first random coil runs from the 8th amino acid to the 26th amino acid in OVCA1, and the second random coil runs from the 30th to the 33rd amino acid.

The amino acid sequences of 321P OVCA1 and 321S OVCA1 were inputted into the software tool independently. As we can see from Figure 7, the secondary structure status in close proximity to amino acid 321 (indicated in blue), does not change with the polymorphic change: it is the first amino acid within a random coil in both predictions. But interestingly, we found that there are five other positions (indicated in red) where the secondary structure status differs between 321P OVCA1 and 321S OVCA1 (Figure 7). The prediction result implies that this amino acid substitution might induce structural changes at other locations within the protein, thereby inducing a global protein structure change.
Based on the secondary structure prediction result, we hypothesized that this polymorphic change might lead to 3-D structure change of the protein. Thus, we further explored different protein 3-D structure prediction tools to test our hypothesis. There are three major categories of protein 3-D structure prediction tools: Homology modeling, Threading, and \textit{Ab initio}. The first two methods are based on homology analysis between the subscribed protein and the already-existing proteins in the Protein Data Bank (PDB). Those proteins in PDB have their 3-D structures obtained by X-ray crystallography or NMR spectroscopy. While the third method, \textit{Ab initio}, predicts 3-D structures from scratch, by first forming local structures amino acid by amino acid, and then aligning these local structures to form the global structure.

For our purpose of predicting and comparing 321P and 321S OVCA1 structures, only the \textit{Ab initio} methods are applicable, because 1) there are no proteins in the PDB that are similar enough to OVCA1 that can be used for predicting OVCA1’s structure using homology methods; 2) since we are predicting two proteins that only differ at one amino acid, using homology methods would result in exactly the same structure.

Among the few \textit{Ab initio} methods, the Rosetta method is the most successful in the 7\textsuperscript{th} CASP (Critical Assessment of Techniques for Protein Structure Prediction) competition (http://predictioncenter.org/casp7/). Although it is quite applicable for predicting the structure of small proteins, it takes extensive time (sometimes more than a year) for predicting large protein structures since it requires vast computational resources.

We submitted 321P OVCA1 sequence to the Robetta server (http://robetta.bakerlab.org/), and viewed the Rasmol model obtained two months after submission with
DeepView/Swiss-PdbViewer. Figure 8 is an image showing the 3-D structure of this protein. In this image, the proline at the 321st position is highlighted in red. As we can see from this image, the proline lies in a connecting position that links the two subunits of the protein. Given its unique position in the 3-D structure of the whole protein, it is highly possible that an amino acid switch at the 321P location would affect the global structure.

In fact, the 3-D structure corresponds with the 2-D structure in Table 2 very well: 1) the 321st amino acid is at the beginning of a coil; 2) the five positions of secondary structure changes are in close proximity to the 321st amino acid, although scattered in the three subunits in the 3-D image.

In Figure 8, the 321P together with the two adjacent amino acids in light blue (322 Glu and 323 Ile) form a loop that is buried inside of the global protein structure and “faces” the helix structure in purple blue (179 Ile to 194 Ala). The 3-D structure of 321S OVCA1 (Figure 9) is altered in comparison to the 321P protein. Instead of being buried inside, the loop where 321S (red) is located is on the surface of the global protein structure, and dissociated from the same helix (in purple blue).
Secondary structure

Proline
- CEEEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC

Serine
- CEEEEECCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC

H: helix
E: strand
C: random coil

Figure 7. Predicted secondary structures of 321P OVCA1 and 321S OVCA1 (by JPred).

The 321st amino acid position is highlighted in blue, and the 5 places where the secondary structures differ are indicated in red.
Figure 8. Predicted 3-D structure of 321P OVCA1 by Robetta server. The proline 321 is highlighted in red. Two adjacent amino acids (322 Glu and 323 Ile) are shown in light blue. Helix forming amino acids from 179 Ile to 194 Ala are shown in purple blue. Only the backbone atoms are shown.
Figure 9. Predicted 3-D structure of 321S OVCA1 by Robetta server. Serine 321 is highlighted in red. Two adjacent amino acids (322 Glu and 323 Ile) are shown in light blue. Helix forming amino acids from 179 Ile to 194 Ala are shown in purple blue. Only the backbone atoms are shown.

3.4 Overexpression of Ovca1 leads to increase in BRCA1 level in MCF-7 cells

In a previous study by Atalay et al (2002), Ovca1 was found to be a downstream gene of Brca1: ectopic expression of Brca1 in MCF-7 cells increased the Ovca1 transcripts level. BRCA1 is a transcription factor that has multiple downstream targets. This suggests that Ovca1 is one of the target genes transactivated by BRCA1. But since it has been suggested
that OVCA1 has multiple functions both in translation and transcription, we further investigated the effect of introducing exogenous \textit{Ovca1} into MCF-7 cells. If OVCA1 has effects in transcriptional regulation, the overexpression of \textit{Ovca1} should affect the expression level of BRCA1.

We transfected MCF-7 (326S OVCA1) cells with pcDNA3.0-OVCA1 (321P) plasmid and with pcDNA3.0 empty plasmid as the mock treatment. Western blotting for BRCA1 was performed using total cellular proteins harvested 24 hours after transfection. With β-actin as the loading control, it is obvious that overexpression of \textit{Ovca1} significantly increased the level of BRCA1 (Figure 10).

We previously showed that transfecting 321S OVCA1 into 129 (321P) cells changes the localization of OVCA1 from diffused to punctate (Liang et al., 2008). However, the negative control was not included in this study. As a pseudo control to determine whether the effects observed in the transfection experiments in MCF-7 cells were due to the polymorphic change or just increasing the dose of OVCA1, a negative control for the original observation by immunofluorescence was done in 129 cells. 321P OVCA1 was transfected into 129 (321P) cells, and the localization of OVCA1 remained diffused in the cell (data not shown). This suggests that in the transfection experiments, where 321P OVCA1 transfected into MCF-7 (326S) caused BRCA1 increase are likely due to the polymorphic change, not merely an increase in the amount of OVCA1 expressed.

In conclusion, BRCA1 transactivates \textit{Ovca1} gene, and OVCA1 protein can positively influence the expression of \textit{Brca1} as well, possibly through some transcriptional regulation mechanism.
<table>
<thead>
<tr>
<th>Total Protein</th>
<th>50 ug</th>
<th>75 ug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>OVCA1</td>
<td>Mock</td>
</tr>
</tbody>
</table>

Figure 10. Expression of BRCA1 and OVCA1. Overexpression of *Ovca1* increases the protein level of BRCA1 in MCF-7 cells.

### 3.5 OVCA1 level shows an early response to UV stimulus in MCF-7 cells

A major mechanism of BRCA1 is that it is directly involved in the DNA damage repair process (reviewed by Huen *et al.*, 2010). To further understand the interaction between BRCA1 and OVCA1, we investigated the influence of ultraviolet radiation (UV) on the OVCA1 status in MCF-7 cells. We started with testing the OVCA1 level at different time points after UV treatment. MCF-7 cells were treated with UV, and total proteins are harvested at 0 hour (control, no UV treatment), 2 hours, 4 hours, 8 hours, 12 hours and 24 hours after UV. Western analysis was performed using OVCA1 antibody and also BRCA1 antibody (as a positive control). B-actin was used as loading control.
As we can see in Figure 11, OVCA1 protein level shows an early response to UV: by two hours after UV treatment, the OVCA1 level decreased significantly then remained unchanged through 24 hours. This indicates that OVCA1 levels are affected by UV and that this response is relatively early. We also noticed that BRCA1 level increased two hours after UV treatment then returned to the same basal level as the non-UV control. Therefore, it is possible that OVCA1 is involved in the BRCA1 DNA damage-repair pathway at an early time point after cellular insult like UV.

Figure 11. Expression of BRCA1 and OVCA1 after UV treatment. Western analysis was done to detect the BRCA1 and OVCA1 protein level at different time points (2 hour, 4 hour, 8 hour, 12 hour and 24 hour) after UV treatment in MCF-7 cells. Cells without UV treatment (0 hour) were used as control.
3.6 The effect of UV on the subcellular localization of OVCA1 protein in MCF-7 cells

Since it is suggested that OVCA1 has a role in both transcription and translation, we thought it interesting to investigate the protein in both the nuclear and the cytoplasmic compartments. Following the last experiment, we wanted to know in which compartment the UV-induced decrease of OVCA1 takes place, and also the relationship between cytosolic and nuclear OVCA1 protein.

We investigated the effect of UV treatment on OVCA1 protein by studying the subcellular localization of OVCA1 after UV treatment by immunofluorescence. MCF-7 cells were treated with UV under the same condition as in section 3.5. Immunofluorescence was performed 24 hours post-UV. Cells without UV treatment were used as the control.

As shown in Figure 12, OVCA1 is located primarily in the cytoplasm of the MCF-7 cells without UV treatment. Although the cell number decreased significantly 24 hours after UV treatment, it is still apparent that cytoplasmic OVCA1 decreased dramatically. Since the two images of OVCA1 staining in UV and non-UV cells were taken under the same exposure time, they can also be used to semi-quantitatively compare the level of OVCA1.

As we can see, there is significant decrease of overall cellular OVCA1 with UV treatment, which is exclusively due to the dramatic decrease in cytoplasmic OVCA1.

Western analysis of cytoplasmic and nuclear fractions was done to confirm the immunofluorescent experiment. MCF-7 cells were treated with UV under the same condition as in section 3.5. We used a nuclei extraction kit to extract the cytoplasmic proteins and nuclear proteins 24 hours post UV treatment. With UV treatment, cytosolic
OVCA1 decreased dramatically, while the nuclear OVCA1 remains almost the same (Figure 13). This suggests that the decrease of cytosolic OVCA1 accounts for the majority of the overall decrease in OVCA1 protein after UV treatment in MCF-7 cells.

Figure 12. Subcellular localization of OVCA1 after UV treatment. Subcellular localization of OVCA1 (green) in the control and UV treated MCF-7 cells. DAPI (blue) was used to stain the nucleus.
It is interesting to note that even though the nuclear proteins were 4 times the amount of cytosolic proteins, the β-actin was approximately equal, suggesting that β-actin is more abundant in the cytoplasm than in the nucleus. Moreover, if we have loaded equal amount of total nuclear and cytosolic proteins, we would expect an even more dramatic decrease in OVCA1, comparing Lane 1 and Lane 2, which means the overall decrease of OVCA1 happens almost exclusively in the cytoplasm. The immunoblotting data coincide very well with the immunofluorescent data.

Interestingly, we noticed that in the cytosolic samples (Lane 1 and Lane 2), the OVCA1 protein appears as double bands. These double bands were consistently detected only in the cytosolic fractions in subsequent experiments. Notably, the decrease of OVCA1 with UV in the cytosolic samples occurs only in the upper band, not the lower band. These double bands are suggestive that OVCA1 is modified, possibly by phosphorylation, although there has not been any report about the post-translational modifications of OVCA1.
### Table

<table>
<thead>
<tr>
<th>Total Protein UV</th>
<th>Cytosolic</th>
<th>Nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>25ug</td>
</tr>
<tr>
<td></td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>100ug</td>
<td>100ug</td>
</tr>
<tr>
<td></td>
<td>_</td>
<td>+</td>
</tr>
</tbody>
</table>

**Lane:** 1, 2, 3, 4

Figure 13. Western analysis of cytosolic and nuclear OVCA1 after UV treatment. MCF-7 cytosolic protein samples (25 ug) and nuclear protein samples (100 ug) before (-) and 24 hours after (+) UV treatment using OVCA1 antibody. B-actin was used as a loading control.

#### 3.7 OVCA1 is post-translationally modified by phosphorylation in the cytoplasm in MCF-7 cells

Based on the results obtained in section 3.6 (Figure 13), we aimed to investigate whether the double bands of OVCA1 in the cytosolic fractions were due to phosphorylation of OVCA1. The strategy was to use antibodies that are specific for phosphorylated proteins to immunoprecipitate all the phosphorylated cellular proteins, then use OVCA1 antibody for immunoblotting to see if OVCA1 is among those phosphorylated proteins. Cultured MCF-7 cells were UV-treated (with the same condition as mentioned in sections above) or not UV-treated (control). At 4 hours, 12 hours, and 24 hours after UV treatment,
cytoplasmic fractions of the cellular proteins were extracted. Equal quantity of total protein was immunoprecipitated with phosphor-Tyr antibodies. We used two different p-Tyr antibodies, PY99 and 2C8, in independent experiments (Figure 14). The immunoprecipitated phosphorylated proteins were separated by SDS-PAGE then subjected to western blotting using OVCA1 antibody. The supernatant after immunoprecipitation was saved for future use (see below).

The detection of OVCA1 bands in the western blots suggests that OVCA1 does get phosphorylated. In addition, the phosphorylated OVCA1 decreases from 0 to 24 hours post-UV treatment (Figure 14). This decreasing trend is consistent between independent experiments using two different p-Tyr antibodies. It is also consistent with Figure 13, which shows that the “upper band” of OVCA1 (phosphorylated OVCA1) decreases dramatically compared to the non-UV cytosolic fraction in the 24 hour post-UV cytosolic fraction.

As an internal control, the supernatants saved from the immunoprecipitation (see above) were used in a western blot with β-actin antibody. β-actin is equal among the four samples, suggesting that equal amount of immunoprecipitated proteins were used for the OVCA1 western blotting, which justifies the decreasing trend of phosphorylated OVCA1 after UV in the cytoplasmic fractions (Figure 14).
3.8 OVCA1 regulates cell cycle at G1/S checkpoint: pRB protein level increases with overexpression of Ovca1 in ovarian cancer cell line A2780

As shown in Behringer’s study using Ovca1 knock-out mice, Ovca1 regulates cell proliferation and cell cycle (Chen and Behringer, 2004). In their cell cycle analysis using Ovca1 knockout MEF cells, it was found that the S phase cell population decreased considerably more in the Ovca1-/- MEF than in the Ovca1+/+ MEF. Also, there is a decrease in the phosphorylated RB (pRB) protein in Ovca1 knockout MEFs compared to
wild type MEF. These results suggest that OVCA1 functions at the G1/S transition during cell cycle: down regulation of *Ovca1* prevents cells from processing to S phase.

In our study, we introduced exogenous *Ovca1* into ovarian cancer cells A2780. A2780 has low expression level of *Ovca1*, but it has wild type p53. We were interested in the pRB level in the *Ovca1* transfected cells. pcDNA 3.0-*Ovca1* was transfected into A2780 cells and the “mock” was transfected with empty plasmid pcDNA3.0. When using an antibody specific for phosphorylated RB for western blotting, we found that the pRB level increased in the *Ovca1* transfected cells (Figure 15). Interestingly, this is consistent with the results from Behringer’s study: the expression level of OVCA1 is related to the pRB level, leading to OVCA1’s role in controlling the G1/S transition during cell cycle.

![Mock Ovca1](image)

Figure 15. Western analysis for pRB level in A2780 ovarian cancer cells. Overexpression of *Ovca1* increase the pRB protein level in A2780 cells. β-actin was used as a loading control.
3.9  **Cell cycle study with the overexpression of Ovca1 in A2780 cells**

Next, we analyzed the cell cycle profile of *Ovca1* transfected A2780 cells using cell flow cytometry. Again, pcDNA3.0-*Ovca1* was transfected into A2780 cells and empty plasmid pcDNA3.0 was transfected into the “control”. In order to determine the transfection efficiency and also to select for the *Ovca1* transfected cells, we co-transfected green fluorescent protein (GFP) with *Ovca1* into the cells. GFP plasmid pEGFP-N1 (4.7 kb) was a generous gift from Dr. Shiyong Wu (Ohio Univeristy). The ratio of GFP plasmid:*Ovca1* plasmid was 1:20; therefore, any cells that are positive for GFP should also contain *Ovca1*.

Twenty-four hours after the co-transfection, cells were fixed and stained with propidium iodide (PI) and then subjected to cell flow cytometry. For the analysis, we first gated the channel for cells that were GFP positive, thereby guaranteeing that it was only the cell cycle of the *Ovca1* transfected cells that we were analyzing. Three independent experiments were performed both for the *Ovca1* transfected A2780 and the control. The raw data collected from the FACS machine were analyzed using ModFit tool. Using the average data from three independent trials, Figure 16 shows the percentage of cells in G1, S and G2 phases in the two groups. In the control group, the average percentage for G1, S and G2 were 27.12%, 24.39% and 48.49%, respectively. In the *Ovca1* transfected cells, the average percentages for G1, S and G2 were 21.05%, 33.96% and 44.99% respectively. As demonstrated in Figure 16, overexpression of *Ovca1* in A2780 induced an increase in the S phase cell population (p value: 0.0257), which is consistent with Behringer’s cell cycle study with *Ovca1* knock-out MEFs.
3.10 **OVCA1 function might be associated with the cytoskeleton structure, the function of RNPs, and the apoptosis pathways.**

To better understand the cellular function of OVCA1, it is important to explore the proteins that might be associated and interacting with OVCA1. It has been suggested that OVCA1 binds to other proteins to carry out its function in transcription and translation, and OVCA1 was found to interact with two RNA binding proteins in a yeast two hybridization study (Salicioni *et al*, 2000).

We used OVCA1 antibody to immunoprecipitate OVCA1 binding proteins, then these proteins were run on a SDS-PAGE gel. Samples without adding the OVCA1 antibody were used as controls. The protein bands that are present in the “OVCA1 lane” (samples...
with OVCA1 antibody added) but absent in the “control lane” were cut out of the gel for mass spectroscopy analysis. We used cultured MCF-7 cells (both untreated and UV treated), and CE and 129 kidney cells for analysis. The gel images are shown in Figures 17, where protein bands of interest are marked. Sample information for each marked protein bands is listed in Table 2. Mass Spectroscopy analysis results from proteins with significant Mascot scores are shown in Tables 3 and 4. Other proteins isolated from the gels are not significant (data not shown).

In untreated MCF-7 cells, putative OVCA1 binding proteins were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), serine protease HTRA2, microtubule-actin cross-linking factor 1 (MACF1), heterogeneous nuclear ribonucleoprotein H3 (HNRNPH3), cytoplasmic actin, tropomyosin, and glucose-regulated protein precursor GRP78. Another OVCA1 interacting protein in UV treated MCF-7 cells was Vimentin, which is also an OVCA1 interacting protein in CE kidney cells. Tubulin and cytoplasmic actin interacted with OVCA1 in 129.

These putative OVCA1 interacting proteins include cytoskeleton structural proteins like MACF1, actin, vimentin, tubulin, tropomyosin, RNP (ribonucleoprotein) proteins like hnRNP H3, and proteins related to apoptosis pathways like HTRA2, GAPDH. These results suggest that OVCA1 function might be associated with the cytoskeleton structure, the function of RNPs, and the apoptosis pathways.
Figure 17. Original SDS-PAGE gel picture showing the potential OVCA1-binding protein in MCF-7 cells. The lane in the middle is the experimental sample (with OVCA1 antibody), and the right lane is the negative control (without OVCA1 antibody). Protein bands that were isolated from the gels are marked.
Table 2.

*Sample information for corresponding lanes in the 3 gels shown in Figure 17.*

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCF-7</td>
<td>8</td>
<td>129</td>
</tr>
<tr>
<td>2</td>
<td>Control (MCF-7)</td>
<td>9</td>
<td>Control (129)</td>
</tr>
<tr>
<td>3</td>
<td>MCF-7</td>
<td>10</td>
<td>MCF-7</td>
</tr>
<tr>
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<td>11</td>
<td>MCF-7 UV</td>
</tr>
<tr>
<td>5</td>
<td>Control (MCF-7)</td>
<td>12</td>
<td>CE</td>
</tr>
<tr>
<td>6</td>
<td>CE</td>
<td>13</td>
<td>129</td>
</tr>
<tr>
<td>7</td>
<td>Control (CE)</td>
<td>14</td>
<td>Control (129)</td>
</tr>
</tbody>
</table>
Table 3.

_Potential OVCA1 binding proteins in MCF-7 cells._

<table>
<thead>
<tr>
<th>Identified Proteins</th>
<th>*Score</th>
<th>Database</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>93</td>
<td>Sprot: G3P_HUMAN</td>
</tr>
<tr>
<td>Serine protease HTRA2</td>
<td>134</td>
<td>Sprot: HTRA2_HUMAN</td>
</tr>
<tr>
<td>Microtubule-actin cross-linking factor 1</td>
<td>154</td>
<td>Sprot: MACF1MOUSE</td>
</tr>
<tr>
<td>Hetergeneous nuclear ribonucleoprotein H3</td>
<td>84</td>
<td>MSDB: AAF68844</td>
</tr>
<tr>
<td>78 kDa glucose-regulated protein precursor</td>
<td>91</td>
<td>Sprot: GRP78_HUMAN</td>
</tr>
</tbody>
</table>

*Score: Mascot Score. Considered significant if greater than 75.

Table 4.

_Potential OVCA1 binding proteins in CE and 129 kidney cells, and UV treated MCF-7 cells._

<table>
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<tr>
<th>Sample</th>
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<th>*Score</th>
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</tr>
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<tbody>
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<tr>
<td>129 kidney</td>
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<td>Sprot: TBB2C_HUMAN</td>
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<tr>
<td>129 kidney</td>
<td>Actin, cytoplasmic</td>
<td>143</td>
<td>Sprot: ACTB_HUMAN</td>
</tr>
<tr>
<td>MCF-7 UV</td>
<td>Vimentin</td>
<td>238</td>
<td>Sprot: VIME_HUMAN</td>
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</tbody>
</table>

*Score: Mascot Score. Considered significant if greater than 75.
CHAPTER 4 DISCUSSION

Genetic modification is a relatively new concept that is used to describe the effect of different genetic backgrounds on the variable phenotypes observed in traits that are inherited in Mendelian fashion. Of particular interest are the genetic modifiers that can modify the phenotypes of a disease. Although genetic modification events occur very frequently in humans, the underlying genetic basis for the modification is poorly understood. Therefore, understanding of how the genetic modifiers work, at what hierarchy the modification effect takes place, and what other factors and signaling pathways are involved will shed light on the predisposition, progression and possible treatment of some genetic diseases.

In our previous studies to discover novel genetic modifiers of p53 in individuals with Li-Fraumeni Syndrome, 3 modifiers that are involved in carcinogenesis and embryonic development were identified: mop1, mop2, and mop3 (Evans et al, 2004; Ayanga et al, 2006; Liang et al, 2008). After mapping and sequencing studies, the tumor suppressor gene Ovca1 was identified to be the critical modifier gene in the modifier mop2, harboring a 321P to 321S polymorphic change in the OVCA1 protein that is of particular interest. This polymorphic change of the OVCA1 protein, resulting from a single nucleotide polymorphism (SNP) of the Ovca1 gene, leads to increased Ovca1 expression at both RNA and protein levels, increased proliferation, decreased G1 arrest, and punctate subcellular localization of OVCA1 protein (Liang et al, 2008).

How can 321P OVCA1 and 321S OVCA1 have such different cellular functions? What other molecular differences do these two proteins have? How does this one amino acid
difference impact the structure of the protein, and how is this structural change linked to their functional differences?

In this study, we first looked into the increased expression level of 321S OVCA1. Since the increased expression is both at the mRNA level and protein level, stability experiments were done to determine whether the higher level of 321S OVCA1 in CE cells were due to higher stability of 321S OVCA1, or simply due to the higher mRNA level. If 321S OVCA1 (CE) is more stable than 321P OVCA1 (129), the higher expression level of 321S OVCA1 could be the result of the longer OVCA1 protein half-life; whereas, if 321P OVCA1 (129) is more stable than 321S OVCA1 (CE), the higher expression level of 321S OVCA1 could only be explained by the higher CE Ovca1 expression at the mRNA level. From the results of the protein half-life experiment, it is obvious that 321P OVCA1 (129) is more stable than 321S OVCA1 (CE), therefore, the increased OVCA1 protein level is a result of the higher Ovca1 mRNA level, which may result from elevated transcription or stability of mRNA. Since the stability of mRNA is predominantly due to the specific sequences in the 3’ UTR and the polymorphic change is in the coding region not the 3’UTR region of the gene, it is unlikely that the elevated OVCA1 protein level is a result of a more stable mRNA. Therefore, a plausible explanation would be increased transcription level of Ovca1 leads to a higher level of Ovca1 mRNA, which results in the increased OVCA1 protein level in the CE (321S OVCA1) cells.

We also used the I-mutant 2.0 tool to confirm the protein stability change from 321P OVCA1 to 321S OVCA1. In this prediction (section 3.1), we assigned the 321P OVCA1
as the “wildtype” protein and the 321S OVCA1 as the “mutant” protein. But we have to keep in mind that this was an arbitrary assignment, as we cannot determine which of these two polymorphic forms of the protein occurs more frequently in the population unless we sequence an adequate number of DNA samples. When we switch the input for the “wildtype” and “mutant” protein and performed the prediction again, the same conclusion about the protein stability was drawn. I-Mutant2.0 successfully predicts around 80% of the thermodynamic data in a large experimental database with a cross-validation procedure (Capriotti et al., 2005). As for the prediction of DDG values, the correlation of predicted with the experimental value from the database is 0.62 for a sequence-based prediction (Capriotti et al., 2005). The DDG values and the reliability index are well within the valid ranges.

Although the biochemical function of OVCA1 protein is not fully understood, studies show that OVCA1 is a component of the very complex biosynthetic pathway of a unique amino acid in eEF-2, diphthamide. The post-translational modification of a histidine converting to a diphthamide in eEF-2 requires 3 step-after-step reactions (Liu, Milne, Kuremsky, Fink, & Leppla, 2004). It is suggested that lacking diphthamide in eEF2 will not result in a generalized translational downregulation, but it can lead to translational failure of some specific proteins (Webb et al., 2008). In this research by Webb et al., it was found that DPH4, one of the five yeast proteins (DPH1 to DPH5) that function to process a histidine residue to form the diphthamide on eEF2, is not part of a proposed protein complex which only contains DPH1, DPH2 and DPH3. However, mice that were homozygous for DPH4 deletion were retarded in growth and development, and most of
the embryos died before birth. The embryos that survive long enough had preaxial polydactyly. These defects were seen in embryos that were homozygous for deletion of DPH1, therefore, it was proposed by the authors that lacking of diphthamide on eEF2 result in translational failure of specific proteins. However, none of these “specific proteins” was identified or discussed further by the authors. Because upregulation in the translational machinery (eEF1α2 specifically) correlates with oncogenic transformation in ovarian cancers (Anand et al., 2002), and that OVCA1 is involved in the elongation process, we speculate that OVCA1 might regulate the ovarian carcinogenesis through eEF-2 and the specific polymorphic change in OVCA1 protein might alter its interaction with eEF-2. In our colocalization study of OVCA1 and eEF-2, 321P OVCA1 co localizes uniformly with eEF2 in the cytoplasm, while 321S OVCA1 has a punctate distribution, predominantly in the cytoplasm, which does not perfectly colocalize with eEF2. This suggests that the structural alteration in OVCA1 protein due to the $321P \rightarrow S$ change might affect the diphthamide biosynthesis, thereby leading to translational failure of some specific proteins while other proteins are successfully translated. This specificity in translated or untranslated proteins might result in the non-uniform colocalization of OVCA1 and eEF2. That is, the punctate distribution of 321S OVCA1 which does not colocalize with eEF2 might be at the ribosomes where the specific mRNAs fail to be translated.

A number of facts and evidence suggests the interaction between OVCA1 and BRCA1 signaling. First of all, Ovca1, Brca1 and p53 are all located in close proximity within the cancer-related hotspot on human chromosome 17. Ovca1 can modify p53-induced tumor
formation, while there is strong interaction between Brca1 and p53 in apoptosis, cell cycle and tumorigenesis (Xu et al., 2001). Secondly, Brca1 mutations increase the risk of not only breast cancers, but also ovarian cancers; loss of Ovca1 correlates with both ovarian and breast cancers (Chen et al., 2004). Thirdly, Ovca1 (and Ovca2) expression is directly inducible by Brca1 (Atalay et al., 2002).

In our efforts to explore the interaction between Brca1 and Ovca1, we first studied the effect of Ovca1 on the expression level of Brca1. The transfection and western blotting experiments show that overexpression of Ovca1 increases the level of BRCA1. Previous studies show that overexpression of Brca1 increases the level of Ovca1, indicating that Ovca1 is a direct downstream target of the transcription factor BRCA1. The result of our study likewise suggests that Ovca1 has the similar effect on Brca1. Considering that OVCA1 is not a transcription factor, its effect on Brca1 expression has to be explained by a mechanism other than direct transactivation. Besides its role in translation, it has been proposed that OVCA1 is involved in transcription as well, e.g. yeast homolog of OVCA1 (YIL103W) is found in a protein complex that includes subunits of RNA pol II (Chen et al., 2005). Therefore, overexpression of Ovca1 might lead to the elevated BRCA1 level through transcription regulation by the basic transcription apparatus.

Because the roles of BRCA1 in UV-induced DNA damage repair have been intensely studied, we examined the response of OVCA1 to UV treatment. OVCA1 showed an early response to UV, as does BRCA1, suggesting that OVCA1 might be involved in the initial cellular processes after DNA damage; however, OVCA1 and BRCA1 might play distinct roles in the initial processes because of their opposite response to UV. Also, we observed
that the decrease of OVCA1 upon UV treatment is more predominant in the cytoplasm than in the nucleus. This suggests that OVCA1 might respond to cellular insult like UV via translational regulation in the cytoplasm, not at the transcriptional level in the nucleus.

When mouse Ovca1 gene was cloned, the authors observed four subcellular localization patterns of OVCA1 protein in COS-7 cells (Chen and Behringer, 2001). In this current study of OVCA1, distinct subcellular localization patterns of OVCA1 were observed as well. In various types of 129 cells, OVCA1 was diffused within the cytoplasm, while in CE cells, OVCA1 appeared punctate. Double staining OVCA1 and eEF-2 in CE and 129 kidney cells showed that OVCA1 colocalized perfectly with eEF-2 (diffused in the cytoplasm) in 129 cells, while CE OVCA1 appeared punctate and did not colocalize with eEF-2. In MCF-7 cells, OVCA1 is diffused primarily in the cytoplasm, but after UV treatment, cytosolic OVCA1 level dramatically decreased and a punctate pattern was observed (Figure 12). These indicate that OVCA1 has very versatile subcellular localization patterns, depending on the cell line, the specific polymorphism of OVCA1, and the treatment. In general, OVCA1 is located primarily in the cytoplasm, which supports its role in the protein translation process; however, a very small amount of OVCA1 can be located in the nucleus, which may function in transcriptional regulation. The polymorphic change of OVCA1 protein induces its structural alteration, thereby changing its binding to OVCA1-interacting proteins and leading to a different subcellular localization pattern. Extrogenous factors like UV that can affect OVCA1 signaling can have an effect on the subcellular localization pattern of OVCA1 as well.
There is no previous report about the post-translational modifications of OVCA1 protein. In this study, we found that OVCA1 is phosphorylated in the cytoplasm, but not phosphorylated in the nuclear fraction in MCF-7 cells. This indicates that the phosphorylation of OVCA1 might be related to its role in translation, but not in transcription. Phosphorylation might activate cytosolic OVCA1, thus enabling it to carry out its function in regulating the elongation process via eEF-2. Also, we found that the phosphorylation of cytosolic OVCA1 decreases after UV treatment over time. This trend is the same as the overall decrease of cellular OVCA1 after UV treatment. If OVCA1 is only active when it is phosphorylated, this decrease in phosphorylation might suggest that exogenous stimuli like UV affect the function of OVCA1 in protein translation only through the decrease of the active form of OVCA1. This is likely to be the case, because the lower bands of OVCA1 in the UV and non-UV lanes in Figure 13 remain almost the same, suggesting the UV treatment does not have effect on the non-phosphorylated OVCA1 protein. Nevertheless, the activity of phosphorylated and non-phosphorylated cytosolic OVCA1 protein remains to be investigated.

The Ovca1-2 knockout mice studied by Behringer et al shows that OVCA1 regulates cell proliferation and cell cycle, especially at the G1/S transition. Ovca1 double knockout MEF (mouse embryonic fibroblast) cells have a smaller S phase population than the single knockout MEFs, and this decrease of S phase cells is related to a decrease in the phosphorylated RB level (Behringer et al, 2004). In our study to further investigate the role of OVCA1 in the cell cycle regulation, we chose the well-characterized ovarian cancer cell line A2780, which usually expresses very low levels of OVCA1. When we
increase the *Ovca1* expression in A2780, we saw increased S phase population and also increased level of pRB protein. This is consistent with the role of OVCA1 in cell cycle regulation found by Behringer *et al.* But we have not looked into the effect of increasing *Ovca1* expression on other cell cycle factors like p21 or cyclin D. In the *Ovca1* knockout mouse study, it was noticed that p21, cyclin D, cyclin E, and Cdk4 protein levels were not significantly different in *Ovca1* +/+ , +/−, and −/− MEFs (Behringer *et al.*, 2004). Therefore, in our future study we can transfect *Ovca1* in A2780 to study other cell cycle regulators to better understand the role of OVCA1 in the cell cycle regulation.

In our effort to search for OVCA1 interacting proteins, we found three major categories of proteins that might be physically binding to OVCA1. Among these three categories are cytoskeleton structural proteins, RNP proteins, and proteins related to apoptosis pathways. Of these, only the RNA interacting proteins have been discussed in previous literature. In the study by Salicioni *et al* in 2000 using yeast-2-hybrid system to search for OVCA1 binding proteins, two RNA binding proteins were found to interact with human OVCA1. Our study revealed eight proteins that could be interacting with OVCA1. It is likely that the method used in our study could yield some false positive results, whereas, with the yeast-2-hybrid system, the binding proteins discovered should be interacting with OVCA1 *in vivo*, thus unlikely to yield false positive results. Therefore, the next step is to perform immunoprecipitation experiments on each of these eight proteins to confirm the interaction with OVCA1.

From the results, four out of the eight OVCA1 binding proteins are cytoskeleton structural proteins, suggesting that OVCA1 is associated with the cytoskeleton possibly
to perform its dual role both in transcription and translation. The cytoskeleton may serve as a bridge that mediates and transports the newly synthesized OVCA1 to its target location within the cell. Also, by the interaction between OVCA1 and cytoskeleton structural proteins, the amount of nuclear OVCA1 and cytoplasmic OVCA1 might be closely regulated.

In our previous study (Liang et al, 2008) in which we identified Ovca1 as the modifier gene for mop2, we considered the CE OVCA1 (321S) as the “mutant” form and the 129 OVCA1 (321P) as the “wild type”. However, a more correct term is a “polymorphic change” until we sequence a large number of DNA samples from the population for that particular nucleotide. To date, we know that 321P OVCA1 is present in the mouse strains C57BL/6J and 129, whereas, 321S OVCA1 is present in strains CE, C57BL/6, FVB/N, and in the OVCA1 homolog in S.cerevisiae. In addition, 326S (321S in mice) is present in MCF-7 and two human fetal samples. All the sequence information is from the NCBI database except for CE, 129, and MCF-7, which were sequenced by our lab. Based on the limited sequence information, 321(326) S OVCA1 is the more frequent form.

In summary, we further investigated the functional significance of the Ovca1 SNP discovered by a previous study, and explored the important signaling pathways that OVCA1 might be involved in. The knowledge gained from this study about the OVCA1 cellular network can lead to better understanding of the wide tumorigenesis spectrum in LFS, and further investigation of this specific SNP as a risk factor can contribute to understanding of its possible roles in cancer predisposition.
CHAPTER 5  FUTURE WORK

In this study, we explored the possible involvement of *Ovca1* in the UV-induced DNA damage pathway. We found an interesting mutual enhancement effect between *Ovca1* and *Brca1*. However, distinct regulation mechanisms might be involved in this mutual enhancement, considering that *Brca1* is a well established transcription factor which has many downstream targets, while the major biochemical function of OVCA1 is in translation. Therefore, elucidating the specific mechanism by which *Ovca1* activates *Brca1* will not only shed light on the interaction between these two tumor suppressors, but also help us to better understand the translational regulation as a whole.

In our effort to explore the cellular network in which OVCA1 protein is involved, we found 8 potential OVCA1-binding proteins. Because of the nature of the method we employed in this study, it is possible that some of these 8 proteins are false positive results. Therefore, immunoprecipitation experiments are needed to confirm the direct protein interactions between OVCA1 and these potential binding proteins. From there, we will be able to further investigate the potential involvement of OVCA1 in pathways and mechanisms like apoptosis and RNA binding.

The SNP we discovered on the *Ovca1* gene is of great interest to us because of the cellular functional differences it makes. However, the combined sequence information about this SNP from both the NCBI database and our own study is still very limited. High-throughput sequencing of this SNP in human DNA samples with wide spectrum of cancer-related genetic backgrounds will not only suggest the predominant polymorphic
form, but also help to establish this SNP as a potential risk factor in LFS as well as in sporadic cancers.
REFERENCES


Nobukuni, Y., Kohno, K., & Miyagawa, K. (2005). Gene trap mutagenesis-based forward genetic approach reveals that the tumor suppressor OVCA1 is a component of the


APPENDIX  LIST OF ABBREVIATIONS

129:129-Trp53<sup>tm1Tyj</sup>/Sv mice

ARF: ADP-ribosylation factor

ATM: ataxia-telangiectasia mutated

ATR: ATM and Rad3-related

BASC: BRCA1-associated genome surveillance complex

Bax: Bcl2 (B-cell CLL/lymphoma 2)-associated X protein

Brca1: breast cancer 1, early onset

CASP: Critical Assessment of Techniques for Protein Structure Prediction

CDK: cyclin-dependent kinase

CE: CE/J mouse

Cftr: cystic fibrosis transmembrane conductance regulator gene

CHX: cycloheximide

DAPI: 4',6-diamidino-2-phenylindole

DDG: protein stability free energy change

DMP: dimethyl pimelimidate

DPH2: diphthamide biosynthesis protein 2

DT: diphtheria toxin

eEF1α2: eukaryotic elongation factor 1 α-2

eEF-2: eukaryotic elongation factor 2
ERCC3: excision repair cross-complementing rodent repair deficiency, complementation group 3

FBS: fetal bovine serum

GADD45: growth arrest and DNA damage induced-45

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GFP: green fluorescent protein

HDM2: human double minute 2

Her2/Neu: human epidermal growth factor receptor 2

Hic1: hypermethylated in cancer-1

HIF: hypoxia-inducible factor

HNRNP H3: heterogeneous nuclear ribonucleoprotein H3

HTRA2: HtrA serine peptidase-2

IGF1-R: insulin-like growth factor 1 receptor

IR: ionic radiation

LFS: Li-Fraumeni Syndrome

LOH: loss of heterozygosity

MACF1: microtubule-actin cross-linking factor 1

MDM2: murine double minute 2

MEF: mouse embryonic fibroblast

mop1: modifier of p53-1

mop2: modifier of p53-2

NCBI: national center for biotechnology information
NER: nucleotide excision repair

Nf1: neurofibromin-1

Ngfr: nerve growth factor receptor

NHEJ: non-homologous end joining

Nm23: non metastatic 23

Ovca1: ovarian cancer gene 1

Ovca2: ovarian cancer gene 2

P/S/A: Penicillin/Streptomycin/Amphotericin B

PARP: poly ADP ribose polymerase

PBS: phosphate buffered saline

PBS-T: PBS-Tween

PCNA: proliferating cell nuclear antigen

PDB: protein data bank

pRB: phosphorylated retinoblastoma

PUMA: p53 upregulated modulator of apoptosis

Rara: retinoic acid receptor, alpha

RB: retinoblastoma

RBM8A: RNA binding motif 8A

RBM8B: RNA binding motif 8B

RNP: ribonucleoprotein

RPM: round per minute

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SWI/SNF: SWItch/Sucrose NonFermentable

SNP: single nucleotide polymorphism

Tsp1: thrombospondin 1

UTR: untranslated region

UV: ultraviolet

VEGF: vascular endothelial growth factor

XPB: xeroderma pigmentosum B