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# NPRL2/TUSC4 FUNCTIONS AS A TUMOR SUPPRESSOR BY REGULATING BRCA1'S STABILITY VIA THE E3 UBIQUITINATION PATHWAY

Yang Peng

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# NPRL2/TUSC4 FUNCTIONS AS A TUMOR SUPPRESSOR BY REGULATING BRCA1'S STABILITY VIA THE E3 UBIQUITINATION PATHWAY

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DISSERTATION

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DOCTOR OF PHILOSOPHY

by
Yang Peng, M.S.
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## DEDICATION

I would like to dedicate this dissertation to my wife,

Qian Huang

who has always encouraged me to believe in myself,

and for everything she has been giving me.

To my parents and parents-in-law

Huiyao Peng and Huiping Xiong

Chuanyou Huang and Lirong Liu

for always reminding me to be a good person.

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I would like to thank all my previous and current lab members for all the days and nights we spent together, for all the smiles you put on my face and for all the happiness and sadness we went through together. I appreciate all these great memories that filled in my graduate school career.

NPRL2/TUSC4 FUNCTIONS AS A TUMOR SUPPRESSOR BY REGULATING BRCA1'S STABILITY VIA THE E3 UBIQUITINATION PATHWAY

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Expression of the tumor suppressor protein BRCA1 is frequently lost in breast cancer patients, and the loss of its expression is associated with disruption of various critical functions in cells and cancer development. In the present study, we demonstrate through microarray analysis that cells with tumor suppressor candidate 4 (NPRL2/TUSC4) knockdown show critical changes to cell cycle, cell death pathways and a global impact on cancer development. More importantly, we observed a clear cluster pattern of NPRL2/TUSC4-knockdown gene profiles with established homologous recombination (HR) repair defect signature. Additionally, NPRL2/TUSC4 protein physically interacts with the E3 ligase HERC2 and prevents ubiquitin pathway-mediated BRCA1 degradation. Knockdown of NPRL2/TUSC4 expression enhanced BRCA1 polyubiquitination, leading to BRCA1 protein degradation and a marked reduction in HR repair efficiency. Conversely, ectopic expression of NPRL2/TUSC4 effectively suppressed the proliferation, invasion, and colony formation of breast cancer cells *in vitro* and tumorigenesis *in vivo*. Furthermore, knockdown of NPRL2/TUSC4 expression transformed normal mammary epithelial cells and

enhanced the sensitivity of U2OS cells to the treatment of poly(ADP-ribose) polymerase inhibitors. Therefore, NPRL2/TUSC4 may act as a bona fide tumor suppressor by regulating BRCA1 protein stability and function in breast cancer.

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**Chapter 1**

# INTRODUCTION AND BACKGROUND

## BRCA1 maintains DNA damage response in breast cancer

### Tumor suppressor and cancer development

According to Hanahan and Weinberg's updated review in 2011, they re-defined the six classical hallmarks of cancer development: sustaining proliferative signaling, resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis and evading growth suppressors (Hanahan and Weinberg, 2011). This concept systematically and fundamentally explains the most critical characteristics most cancer cells need to acquire during their development. However, it's unlikely these abnormities would occur synchronically and naturally under normal circumstances, instead it's more likely that the tumor proliferation is regulated sequentially, while any of these defects can be raised. For example, sustaining proliferative signaling also requires cancer cells disrupt the normal growth regulation mechanism, and lead to the evasion of cell growth suppressors, but many of these processes are conducted by tumor suppressor genes. Tumor suppressors operate in numerous ways to limit cell growth and proliferation to prevent cancer. Genome-wide screening has shown that many tumor suppressor genes are deleted or mutated in various types of cancers, with the inactivation patterns of many well-recognized tumor suppressor

genes being well characteristic and non-randomized (Vogelstein et al., 2013). Recent studies also confirmed the mutation of certain tumor suppressor genes confers to selective tumor growth advantages (Xue et,al., 2012).

**BRCA1 and breast cancer**

As the most diagnosed and leading cause of cancer-related deaths in women, breast cancer is responsible for the 180,000 new cases in the United States alone and 1.6 million worldwide, while more than a half million women died in 2011 primarily due to the lack of early detection (Global Health Estimate, WHO 2013). Among those cancer patients, women with BRCA1 mutations are six times more likely to develop cancer before the age of 70 compared to women with normal BRCA1 (NCI, 2014). Thus to investigate the relationship between BRCA1 and breast cancer as well as how BRCA1 is regulated before the early cancer lesions posed extreme challenges and remarkable clinical potentials to current breast cancer research fields.

**DNA damage response and BRCA1**

DNA damage can be caused by various sources, and leads to genomic stress for the cell. To safeguard the integrity and fidelity of genomic information, cells activate the evolutionary conserved DNA damage response pathways to manage the lesions once the lesions are found. Cell cycle checkpoints will stop the damaged cells from dividing and activate the damage repair mechanisms. If the DNA damage lesion can be fixed, cell cycle will restart, otherwise, the cells will be

eliminated by apoptosis pathway (also known as programmed cell death) (Medema and Macurek, 2012). Depends on the damage types, different pathways are responsible for the DNA double strand breaks (DSB) and DNA single-strand break (SSB). Most current understanding indicate that Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3-Related (ATR) are the main players in mediating cells' DSB and SSB responses respectively, although the molecules involved in each pathway can overlap (Ciccia and Elledge, 2010) (Figure 1). Upon the recognition of DNA breaks by sensor proteins, ATM and ATR quickly undergo the autophosphorylation or phosphorylation of their substrates, such as CHK1 and CHK2 (Zhou and Elledge, 2000). Histone protein H2AX will rapidly get phosphorylated at Ser-139 by ATM or ATR and produce γ-H2AX at the damage sites, in turn recruit other damage repair proteins to the broken DNA. ATM and ATR also phosphorylates their own unique substrates to stimulate DSB and SSB responses respectively. More specifically, ATM phosphorylates checkpoint kinase 2 (CHK2) at Thr-68 and ATR phosphorylates checkpoint kinase 1 (CHK1) at Ser-317 and Ser-345. CHK2 phosphorylation in turn leads to p53 phosphorylation, MDC1, RNF8, RNF168, BRCA1 and 53BP1 recruitment to the damage sites while CHK1 phosphorylation is followed by the phosphorylation of Cdc25A and Tlk1/2. These processes induce downstream processes that includes damage-induced transcription, DNA repair, cell cycle arrest/delay, apoptosis and chromatin remodeling (Bartek and Lukas, 2003).

**BRCA1 safeguards genome integrity and regulates DNA damage repair**

BRCA1 gene was first identified and cloned in 1994. BRCA1 protein contains 1863 amino acids and located on chromosome 17q21. Its C-terminus has BRCT motif to recognize and interact with various DNA repair proteins, while its N-terminus contains a ring-finger domains allowing proteins interactions such as BRCA1-associated RING domain-1 protein (BARD1) to form heterodimer, and this complex was confirmed to carry ubiquitination ligase activities (Xia et al., 2003). BRCA1 plays multiple roles to regulate normal molecular and cellular functions such as cell cycle checkpoint control, mRNA transcription regulation and DNA damage repair (Kennedy et al, 2004)(Figure 3). Loss-of-function mutation for BRCA1 correlates with approximately 82% risk of developing breast cancer (King et al., 2002) (Figure 2) and low BRCA1 expression is associated with significant increase of sporadic cancer incidences (Couch et al., 1997; Wang et al., 2004).

There are two major DNA damage repair mechanisms involve in mammalian cells, homologous recombination (HR) repair and non-homologous end joining repair. Previous reports indicated that BCRA1 play important roles in both repair pathways and it involves intensively in HR repair to protect cells to repair DNA double-strand break (DSB) in a least error-prone manner (Figure 4).

**Role of BRCA1 in homologous recombination (HR) repair**

HR repair has been considered as one of the most important mechanism to maintain genomic stability during DSB by mediating error-free repair (Levitt and Hickson 2002). The deficiency of HR repair is associated with cancer development

and sensitize DNA damage-inducing therapy (Alli et al., 2009). When DNA DSB occurs, HR repairs DNA during the S and G2 phases and before M phase of cell cycle. After cell sensing the DSB, BRCA1 will be recruited and form protein complex with Rad50, MRE11 and NBS1 (also known as MRN complex), the BRCA1-MRN complex can take 5'—3' exonuclease activity and expose 3' end of the break DNA, either single-strand annealing or strand invasion will occur during the process of repair (Karran 2000). It is believed that BRCA1 participates in the strand invasion repair system and also physically interact with BRCA2, RAD51 to form repair complex at the DNA damage break sites (Karran 2000; Gilmore et al., 2003), based on the earliest evidence from the previous reports that BRCA1 deficient in stem cells leads in the increased sensitivity to alkylating reagents which commonly used to cause DNA DSB (Moynahan et al., 2001). Recent works supported this statement that mutation or inactivation of BRCA1 will not only impair HR repair efficiency but also decrease the accuracy of NEHJ repair pathway and eventually enlarge the effect of DNA damage toxicity and increase the possibility of early cancer lesions.

**Role of BRCA1 in cell cycle regulation and drug sensitivity**

BRCA1 has been reported to be a cell cycle regulator majorly owing to its role as the substrate of DNA damage kinases such as ATM and ATR, and Checkpoint Kinase 2 (CHK2) (Yarden et al., 2002; Xu et al., 2001; Xu et al., 2002). ATM and ATR mainly respond to DNA double strand breaks such as ionizing irradiation and DNA single strand breaks such as Ultraviolet respectively (Zou and Elledge 2003).

BRCA1 can be phosphorylated by ATM, ATR and CHK2 at various sites, and in turn activate the checkpoint of cell cycle (Cortez et al., 1999; Lee et al., 2000; Tibbetts et al., 2000). It has been suggested that phosphorylated BRCA1 is associated with transcriptional activation of multiple critical checkpoint proteins and stimulate their expression level such as p21, p27 in G1/S phase arrest by inhibiting cyclin-depedent kinase 2(CDK2), but mechanism of how BRCA1 is involved in cell cycle checkpoint remains unclear (Somasundaram et al., 1997).

Additionally, mouse embryonic fibroblast with mutated BRCA1 showed increased DNA damage causing drugs' sensitivity such as Irinotecan and Etoposide which target DNA topoisomerase I and II respectively (Fedlier et al., 2003). Accumulating evidence indicated BRCA1 is important in the inhibition of cell apoptosis after the treatment of DNA-damaging drugs, especially in breast cancer cell lines (Kennedy et al., 2004). Thus, the disruption of BRCA1 will significantly decrease cell's capacity to fix both double-strand break caused by drugs such as Irinotecan, Etoposide, as well as PARP inhibitors, and such increased drug sensitivity in cell is largely owing to their impaired capacity for HR repair (Alan and Ashworth, 2012).

## Regulation of BRCA1 in mammalian cells

### BRCA1 is regulated by poly-ubiquitination pathway

In Ruffner and Verma's report in 1997, BRCA1 was found undergoes hyperphosporylation during G1 and S phase and starts dephosphorylation

immediately after entering M phase, thus they concluded that BRCA1 is regulated during different stages of cell cycle in a qualitative and quantitative manner (Ruffner and Verma, 1997). Additionally, BRCA1's interaction and colocalization with other DNA damage response protein such as BRCA1 Associated Ring Domain 1 (BARD1) also exhibits a cell cycle dependent manner (Jin et al., 1997), so, it's clear that BRCA1's expression is tightly regulated both the transcriptional and protein level during different cell cycle stages, but very little is known about how BRCA1's protein stability is controlled.

Previous publications have identified that F-box protein 44 (FBXO44) mediates BRCA1's degradation in ubiquitination pathway by Skp1-Cul1-F-box-protein 44 ($SCF^{FBXO44}$) complex and such regulation is promoting the development of sporadic breast cancer (Lu et al.,2012). Furthermore, E3 ligase HERC2 can also specifically target BARD1-uncoupled BRCA1 and lead to the degradation of BRCA1 in ubiquitination pathway; the depletion of HERC2 significantly reduced the impact of the inactivation BARD1 and restored BRCA1 expression *in vitro*, suggesting that HERC2 plays an critical role in destabilizing BRCA1 and contributing to the breast carcinogenesis (Wu et al., 2010). Interestingly, N-terminus mediates the both binding between BRCA1 and $SCF^{FBXO44}$, HERC2.

**HERC2 and ubiquitination-proteasome pathway**

Degradation of proteins involve two successive steps: load poly-ubiquitin by covalent bonds to the targeted proteins and degradation of the proteins by 26S proteasome complex. In general, the ubiquitination pathways start with the

activation of ubiquitin by E1 enzyme which is also known as ubiquitin-activating enzyme, then E2 enzyme (also is called as ubiquitin-conjugating enzyme, UBC) will lead the ubiquitin via thiol ester bond to bind the targeted proteins. The specific targeting of E3 ligase is realized by the unique recognition motif and E3 ligase will process the conjugation of ubiquitin to the targeted protein and facilitate the synthesis of the poly-ubiquitin chain in multiple cycles. Even in most cases, E2 enzyme transfer the first ubiquitin moiety to the E3-bound substrate protein, and then conjugate the following ubiquitin moiety to bound the previous one, but RING finger targeted substrate allow E2 enzyme transfer the ubiquitin moiety directly to the substrate. Finally, ubiquitin will be removed by deubiquitinating enzymes (DUBs) for recycle and ubiquitin-tagged proteins will be destructed into small peptides by the 26S proteasome complex (Glickman and Ciechanover, 2002) (Figure 5).

HERC2 gene is highly mutable and it was discovered from the deletion hotspot on human chromosome 15q11-q13, HERC2 gene encodes a large protein with a molecular weight of 528kDa (Lehman et al., 1998). Bioinformatics analysis indicated its highly conserved function domains include RCC1-like domain and COOH-terminal HECT domain, which are responsible for the Ran-dependent membrane trafficking and E3 ligase function respectively By mediating ubiquitin-dependent recruitment of DNA damage repair proteins to damage chromosomes and form complex with RNF8, HERC2 facilitates E2 ligase UBC13 to conjugate with RNF8, in turn to regulate the expression levels of series repair proteins such as 53BP1 and BRCA1 (Bekker-Jensen et al., 2010).

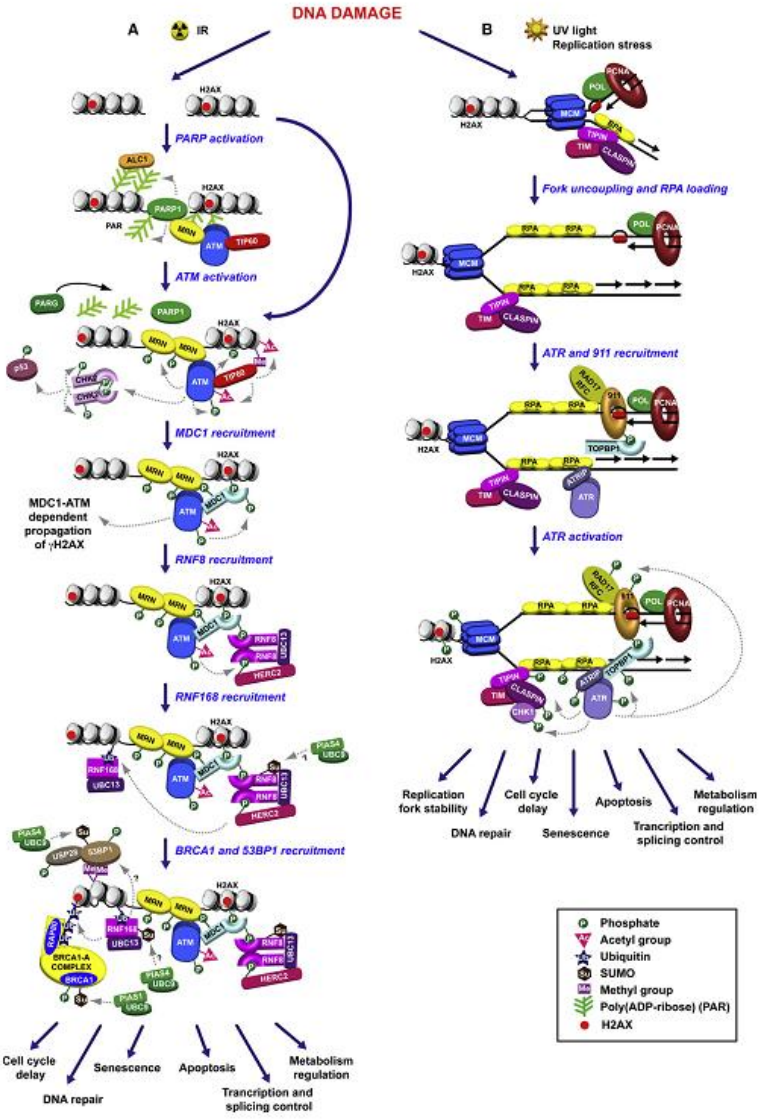



**Figure 1. Schematic Model for major DNA damage response kinase: ATM and ATR Activation**

(A) IR treatment forms DSBs and activates PARP1. Activation of the ATM activity by MRN and TIP60 leads to the phosphorylation of CHK2, p53, and the γ-H2AX as well as downstream signaling cascade, which results in the recruitment of MDC1, RNF8, RNF168, BRCA1, and 53BP1 to damage sites (B) UV or replication stress lead to replication fork stalling and accumulation of RPA to coat on ssDNA, then ATR/ATRIP as well as RAD17/ RFC2-5 complexes will be recruited to damaged ssDNA. ATR kinase recruits the 911-associated protein TOPBP1 and leads to the activation of the ATR signaling cascade and downstream CHK1 phosphorylation. *Adapted from Ciccia and Elledge, Molecular Cell 40, 179-204 (2010) with permission from Elsevier.*

**Functional role of NPRL2/TUSC4 in suppress tumor proliferation and maintain DNA damage response**

Tumor Suppressor Candidate 4 (NPRL2/TUSC4) is also called Nitrogen Permease Receptor 2-like (NPRL2), and it was first identified from lung cancer homozygous deletion region which containing multiple tumor suppressor genes on chromosome 3p21.3. NPRL2/TUSC4 gene contains 3.3kb with 11 exons coding for a 1.5kb mRNA, and various splicing isoforms are expressed abundantly in normal lung and testis tissues, NPRL2/TUSC4 (red in figure) conserved across species ranging from yeast to chimpanzee with 33-66% aligned sequence (Figure 6). Sequencing data indicated 1 out of 40 lung cancer cell lines contains a frameshift mutation of NPRL2/TUSC4 gene which produces a stop codon (CAA to TAA Stop codon 261 in H1514 cell line), NPRL2/TUSC4 gene encodes a 43 kd soluble protein with an unknown protein binding domain (Lerman and Minna, 2000). In a follow up paper in 2002, exogenous expression of NPRL2/TUSC4 by adenovirus significantly inhibited the growth of Non Small Lung Cancer cell lines proliferation *in vitro* and the cancer development *in vivo*, it also increased the apoptotic cells in lung cancer cell lines which NPRL2/TUSC4 expression was originally disrupted, as the programmed cellular response to stress and stimuli, the decreased apoptosis is always considered as result of the inactivation of tumor suppressor, so these results suggested the important roles of NPRL2/TUSC4 are playing in the safeguard and maintenance of genomic stability ( Ji et al., 2002).

Additionally, inactivation of NPRL2/TUSC4 was found in multiple other cancer types including renal and cervical cancers (Li et al., 2004). NPRL2/TUSC4

was also found reciprocally correlated with the sensitivity of cisplatin (CDDP), a commonly used anti-cancer drug which containing platinum and cause apoptosis in cancer cells. Researchers reintroduced NPRL2/TUSC4 expression in NPRL2/TUSC4-null, cisplatin-resistant lung cancer cell line H322, and they were able to resensitize the response of cell to cisplatin and increased the apoptotic cell death, more specifically, the combination therapy with NPRL2/TUSC4-containing nano-particles and cisplatin significantly reduced the tumor size and inhibited cell proliferation compare to cisplatin treatment alone. This result indicated NPRL2/TUSC4's potential role to mediate the DNA damage response and the capacity to predict the clinical outcomes of cisplatin treatment in patients (Ueda at al., 2006). The same research group published in 2010 again proved that the increased sensitivity to cisplatin in NPRL2/TUSC4-null lung cancer cell line was associated increased DNA damage response activities, reintroduction of NPRL2/TUSC4 into cells by nanoparticles not only increased p-ATM, p-CHK1, p-CHK2, γ-H2AX phosphorylated protein levels, which indicated the activation of DNA damage response, but also showed that reintroduction of NPRL2/TUSC4 arrested cells into G2/M phases after cisplatin treatment, suggested cells with NPRL2/TUSC4 would eventually undergo apoptosis process, which shed the light to overcome cisplatin resistance and promote clinical efficacy (Jayachandran et al., 2010).

Based on sequencing and bioinformatics analysis, it was believed an unknown protein-binding domain was contained at the N-terminal of NPRL2/TUSC4 protein. In 2008, Kurata and his colleagues found that

NPRL2/TUSC4 can physically interact with 3-phosphoinositide-depedent protein kinase-1 (PDK1) and prevent the binding of its co-activator Src to bind its phosphorylation sites at tyrosine-9,-373 and -376 residues, thus inhibit the activation of PDK1 and abolish the activity of AKT pathway, the depletion of NPRL2/TUSC4 expression also promoted the cell proliferation (Kurata et al., 2008). Furthermore, NPRL2/TUSC4 can also form complex with NPRL3 and DEPDC5, the inhibition of this complex negatively regulate mTROC1 pathway and leading to cells become more resistance to amino acid deprivation, and such cells are hyperactive in term of growth and hypersensitive to mTROC1 inhibitor rapamycin, this paper confirmed the tumor suppresor role of NPRL2/TUSC4 in the maintenance of genome stability and regulation of cell as well as tumor growth (Bar-Peled et al., 2013).

These studies consistently described that the inactivation of NPRL2/TUSC4 correlates with lower level of DNA damage response kinase activities and elevated cell proliferation, while overexpression of TUCS4 is associated with inhibition of tumor growth in various cancer types. Based on the accumulating evidences that NPRL2/TUSC4 plays tumor suppressor functions *in vitro* and *in vivo* to maintain genomic stability and inhibit tumorigenesis pathways by its binding domains, it's reasonable to suspect that NPRL2/TUSC4 deficiency would lead to a compromised DNA damage repair system such as HR repair and allow the potential increased sensitivity of cells to DNA damaging drugs and therapeutics. And most likely these regulation would occur through protein-binding regulation considering

NPRL2/TUSC4 doesn't contain the domains responsible for phosphorylation or other kinase activities.

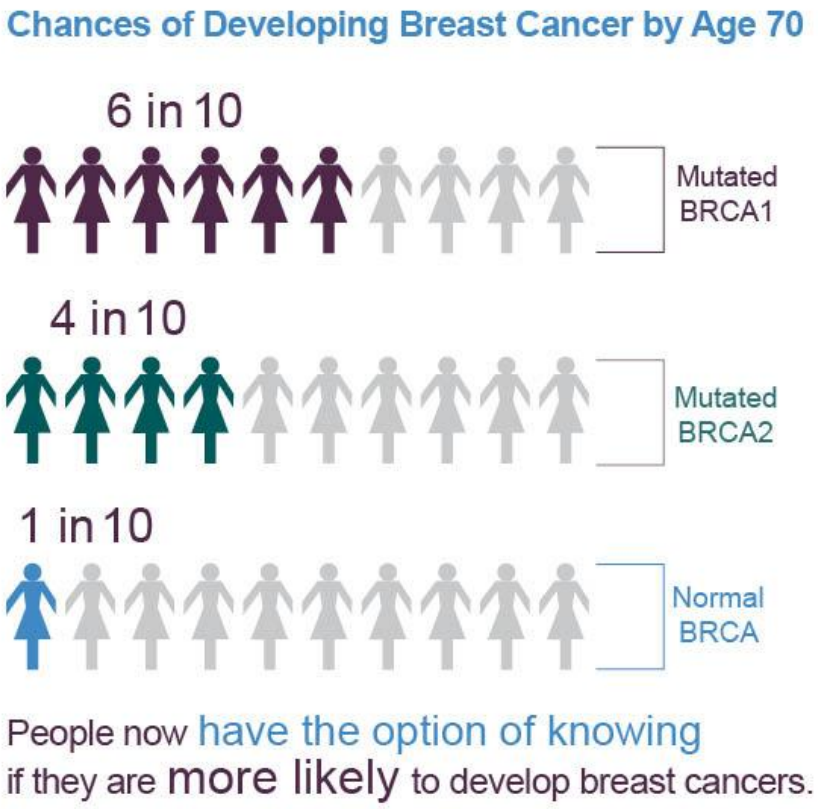



**Figure 2. BRCA1 mutations and breast cancer.**

BRCA1 mutated women have 60% possibility to develop breast cancer before the age of 70**,** while BRCA2 mutated women have 40% possibility and normal BRCA1 women have only 10%. *Adapted from www.cancer.gov/cancertopics/factsheet/Risk/BRCA.*

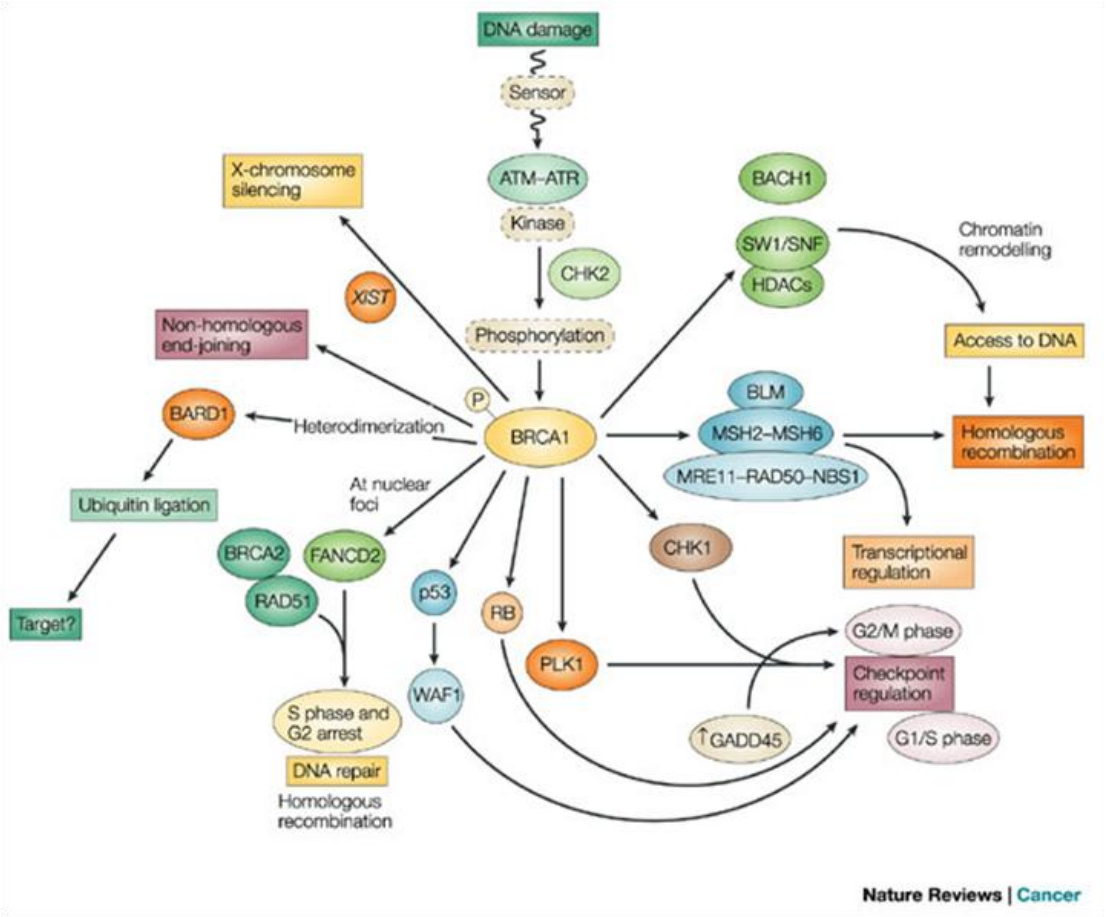



**Figure 3.BRCA1 and its function network.**

BRCA1 is associated with multiple pathways including DNA repair, cell-cycle checkpoint regulation, ubiquitylation and transcriptional regulation. DNA damage response triggers BRCA1 activation. Several damage sensors, including ATM/ATR are activated in response to DNA damage. CHK2 is activated to prevent cell division by mediating phosphorylation of BRCA1 and p53. BRCA2 and RAD51 can form complex with FANCD2, then binds to BRCA1 and promotes S-phase or G2 arrest. BRCA1 forms a heterodimer with BARD1 to activate the ubiquitin-ligase function. DNA repair by homologous recombination is mediated by the BRCA1-associated surveillance complex which contains of BLM, MSH2–MSH6 and MRE11–RAD50–NBS1. BRCA1 has been also shown to mediate X-chromosome silencing, and also to mediate non-homologous end joining during DNA repair. BRCA1 can form complexes to mediate chromatin remodeling and homologous recombination. BRCA1 interacts with CHK1 to regulate apoptosis.
*Adapted from Narod and Foulkes Nature Reviews Cancer 4, 665-676 (2010) with permission from Elsevier.*

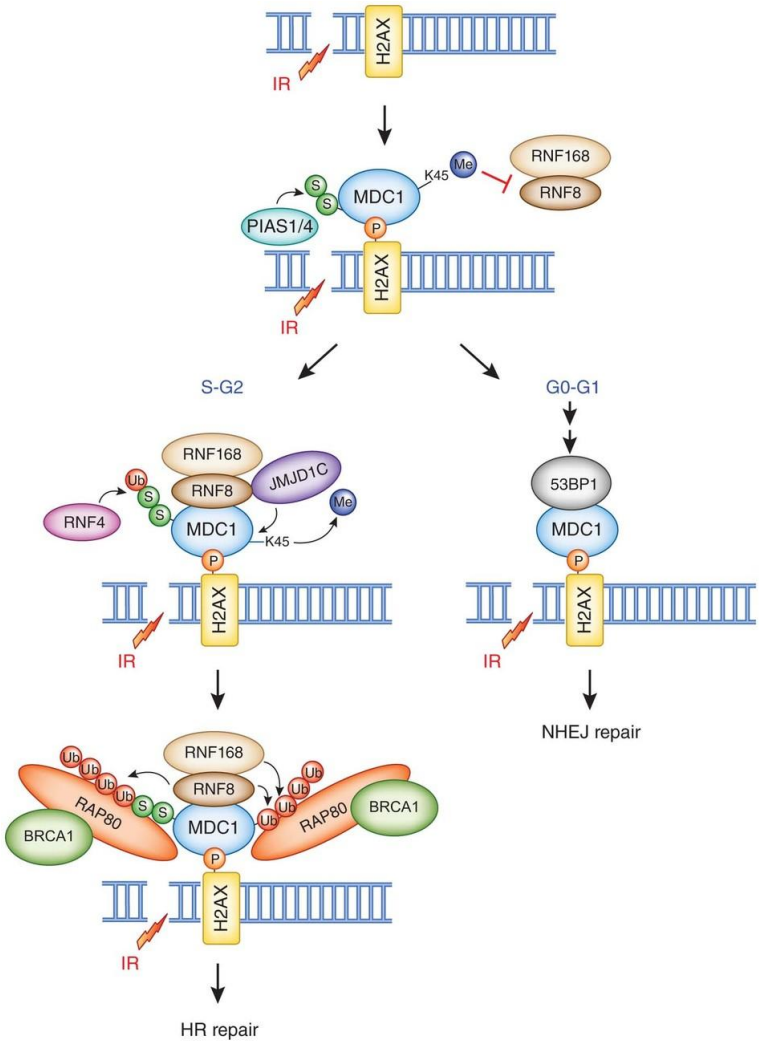



**Figure 4. Schematic Model for major homologous recombination (HR) and Non-homologous end-joining (NHEJ) repair.**

DSBs can be repaired by one of two pathways: homologous recombination (HR) or non-homologous end joining (NHEJ). Both pathways require recruitment of MDC1 and the RNF8 as well as RNF168 ubiquitin E3 ligase to DNA damage sites. The difference between the two pathways is that HR repair requires BRCA1 while HNEJ repair is dependent on 53BP1 recruitment. In HR repair, the RAP80–BRCA1 complex is recruited through 2 pathways: either it binds to K63-linked poly-ubiquitin chains attached to MDC1 or through interactions with hybrid SUMO-ubiquitin chains. Adapted from Lu and Matunis Nature Structural & Molecular Biology 12, 1346-1348 (2013) *with permission from Elsevier.*

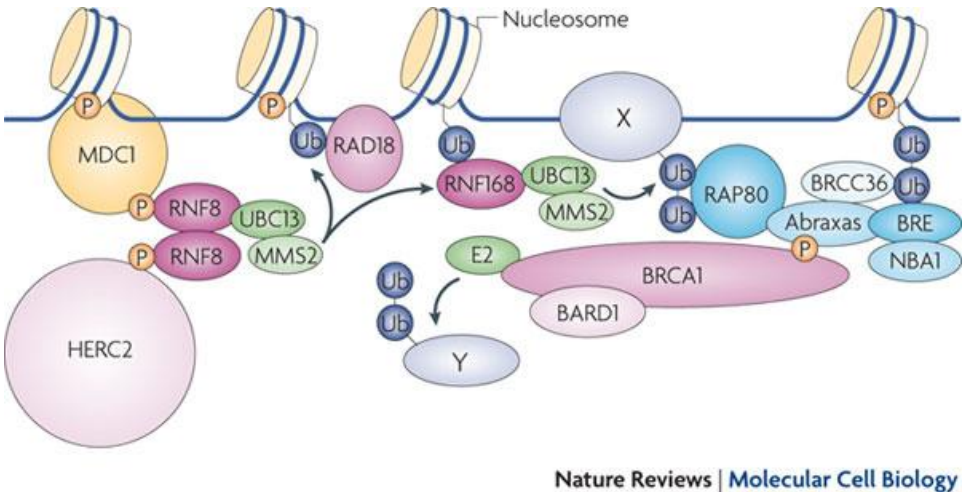



**Figure 5. Ubiquitin signaling of DNA double-strand breaks.**

The signaling cascade of ubiquitylation events. E3 ligases specifically target their substrates , examples are ( RING finger protein 8 (RNF8), HECT domain and RCC1-like domain-containing protein 2 (HERC2), RNF168, BRCA1 and BRCA1-associated RINg domain protein 1 (BARD1)) as shown above. E2 enzymes are the ubiquitin-conjugating enzyme including UBC13, MMS2 and an unspecified E2. X and Y stands for unidentified ubiquitylation targets. While E1 enzyme which is also known as ubiquitin-activating enzyme is not shown above.
*Adapted from Ulrich and Walden, Nature Reviews Molecular Cell Biology 11, 479-489 (2010) with permission from Elsevier.*

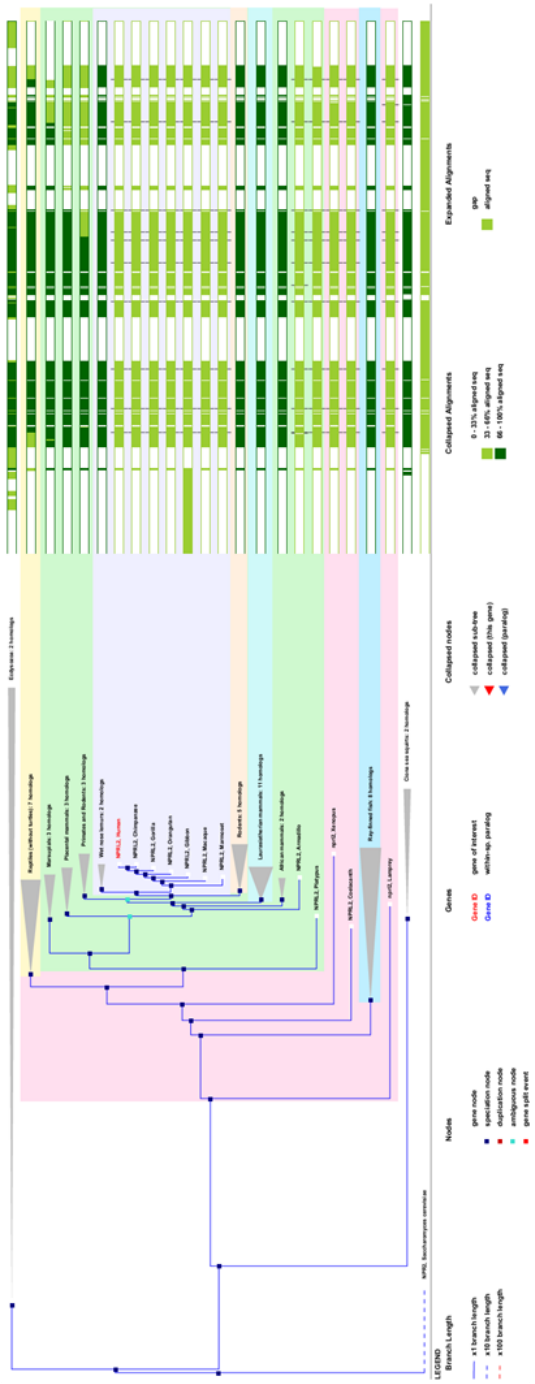


**Figure 6. NPRL2/TUSC4 gene tree.**

Human NPRL2/TUSC4 (red in figure) conserved across species ranging from yeast to chimpanzee with 33-66% aligned sequence. Image was generated by Ensembl genome browser , gene ID: ENSG00000114388

## Chapter 2

# MATERIALS AND METHODS

*Cell cultures and plasmid*

The U2OS, MDA-MB-231, and MCF-10A cell lines were purchased from the American Type Culture Collection. McCoy's 5A medium (CellGro;10-050-CV) supplemented with 10% fetal bovine serum was used to maintain U2OS cells, RPMI 1640 medium (Corning; 10-104-CV) supplemented with 10% fetal bovine serum was used to culture MDA-MB-231 cells, and serum-free mammary epithelial growth medium (Clonetics; CC-3051) containing insulin, hydrocortisone, epidermal growth factor, and bovine pituitary extract was used to maintain MCF-10A cells. All cells were incubated under humidified conditions in 5% $CO_2$. The pCMV5-3 ×Flag vector plasmid was kindly provided by Dr. Funda Meric-Bernstam (The University of Texas MD Anderson Cancer Center). The MD Anderson DNA Sequencing and Microarray Facility confirmed the identities of all plasmids.

*Antibodies and reagents*

An anti-NPRL2/TUSC4 antibody was purchased from Proteintech (10157-1-AP), an anti-BRCA1 antibody was purchased from Santa Cruz Biotechnology (sc-6954), and anti-Flag M2 (F3165) and anti-β-actin (A2066) antibodies were purchased from Sigma. Anti-HERC2 antibodies were purchased from BD Biosciences (612366). MG132 was purchased from EMD Biosciences (133407-82-6), and cycloheximide

was obtained from Sigma (C7698). G418 was purchased from Sigma (A1720). Full-length NPRL2/TUSC4 was amplified using a TOPO TA cloning kit for subcloning (Invitrogen; 45064) with the sense primer for the sequence of 5'-AATGGGCAGCGGCTGCCGCA-3' and anti-sense primer for the sequence of 5'-TCACTTCCAGCAGATGATGA-3'.

*RNA Extraction and RT-PCR*

RNA was extracted by TRIzol reagent (Life Technologies; 15596026) and reverse transcription was conducted using SuperScript III kit (Invitrogen), and BRCA1 was amplified using RT-PCR with the sense primer 5'-CAGCGATACTTTCCCAGAGC-3' and anti-sense primer 5'-CTTGTTTCCCGACTGTGGTT-3'. Cyclophilin was used as internal control.

*RNA interference*

Stable knockdown of NPRL2/TUSC4 expression was established via RNA interference using lentiviral vector short hairpin RNA (Sigma; MISSION; NM660545). NPRL2/TUSC4 was targeted with a lentiviral particle of MISSION short hairpin RNA as well as MISSION nontargeted control particles. Western blotting was performed after transduction to confirm the knockdown efficiency, and puromycin was added to U2OS cell medium to maintain the NPRL2/TUSC4-knockdown specificity. For transient transfection, human NPRL2/TUSC4 siRNA was purchased from Thermo Scientific (On-Target; 10641), and the NPRL2/TUSC4 target siRNA sequences were GCAUCGAACACAAGAAGUA and

GACCCAAGAUCACCUAUCA. Human BRCA1 siRNA was purchased from Thermo Scientific (On-Target; J-003461-09), and the BRCA1 target sequence was CAACAUGCCCACAGAUCAA. Human HERC2 siRNA was purchased from Thermo Scientific (On-Target; J-007180-09, J-007180-10, J-007180-11, and J-007180-12), and the HERC2 target sequences were 5'-GCACAGUAUCACAGGUA-3', 5'-CGAUGAAGGUUUGGUAUUU-3', 5'-GAUAAUACGACACAGCUAA-3', and 5'-GCAGAUGUGUGCUAAGAUG-3', respectively.

*Immonuprecipitation and Immunoblotting*

For immunoprecipitation of HERC2, BRCA1, and NPRL2/TUSC4, U2OS cells were first transfected with an empty vector or FLAG-NPRL2/TUSC4 plasmids. After 72 h of transfection, G418 was added to the medium for selection purposes. After stable clones were isolated from the pool, whole cellular extracts were incubated with RIPA buffer as described previously (Pierce et al,. 1999), and the products were immunoprecipitated with an anti-FLAG M2 Affinity gel (Sigma; A2220) for 8 h at 4°C. After washing, the complexes were eluted with 3×FLAG peptide and evaluated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). For immunoprecipitation of the binding between HERC2 and BRCA1, cell lysates were precleared with A/G Agarose beads (Santa Cruz Biotechnology; SC-2003) and incubated with 1 μg of antibody at 4 °C overnight. Precipitates were then washed and suspended in 5× SDS buffer and submitted to SDS-PAGE and immunoblotting. For immunoblotting, after samples were separated using

electrophoresis, membranes were blocked with 5% milk diluted in Tris buffer with 0.1% Tween 20 for 1h at room temperature. The primary antibody was diluted in 5% bovine serum albumin in phosphate-buffered saline with sodium azide (Sigma; S227) and then incubated with the membranes for 2 h at room temperature. Subsequently, membranes were washed with phosphate-buffered saline with 0.1% Tween 20 and incubated with secondary antibody. Finally, signals of the bound antibody were detected using enhanced chemiluminescence (GE Healthcare; RPN2232).

*In vitro proliferation and PARP inhibition assays*

MTT (Sigma; M5655) was used to evaluate the proliferation of cells. Briefly, cells were counted and seeded in a 96-well flat-bottomed plate. After 96 h, cells were incubated with MTT substrate (Sigma; 20 mg/ml) for 4 h, and the cultures were removed and replaced with dimethyl sulfoxide. The optical density was measured spectrophotometrically at 570 nm. The colony formation assay was performed by seeding 200 cells in six-well plates. Olaparib and rucaparib were added to the culture medium, and the cells were compared with untreated control cells. Colonies were scored after 3 weeks. All experiments were repeated three times.

*Microarray analysis*

mirVana RNA isolation kit (Ambion) was used to isolate total RNA. Five hundred nanograms of total RNA were used with a Sentrix Human-6 Expression Bead Chip (Illumina) for labeling and hybridization. BeadArray Reader was used for chip

scanning (Illumina). As described previously (Peng et al., 2014), the gene expression profile was subjected to normalization and log2 transformation. The NextGENe software program was used to identify genes whose expression differed in two clusters, and a *t*-test was used to separate genes with significantly different expression ($P < 0.001$). The Ingenuity Pathway Analysis system was used for gene enrichment analysis.

*Homologous recombination repair and flow cytometry analyses*

The plasmids DR-GFP, pCAGGS, and pCBASce were gifts from Dr. Maria Jasin (Memorial Sloan-Kettering Cancer Center). U2OS cells were first treated with NPRL2/TUSC4 and BRCA1 siRNA as well as control siRNA for 24 h. BRCA1-containing plasmids were then transfected into NPRL2/TUSC4-knockdown cells to induce re-expression of BRCA1. After 48-72 h, flow cytometry was performed to detect GFP-positive cells using a FACSCalibur and the CellQuest software program (Becton Dickinson). Three independent experiments were performed to obtain mean values and their standard deviations. Cell-cycle analysis was performed at the MD Anderson Cancer Center Flow Cytometry and Cellular Imaging Facility.

*Tumor growth in nude mice and immunohistochemistry*

Six-week-old female nude mice were used in this study. The MD Anderson Institutional Animal Care and Use Committee approved the animal protocol. $5 \times 10^6$ MDA-MB-231 cells with and without NPRL2/TUSC4 overexpression or $1 \times 10^7$

MCF-10A cells with and without knockdown of NPRL2/TUSC4 expression were injected to the mammary fatpads of mice. Tumors were measured from 1 week after MDA-MB-231 cells injection and monitored weekly, whereas MCF-10A tumors were observed 1 month after cell injection. At least five nude mice were used for each group. Human breast tissue samples (Biomax) were embedded in Xylene and 100%, 95%, 70%, 50% ethanol respectively for deparaffinization, slides were then incubated with NPRL2/TUSC4 antibody at 4º overnight followed by antigen retrieval. Then samples were processed and evaluated immunohistochemically under microscope after being dehydrated and stabilized.

*Transfection and ubiquitination assay*

U2OS cell transfection was conducted using Oligofectamine (Life Technologies; 12252-001). Plasmids encoding HA-tagged ubiquitin were transfected in U2OS cells with and without knockdown of NPRL2/TUSC4 expression. Forty-eight hours after transfection, cells were harvested and lysed with RIPA buffer. Cell lysates were then incubated with $Ni^{2+}$ beads (Amersham Pharmacia Biotech) to pull down histidine-tagged BRCA1 with the beads. Precipitated BRCA1 protein was isolated using SDS-PAGE and detected using an anti-HA antibody (Sigma; ab18181).

*Immunoflurosence foci staining*

Cells were cultured on cover slips in 6-well plates before staining for 24 hours, then growth medium was removed and cells were washed by ice-cold PBS twice. Then cells were incubated by cytoskeleton buffer (10mM PIPES, Ph6.8, NaCl 100mM,

Sucrose 300mM, $MgCl_2$ 3mM, EGTA 1mM,Triton X100/ 0.5%) for 3 min on ice, then cells were washed with PBS for 3 times and incubate in stripping buffer (Tris HCL 10mM, NaCl 10mM, $MgCl_2$ 3mM, Tween 20/1%, Sodium deoxychalate 0.25%) for 3 min on ice. After wash with PBS for 3 times, cells were then fixed in 4% PFA overnight at 4 degrees. After that, cells were permeabilized in 1% Triton X-100 and 0.5% NP-40PBS for 30 mins and blocked in 5% horse serum/1% goat serum mixture for 1 hour at room temperature. Samples were incubated with primary antibody in blocking buffer for 2 hours at room temperature, followed by 0.01% Triton-PBS wash and Fluor 488 and 594 conjugated secondary antibody (Life Technologies, 1:500) incubation for 1 hour at room temperature. Lastly, samples were mounted with DAPI antifade medium for microscope analysis.

*Microscopy analysis*

DNA damage foci staining images were captured with fluorescent microscope (Nikon, Eclipse E800). 60X oil objective lens (Plan Fluor, NA 1.3) and CCD camera (QImaging, Qiclick F-M-12) were equipped to facilitate the analysis and images capture were processed by the Nikon NIS-Elements system.

*HU recovery and checkpoint assay*

Cells were incubated in medium with or without HU (2 mM) for 24 hours. Then HU was removed, and cells were harvested at 0, 8, and 16 hours after release. Harvested cells were fixed in ethanol, stained with propidium iodide, and analyzed by FACS. For HU followed by Taxol, cells were untreated or treated with HU (2

mM) for 24 hours. Then HU was removed by replacing fresh medium, Taxol was added and harvested cells at 0, 8, 16 hours, cells were then fixed in ethanol and stained using phospho-histone H3 (Ser10)-specific antibody (p-H3) and propidium iodide (PI).

*Statistical Analysis*

All statistical analyses were performed with a two-tailed Student's *t*-test.

## CHAPTER 3

# NPRL2/TUSC4 REGULATES BRCA1'S STABILITY BY BLOCKING ITS PHYSICAL INTERACTION WITH HERC2

## Results

### NPRL2/TUSC4 expression is reduced in breast cancer and correlates with breast cancer progression

To characterize whether NPRL2/TUSC4 expression is associated with breast cancer, we performed Western blotting to measure the expression of NPRL2/TUSC4 in non-transformed breast cell lines, including HMEC, MCF-10A, and MCF-12A, and breast cancer cell lines with both luminal and basal subtypes (Figure 7A). We observed that NPRL2/TUSC4 expression was markedly higher in the non-transformed cell lines than that in breast cancer cell lines. We also evaluated NPRL2/TUSC4 expression in normal breast tissue and breast carcinomas using immunohistochemical staining, and found that NPRL2/TUSC4 expression was lower in the tumors than that in matched adjacent normal breast tissue (Figure 7B). Furthermore, The Cancer Genome Atlas-based analysis of mRNA expression in invasive breast carcinomas demonstrated a significant difference between the survival rates in patients with unaltered NPRL2/TUSC4 expression and those with downregulated NPRL2/TUSC4 expression ($P$ = 0.000005). Specifically, the survival rate of 100 months was 24.7% in 889 patients with downregulation of NPRL2/TUSC4 expression (Z-score threshold, ±1), while the survival rate in these patients dropped sharply to 0% after 100 months. In

comparison, the survival rate was 40% in patients with unaltered NPRL2/TUSC4 expression after 200 months, with a *P* value less than 0.0001 (Figure 8A). Comparison of patients with upregulated and unaltered NPRL2/TUSC4 expression did not demonstrate any significant differences in survival rate (Figure 8B). Total of 889 breast carcinoma patients has 65.20% without TUSC4 alteration, 24.70% with TUSC4 level down-regulation, 9.40% up-regulation and 0.6% mutation (Figure 8C). These results strongly suggested that low NPRL2/TUSC4 expression is associated with the cancer phenotype, indicating that NPRL2/TUSC4 may play an important role as a tumor suppressor in breast cancer patients. Based on the sequencing data of first 467 patients, there were 79 basal-like breast carcinoma patients, 203 luminal A patients and 112 luminal B patients which account for 16.9%, 43.5%, 24% of the total patients number respectively. Further sequencing data is necessary to complete the subtypes profiles for the rest of the patients. Future studies will need to establish the relationship between TUSC4 expression level and different breast cancer subtypes in terms of patients' survival rates.

To systematically evaluate the tumor-suppressive function of NPRL2/TUSC4, we performed microarray analysis comparing NPRL2/TUSC4-knockdown cell lines and control cell lines (Figure 9B). We then examined the differentially expressed genes in these cells using the Ingenuity Pathway Analysis system (QIAGEN). Comparison of the NPRL2/TUSC4-knockdown and control cells ranked cancer as one of the top disease and disorder pathways, further suggesting that NPRL2/TUSC4 functions as a tumor suppressor gene (Figure 9C). Additionally, high portion of genes in NPRL2/TUSC4-deficient gene signatures

were involved in canonical pathways such as DNA damage response and breast cancer regulation (Figure 9D).

Owing to the low NPRL2/TUSC4 expression in breast cancer and the association with poor breast cancer patient survival rates, we explored the role of NPRL2/TUSC4 in the development of breast cancer and the possible functional pathways that NPRL2/TUSC4 is involved in. It has been previously reported that multiple cancers have mutations in or epigenetically silenced HR related genes, which indicated potential association between HR repair deficiency and cancer development (Deng and Wang 2003; Lord and Ashworth 2002 ). Thus, we suspected that low expression of NPRL2/TUSC4 contributes to the deficiency of HR repair, which also drives genomic instability in breast cancer development. We performed a cluster analysis of a NPRL2/TUSC4-knockdown microarray signature with previously established HR repair deficiency (HRD) gene signatures (Peng et al, 2014). The heat map demonstrated that NPRL2/TUSC4-deficient cells formed a cluster with HRD gene signatures (Figure 9A), whereas the control cells separated from NPRL2/TUSC4-knockdown samples. Considering the fact that the HRD signature described above was discovered under the condition of loss of tumor suppressor BRCA1, the results suggested a potential correlation and molecular similarity between NPRL2/TUSC4 knockdown cells and BRCA1-deficient HR repair deficiency.

By analyzing the well defined triple negative breast cancer cell lines makers, we also identified that NPRl2/TUSC4 has lower expression in basal-like cell lines compare to luminal types (Figure 9 E,F). Furthermore, RPPA analysis indicated

knockdown of TUSC4 U2OS cells exhibited similar gene clusters with BRCA1 knockdown cells compare to control cells (Figure 9G).

**NPRL2/TUSC4 is widely expressed in both cytoplasm and cellular nucleus**

We next examined the distribution of NPRL2/TUSC4 in cells. First, by damage foci staining, we found NPRL2/TUSC4 can form DNA damage foci in cells after both IR and UV, indicated the potential functions of NPRL2/TUSC4 in DNA damage response pathways (Figure 10). Additionally, chromatin fractionation assay indicated NPRL2/TUSC4 is widely distributed in both cytoplasm and nucleus, furthermore, data also identified NPRL2/TUSC4 is a chromatin binding protein, further suggested its possible functions in DNA damage response (Figure 11).

**NPRL2/TUSC4 knockdown impairs HR repair by downregulation of BRCA1 expression**

The major conserved pathway used in mammalian cells to maintain genetic integrity and DNA fidelity is HR repair (Sung and Klein 2006; Lieber et al., 2003). Here, we have identified an association between expression profile of NPRL2/TUSC4 knockdown and BRCA1 deficient HRD gene signature. We suspected that loss of NPRL2/TUSC4 will also affect the foci formation of BRCA1, so we carried out phenotypic examination to test whether NPRL2/TUSC4 is required for BRCA1 foci formation by immunostaining. We performed BRCA1 foci staining followed by IR and UV irradiation. NPRL2/TUSC4 knockdown significantly

demolished the BRCA1 foci formation after irradiation, whereas control small interfering RNA (siRNA) did not affect the formation of BRCA1 foci (Figure 12, 13). We further evaluated HR repair efficiency by the standard HR repair analysis system (Pierce et al, 1999; Peng et al., 2009) with NPRL2/TUSC4-deficient U2OS model cells. More specifically, the DR-GFP reporter substrate was incorporated into cellular genomic DNA. SceGFP contains an I-SceI endonuclease site within the coding region, which abolishes GFP expression. iGFP is a truncated GFP, which contains homologous sequence for the SceGFP. Expression of I-SceI induces a single DSB in the genome. in normal cells, this DSB can be repaired by HR repair , the expression of GFP can be restored, while in HR deficient cells, the DSB cannot be repaired, the expression of GFP will be significantly reduced and analyzed by flow cytometry to indicate the efficiency of HR repair (Figure 14B). We found that NPRL2/TUSC4-knockdown cells had a significant decrease of HR reporter activity compared to control cells, which suggested impaired HR repair efficiency (Figure 14A). We used BRCA1-knockdown cells as a positive indicator of homologous recombination repair to confirm the HR repair efficiency. NPRL2/TUSC4-knockdown cells presented comparative reduction in HR repair efficiency as BRCA1-knockdown cells, which were around 40-50% lower than that in control cells. To confirm that the defective HR repair efficiency was not caused by transfection efficiency or inaccurate efficiency from I-*Sce*I, we reintroduced BRCA1 expression into the NPRL2/TUSC4-knockdown cells and observed a significant increase in HR repair efficiency over that in NPRL2/TUSC4-knockdown-only cells ($P < 0.05$).

Surprisingly, we found that knockdown of NPRL2/TUSC4 expression reduced the BRCA1 protein expression (Figure 14C), indicating that the decrease in HR repair efficiency in the NPRL2/TUSC4 knockdown cells may have resulted from abnormal BRCA1 protein expression. These results are consistent with our above findings that NPRL2/TUSC4-knockdown cells have HRD gene expression patterns similar to those in BRCA1-deficient cells which were used to generate our HRD gene signatures. These results revealed for the first time a novel function of NPRL2/TUSC4 in that disruption of its expression decreases BRCA1 expression and functions. Furthermore, BRCA1 and NPRL2/TUSC4 knockdown significantly reduced the HR repair efficiency compare to control, while NPRL2/TUSC4 knockdown with BRCA1 overexpression restored the HR repair, double knockdown of both genes further reduced HR repair efficiency (Figure 27).

Additionally, about 1% of patients carry BRCA1 mutation, and the log-odds ratio between BRCA1 mutation and down-regulated NPRL2/TUSC4 is 0.875, which indicates the tendency of co-occurrence. However, the p-value is 0.102, suggesting there in no significant correlation. Thus, the BRCA1 mutation and down-regulated NPRL2/TUSC4 are not mutually exclusive nor co-occurring.

**NPRL2/TUSC4 regulates BRCA1 protein stability**

We then investigated whether BRCA1 mutation is associated with NPRL2/TUSC4 expression level, as we expected, NPRL2/TUSC4 is not correlated with BRCA1 mutation in breast cancer patients (Figure 15A). We next sought to determine how NPRL2/TUSC4 affects BRCA1 protein expression. To that end, we

first sought to determine whether reduced BRCA1 protein expression after NPRL2/TUSC4 knockdown was caused by altered cell-cycle distribution because BRCA1 expression has known to be cell-cycle regulated. We carried out a cell-cycle analysis and did not observe a significant difference in G1-, G2/M-, or S-phase distribution between control and NPRL2/TUSC4-knockdown cells (Figure 15B), indicating that decreased BRCA1 expression after NPRL2/TUSC4 knockdown was not resulted from the cell-cycle shift. To further determine whether such changes occur through transcriptional regulation, we performed quantitative reverse transcriptase-polymerase chain reaction to measure the BRCA1 mRNA expression in control cells and cells with NPRL2/TUSC4 knockdown. We identified no significant BRCA1 mRNA differences after NPRL2/TUSC4 knockdown (Figure 15C), thus ruling out the possibility that BRCA1 expression by NPRL2/TUSC4 was regulated at the mRNA level**.**

Next, we sought to determine if NPRL2/TUSC4 regulates BRCA1 protein stability. To answer this question, we conducted BRCA1 protein stability experiments by treating control and NPRL2/TUSC4-knockdown U2OS cells with cycloheximide**,** for the purpose of blocking protein synthesis. As shown in Figure 16A, NPRL2/TUSC4 knockdown reduced the half-life of BRCA1 from about 20 h to less than 6 h, suggesting that NPRL2/TUSC4 plays an essential role in stabilizing BRCA1 at the protein level (Figure 16 B,C). Additionally, to determine whether BRCA1 protein stability is regulated by NPRL2/TUSC4 via the proteasome pathway, we treated control and NPRL2/TUSC4-knockdown U2OS cells with the proteasome inhibitor MG132. As shown in Figure 17, MG132-based treatment

restored the BRCA1 expression in cells with NPRL2/TUSC4 knockdown but only slightly increased the BRCA1 protein expression in control cells. This result suggested that NPRL2/TUSC4 regulates BRCA1 protein stability via the proteasome-dependent pathway.

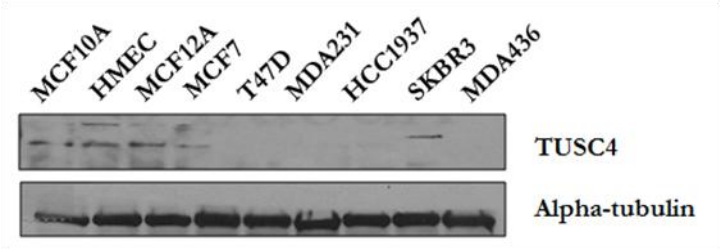
**NPRL2/TUSC4 regulates BRCA1 protein stability via ubiquitination pathway**

It has been previously reported that HERC2 is an E3 ligase that targets BRCA1 for degradation **(**Jin et al., 1997). To determine whether NPRL2/TUSC4 regulates BRCA1 stability via HERC2, we performed ubiquitination assay by transfecting hemagglutinin (HA)-tagged ubiquitin plasmids into cells with or without NPRL2/TUSC4 knockdown, followed by immunoprecipitation with control IgG or an anti-BRCA1 antibody. Surprisingly, we observed no signs of ubiquitination regardless of the NPRL2/TUSC4-knockdown status. However, after treatment with MG132, Western blotting for HA-tagged ubiquitin showed that NPRL2/TUSC4-knockdown cells underwent heavy ubiquitination, whereas control cells exhibited only a light polyubiquitination ladder (Figure 18), indicating that knockdown of NPRL2/TUSC4 expression caused a robust increase in BRCA1 protein polyubiquitination. We also confirmed previous findings that downregulation of expression of HERC2 led to increased expression of BRCA1 regardless of the presence of NPRL2/TUSC4 (Figure 19A, B). Under both conditions, BRCA1 expression was markedly upregulated after depletion of HERC2.

The next question to be answered was whether NPRL2/TUSC4 participates in ubiquitination of BRCA1 via HERC2 indirectly or stabilizes BRCA1 directly. We

performed immunoprecipitation with established NPRL2/TUSC4-overexpressing U2OS cell lines to determine the relationships among NPRL2/TUSC4, BRCA1, and HERC2. Reciprocally, we found that NPRL2/TUSC4 physically interacts with HERC2 but not with BRCA1 (Figure 20), which strongly suggested that NPRL2/TUSC4 regulates BRCA1 stability via interaction with HERC2. Considering the previously reported binding functions of NPRL2/TUSC4 (Ueda et al., 2006; Jayachandran et al, 2010) (Figure 22), we suspected that NPRL2/TUSC4 may prevent physical interaction between BRCA1 and HERC2. The binding between these two proteins (Wu et al, 2010) has been previously described, so we performed further immunoprecipitation to determine whether overexpression of NPRL2/TUSC4 weakens this binding. As shown in Figure 21, endogenous HERC2 physically bound to BRCA1. Intriguingly, overexpression of NPRL2/TUSC4 interrupted the binding between HERC2 and BRCA1, indicating that NPRL2/TUSC4 may regulate BRCA1 stability by preventing physical interaction between BRCA1 and HERC2. Additionally, BRCA1 expression level is negatively associated with HERC2 expression level in breast cancer cell lines (Figure 27).

**A.**




**B.**

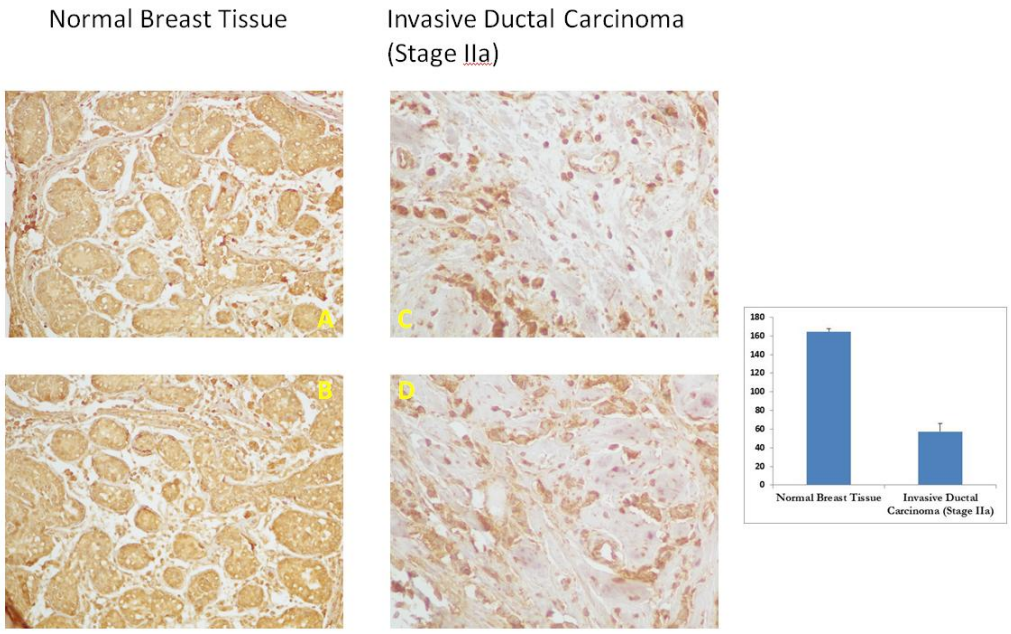
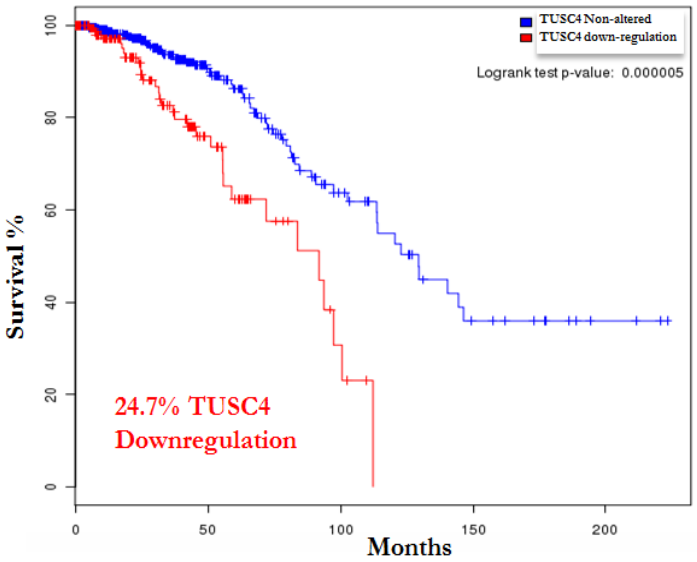


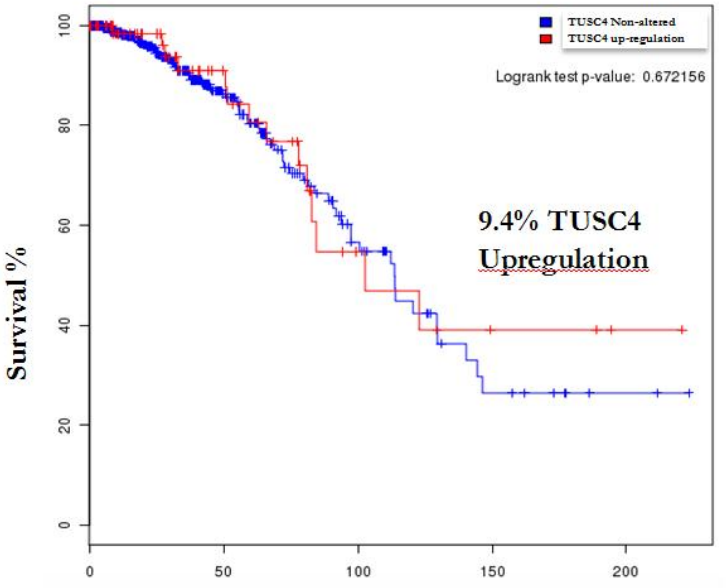

**Figure 7. Low NPRL2/TUSC4 expression level in breast cancer**

A. Lower NPRL2/TUSC4 expression levels was found in both luminal and basal types of breast cancer cell lines, while non-transformed breast cell lines (HMEC, MCF-10A and MCF-12A) exhibited higher NPRL2/TUSC4 level.
B. IHC staining indicated normal breast tissue (left) expressed higher NPRL2/TUSC4 protein level than breast cancer tissues (right), and bar graphs also indicated the quantified NPRL2/TUSC4.

**A.**


TUSC4 Non-altered
TUSC4 down-regulation
Logrank test p-value: 0.000005
Survival %
Months
24.7% TUSC4
Downregulation
0
20
40
60
80
100
0
50
100
150
200

B.


TUSC4 Non-altered
TUSC4 up-regulation
Logrank test p-value: 0.672156
Survival %
9.4% TUSC4
Upregulation
0
20
40
60
80
100
0
50
100
150
200

C.

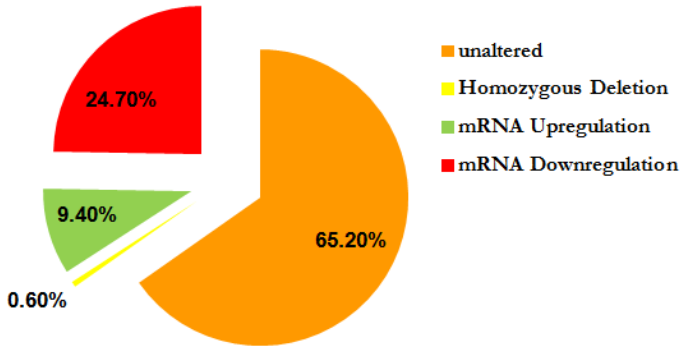
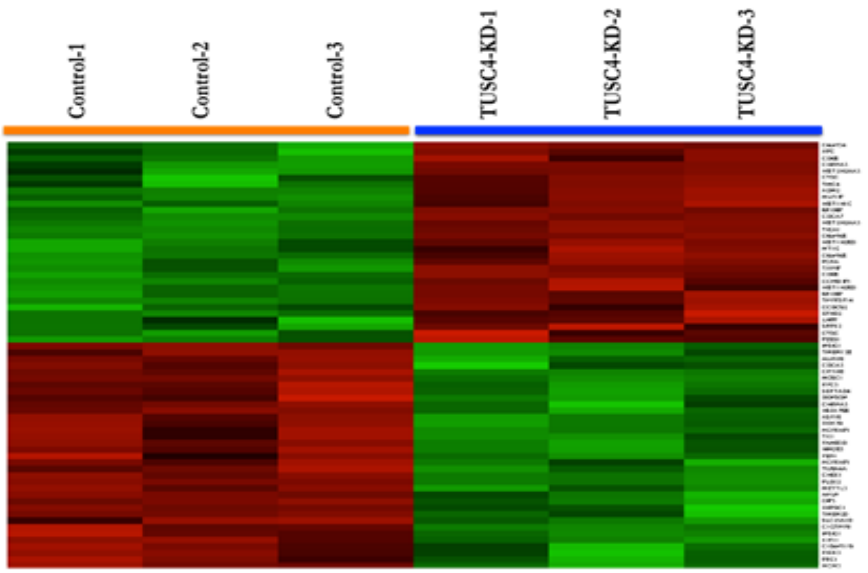


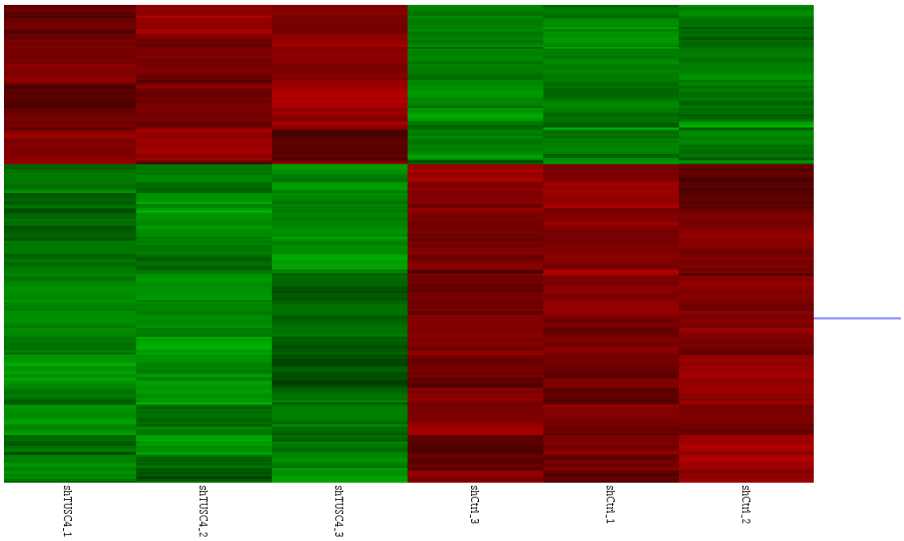

**Figure 8. Low NPRL2/TUSC4 expression level is associated with poor survival rate**

A. 24.7% breast cancer patients showed low NPRL2/TUSC4 expression level, and the low NPRL2/TUSC4 level correlates with poor survival rate ($p$=0.00005, data adapted from cBio portal for Cancer Genomics). B.9.4% of up-regulation of NPRL2/TUSC4 expression patients out of 889 in total didn't show significant change of survival rate compared to TUSC4 non-altered patients, with $p$ value of 0.672; C.total of 889 breast carcinoma patients has 65.20% without NPRL2/TUSC4 alteration, 24.70% with NPRL2/TUSC4 level down-regulation, 9.40% up-regulation and 0.6% mutation.

**A.**


Control-1
Control-2
Control-3
TUSC4-KD-1
TUSC4-KD-2
TUSC4-KD-3

**B.**


shTUSC4.1
shTUSC4.2
shTUSC4.3
shCtrl.3
shCtrl.1
shCtrl.2

**C.**

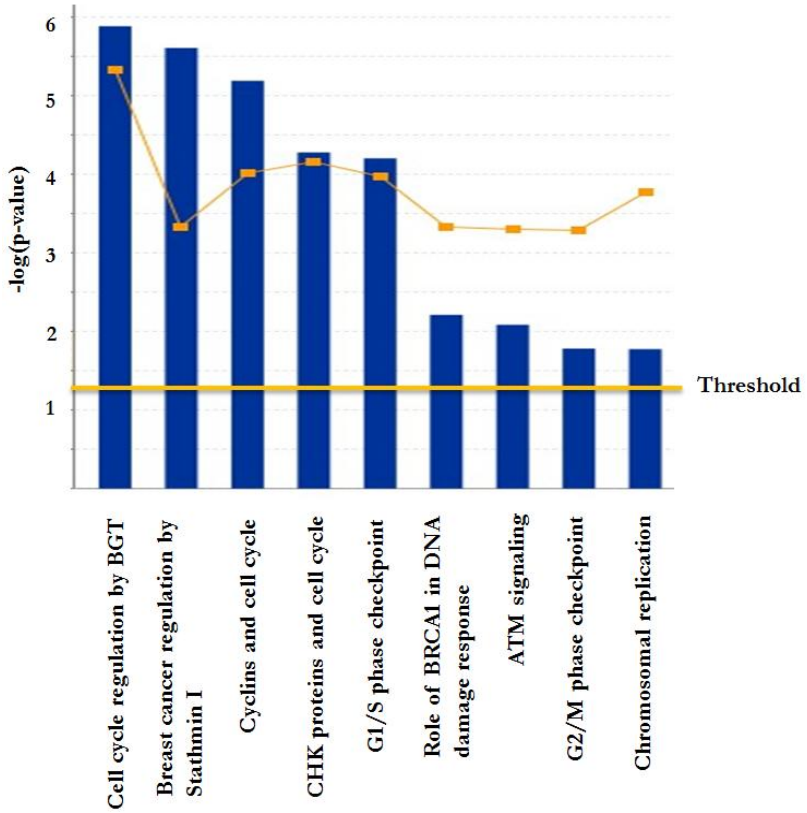
**Diseases and Disorders**

| Name | p-value |
|---|---|
| Cancer | 6.47E-05 - 2.56E-02 |

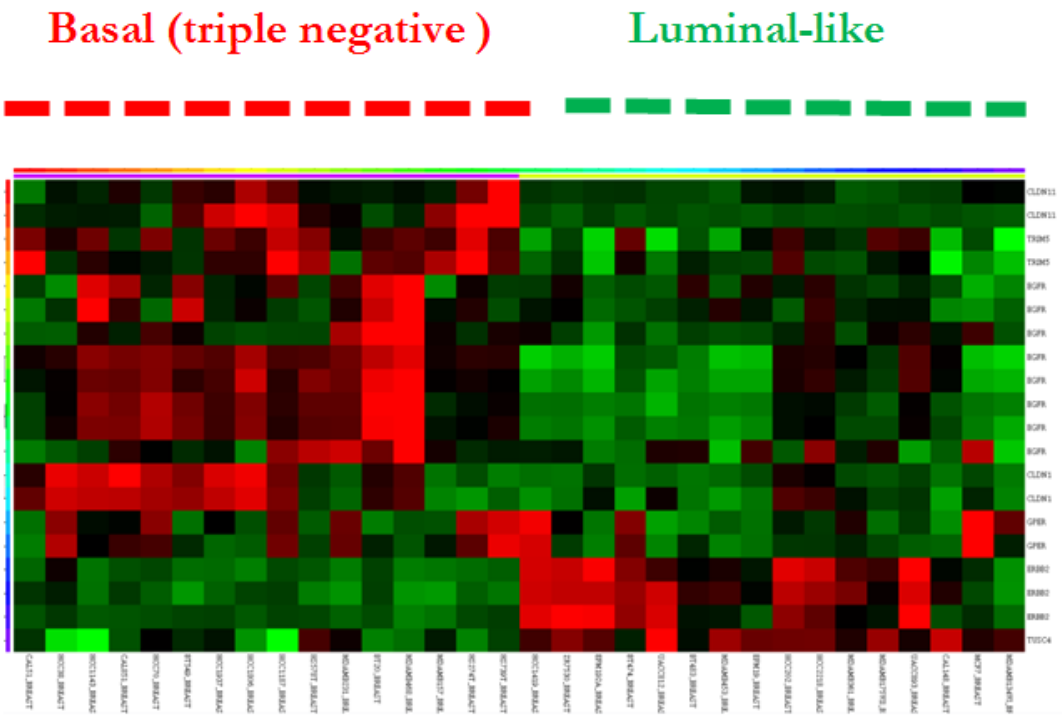
**Molecular and Cellular Functions**

| Name | p-value |
|---|---|
| Cell Death and Survival | 7.38E-06 - 2.71E-02 |
| Cell Cycle | 1.54E-04 - 2.67E-02 |
| Cellular Development | 4.28E-04 - 2.54E-02 |
| Cellular Assembly and Organization | 7.10E-04 - 2.41E-02 |
| Cellular Function and Maintenance | 7.10E-04 - 2.21E-02 |

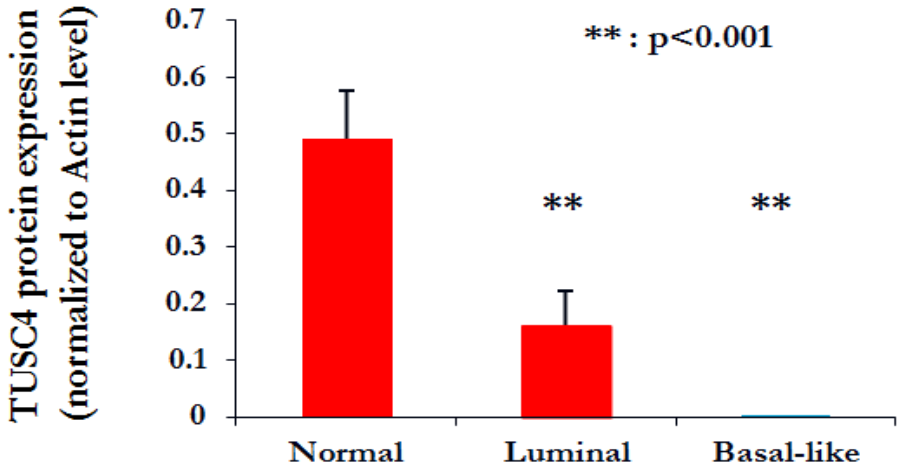
**D**



**E**


Basal (triple negative )
Luminal-like

**F**


TUSC4 protein expression
(normalized to Actin level)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
** : p<0.001
**
**
Normal
Luminal
Basal-like

**G**

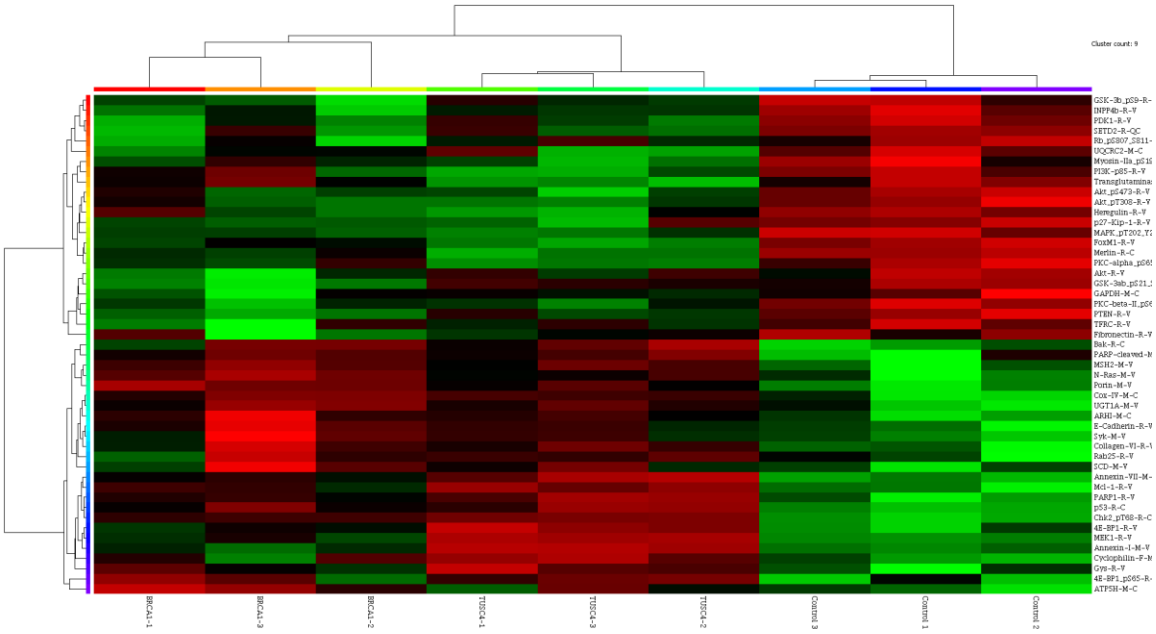


**Figure 9. NPRL2/TUSC4 is associated with HR defect genes and breast cancer phenotypes.**

A**.** NPRL2/TUSC4 knockdown microarray gene expression profiles were clustered with previously identified 230 homologous recombination defect gene signature, genes with $p$<0.001 and log ratio 0.1 were separated to generate heat map between NPRL2/TUSC4 knockdown cells and control cell lines.

B. Gene expression analysis identifies NPRL2/TUSC4 knockdown gene signature. The analysis was performed using NexusExp3 array tools. Heat map of the NPRL2/TUSC4 knockdown gene signature, consisting of 263 genes whose expression differed from control cell lines. Microarray was conducted in three independent samples of control cells and three independent samples of NPRL2/TUSC4 knockdown cell line. Student's t-test was conducted between the average of control cells and that of knockdown cells ($p$<0.00001).

C. IPA analysis of NPRL2/TUSC4 knockdown cells' top pathways changed.

D. Top canonical pathways in terms of number of genes in NPRL2/TUSC4-deficient gene signature on analysis with Ingenuity Pathway Analysis Software. Genes with ±1.25 fold change and $p$ value less than 0.05 were selected and

imported into IPA system. Significance refers to the log (*p* value), which was calculated by IPA program using Fisher's exact test. Threshold is at *p*= 0.05.

E. NPRL2/TUSC4 is associated with different phenotypes of breast cancer cell lines.

F. Low NPRL2/TUSC4 expression level is associated with breast cancer phenotypes. Normal or non-transformed breast cell lines has relatively higher expression level than luminal and basal-like breast cancer cell lines, the p value is less than 0.05.

G. RPPA analysis indicated NPRL2/TUSC4 knockdown cells shared similar cluster patterns with BRCA1 knockdown cells.

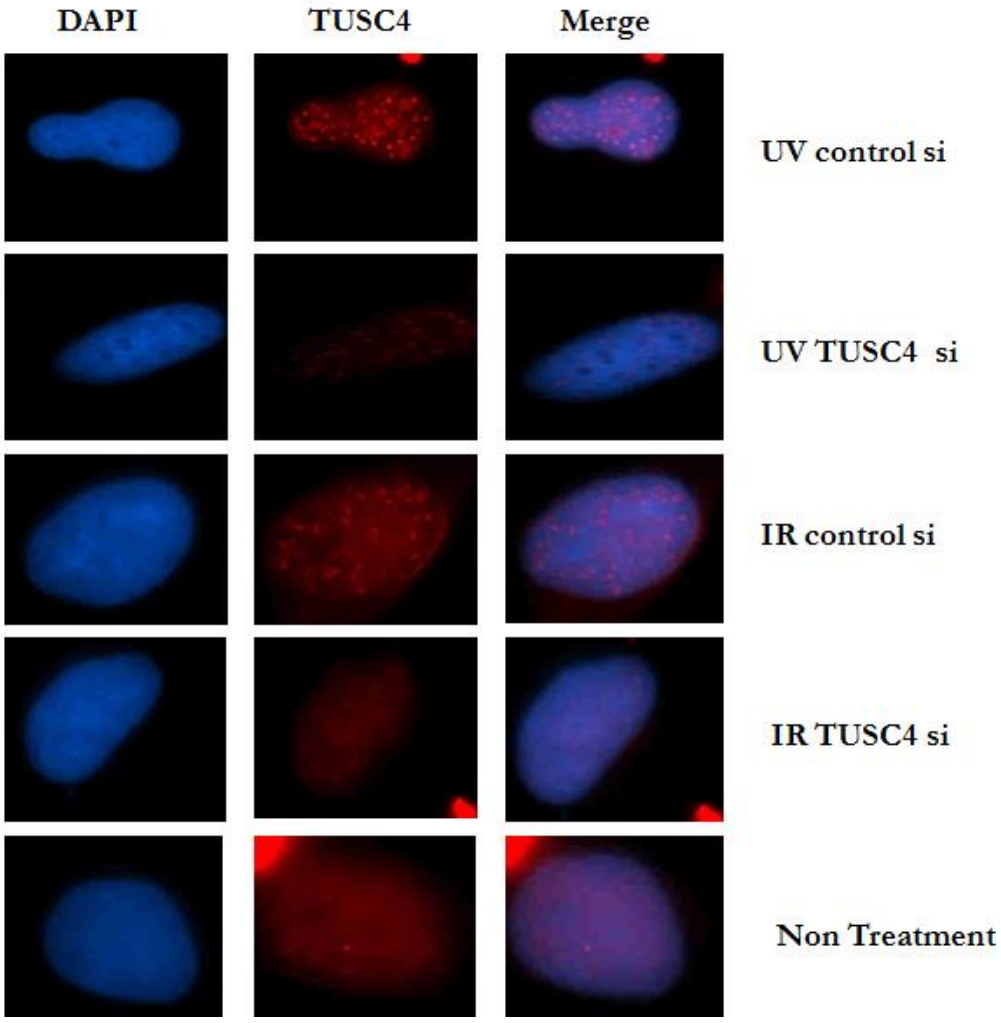



**Figure 10. NPRL2/TUSC4 forms DNA damage foci after irradiation**

NPRL2/TUSC4 forms DNA damage foci after both types of irradiation in U2OS cells, under the conditions of both IR and UV, while the foci were not observed in the NPRL2/TUSC4 knockdown cells, as well in the non treated cells.

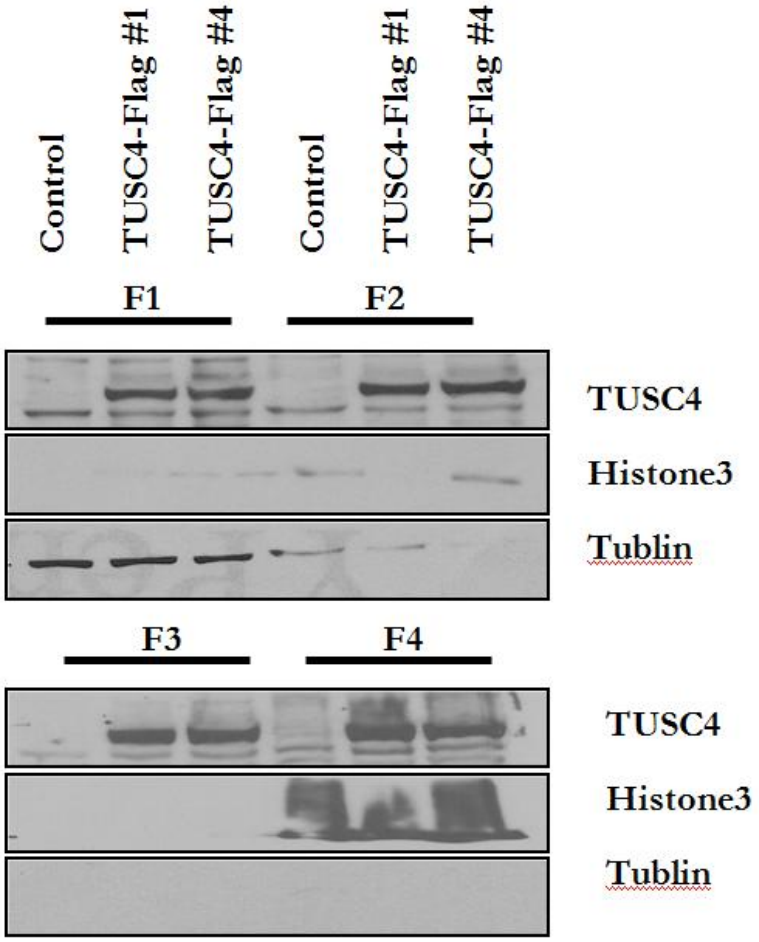



**Figure 11. NPRL2/TUSC4 widely spreads in both cytoplasm and nuclear**

Nuclear fractionation definitions are described below, F1-Cytoplasm, F2- Washing supernatant from Cytoplasm, F3-Soluble Chromatin proteins, F4-Chromatin bound proteins. U2OS control and NPRL2/TUSC4 Flag tag overexpression cells were used from left to right.

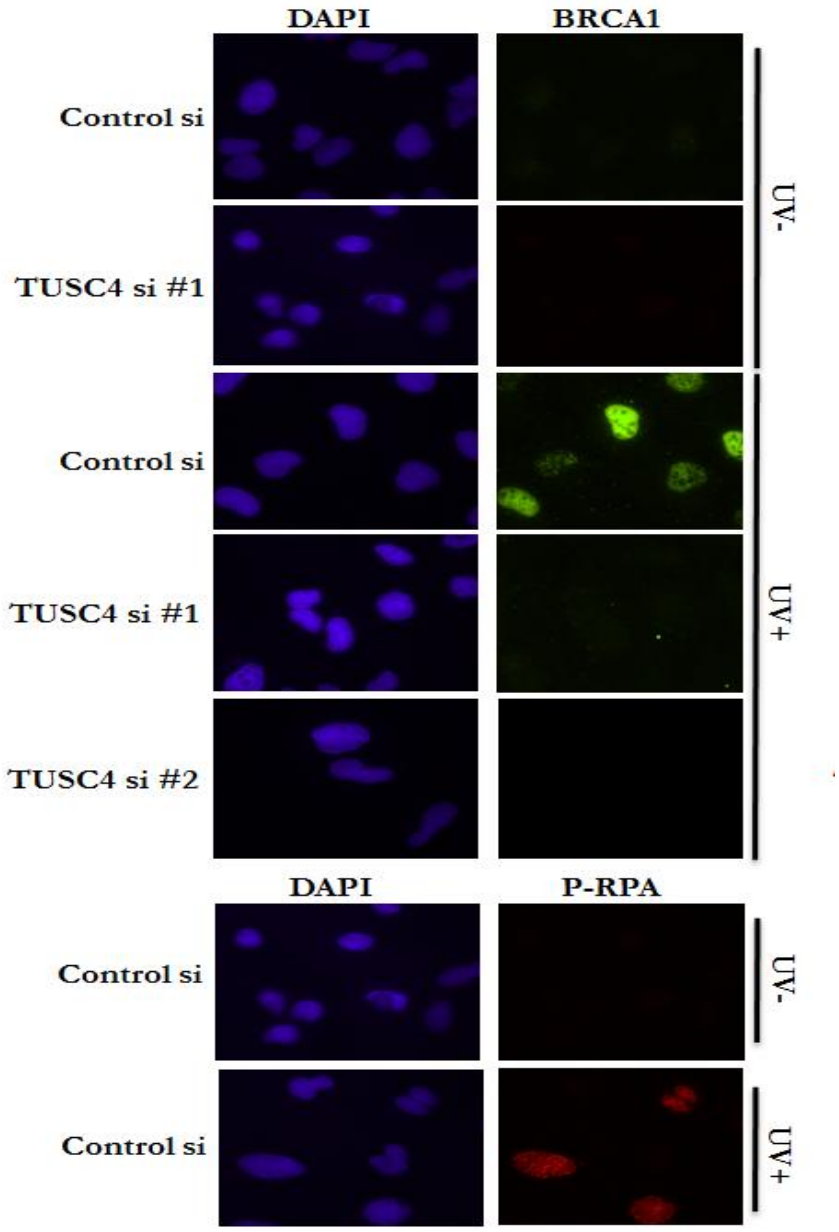



**Figure 12. NPRL2/TUSC4 knockdown reduced BRCA1 protein foci after UV**

NPRL2/TUSC4 knockdown reduced BRCA1 foci formation after UV, while control cells (NT) didn't have such effect. p-RPA indicated the efficiency of irradiation.

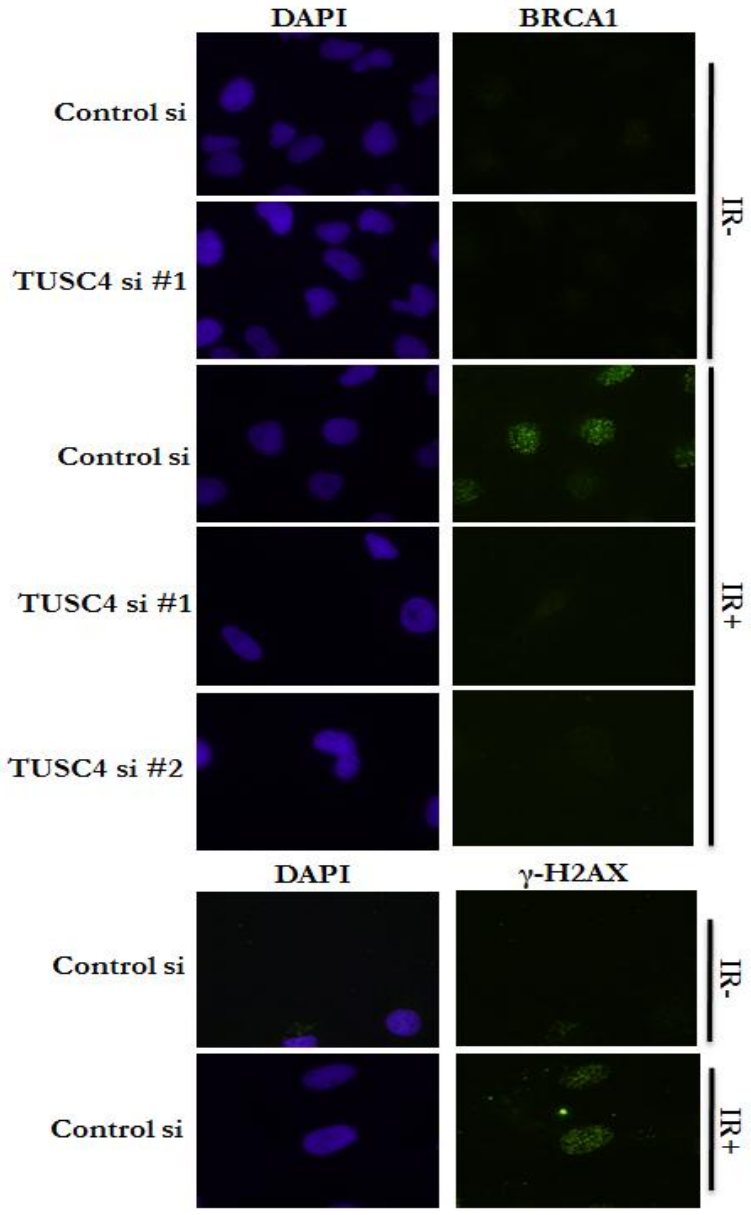
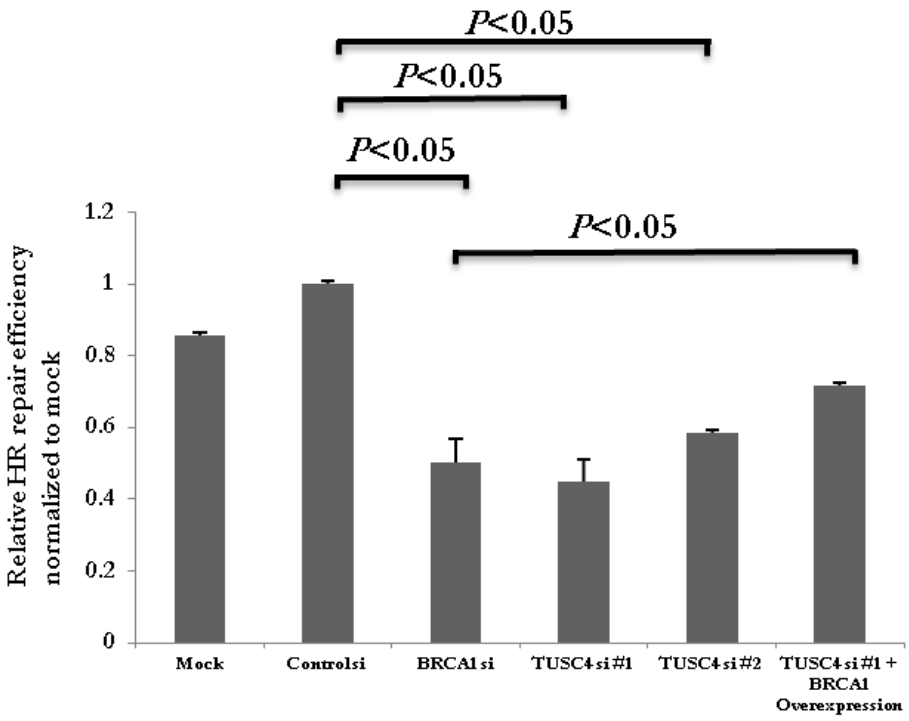



**Figure 13. NPRL2/TUSC4 knockdown reduced BRCA1 protein foci after IR**

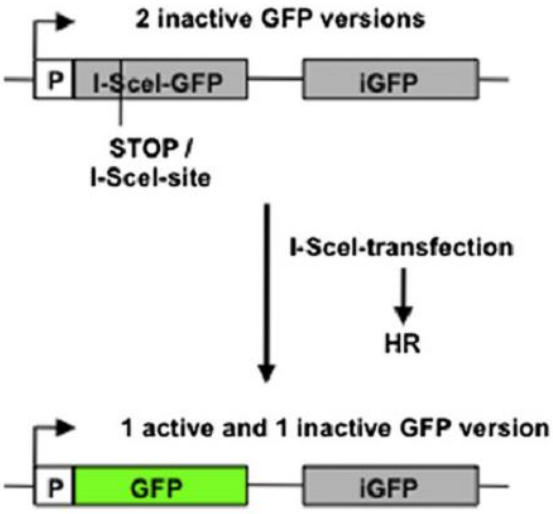
NPRL2/TUSC4 knockdown reduced BRCA1 foci formation after IR, while control cells didn't have such effect (NT). γ-H2AX indicated the efficiency of irradiation.

**A.**




**B.**

**NPRL2/TUSC4 knockdown reduced HR repair efficiency and BRCA1 protein expression level**



**C.**

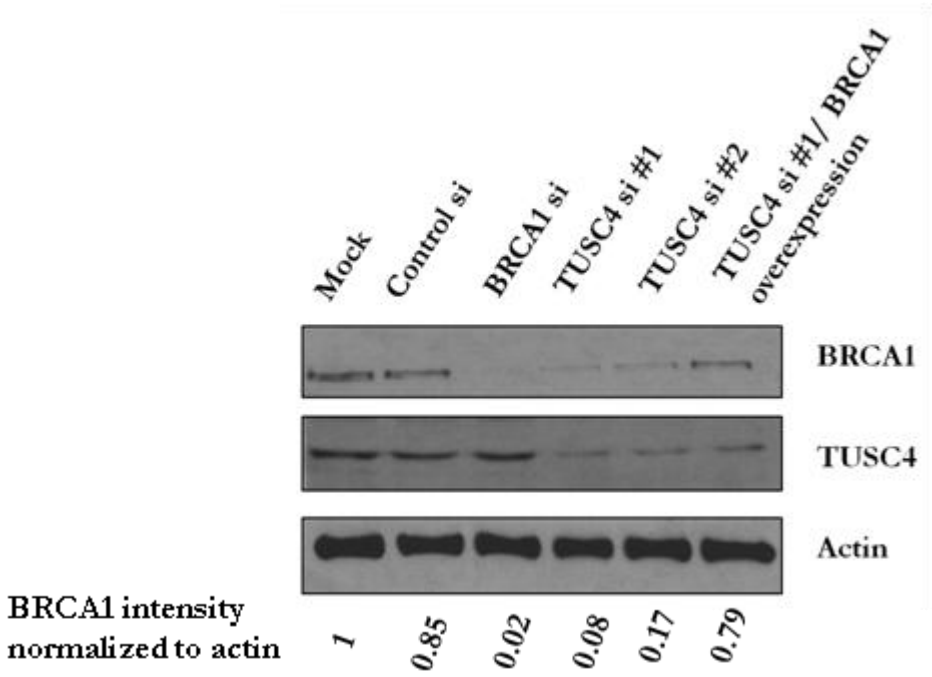
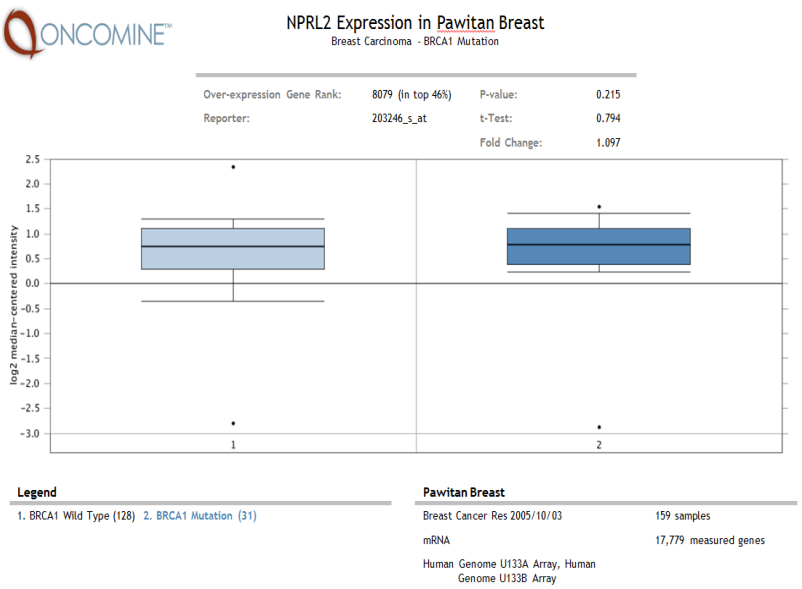


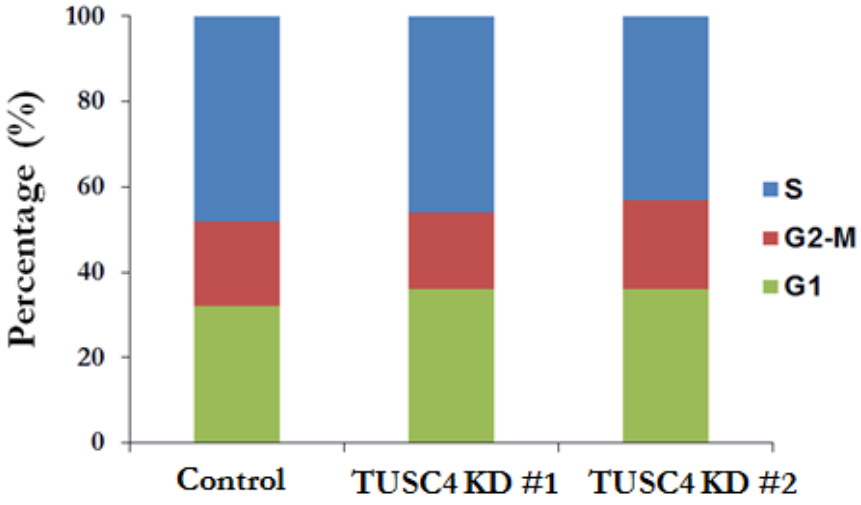

**Figure 14. NPRL2/TUSC4 knockdown reduced HR repair efficiency and BRCA1 protein expression level**

A. NPRL2/TUSC4 knockdown significantly reduced HR repair efficiency compared to control ($p$<0.05), while reintroduction of BRCA1 expression into NPRL2/TUSC4-knockdown cells rescued the HR repair efficiency ($p$<0.05).
B. Simplified schematic of the DR-GFP reporter assay for HR repair, *Adapted from Lutzmann, Molecular Cell 47, 523--534 (2012) with permission from Elsevier.*
C. Western blotting confirmed NPRL2/TUSC4, BRCA1 knockdown and overexpression of BRCA1 after NPRL2/TUSC4 knockdown. BRCA1 intensity normalized to NPRL2/TUSC4 was measured by Image J.

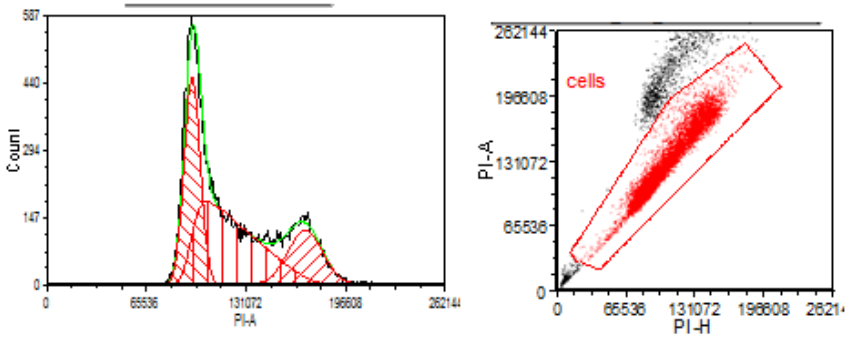
**A.**


ONCOMINE
NPRL2 Expression in Pawitan Breast
Breast Carcinoma - BRCA1 Mutation
Over-expression Gene Rank: 8079 (in top 46%)
P-value: 0.215
Reporter: 203246_s_at
t-Test: 0.794
Fold Change: 1.097
log2 median-centered intensity
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
1
2
Legend
1. BRCA1 Wild Type (128) 2. BRCA1 Mutation (31)
Pawitan Breast
Breast Cancer Res 2005/10/03
159 samples
mRNA
17,779 measured genes
Human Genome U133A Array, Human Genome U133B Array

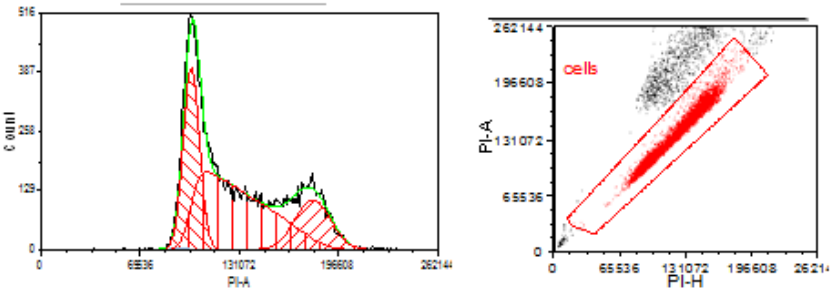
**B.**


Percentage (%)
100
80
60
40
20
0
Control
TUSC4 KD #1
TUSC4 KD #2
S
G2-M
G1

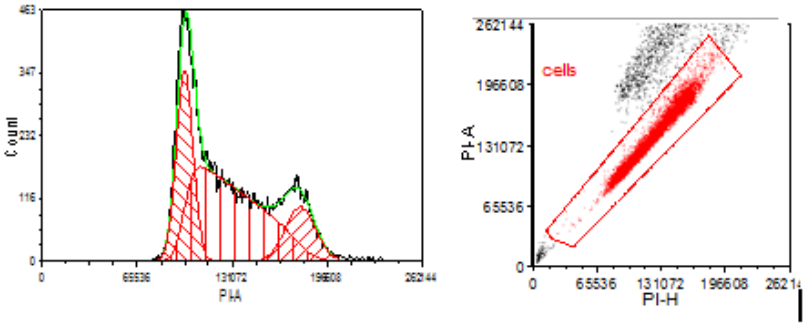
**Control**



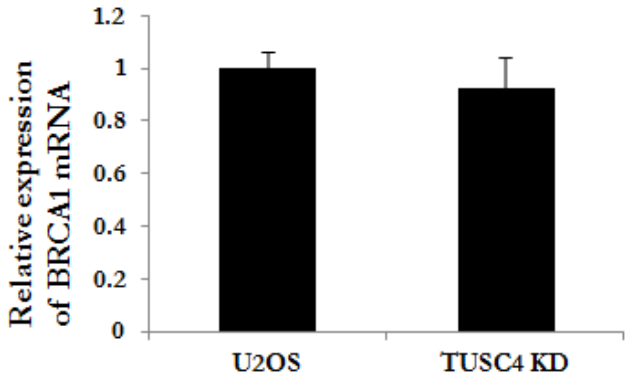

**TUSC4 KD #1**




**TUSC4 KD #2**




**C.**



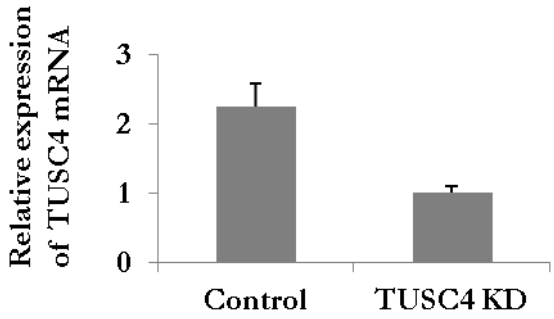



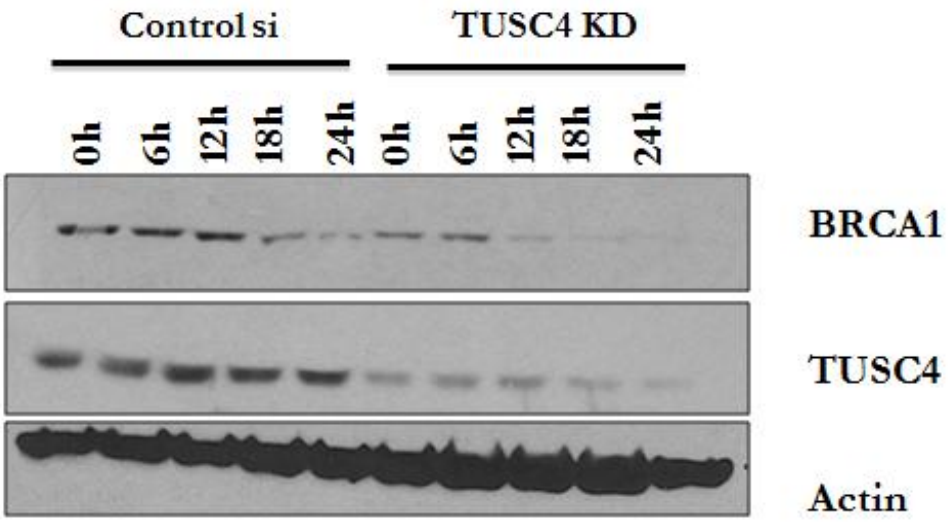

**Figure 15. NPRL2/TUSC4 knockdown decreased BRCA1 is not through cell cycle distribution**

A. BRCA1 mutation is not associated with NPRL2/TUSC4 expression level. Data was generated from database Oncomine, Pawitan breast cancer cell samples;

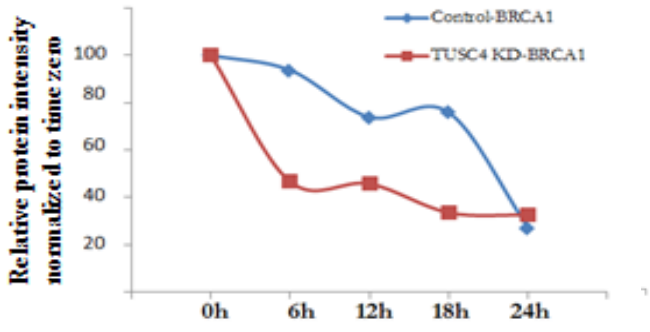
B. Knockdown of NPRL2/TUSC4 didn't significantly change the cell cycle distribution of U2OS cells compared to control cells. G1, G2/M and S phases cells were indicated by percentage of total cell numbers.

C. NPRL2/TUSC4 knockdown cells didn't show significant decrease of BRCA1 mRNA by qRT-PCR compared to control cells. The values of each column were normalized to the value of cyclophilin.

**A.**


Control si
TUSC4 KD
0h
6h
12h
18h
24h
0h
6h
12h
18h
24h
BRCA1
TUSC4
Actin

**B.**


Control-BRCA1
TUSC4 KD-BRCA1
Relative protein intensity normalized to time zero
100
80
60
40
20
0h
6h
12h
18h
24h

**C.**

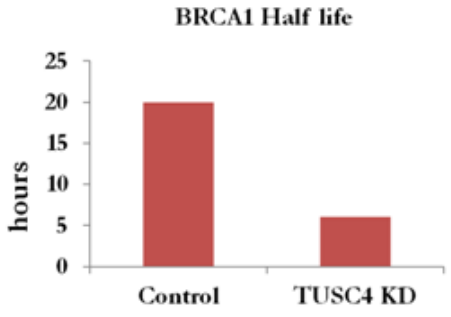

BRCA1 Half life
hours
25
20
15
10
5
0
Control
TUSC4 KD

**Figure 16. Knockdown of NPRL2/TUSC4 decreased BRCA1 stability in CHX assay**

A. NPRL2/TUSC4 knockdown U2OS cells showed shortened half-life of BRCA1 level compared to control cells. All cells were treated with 1μM of CHX for 0 to 24 hours.
B. Curves of BRCA1 level normalized to 0h after CHX treatment, while blue curves indicated control cells, and red curves indicated NPRL2/TUSC4 knockdown cells.
C. Bar graph of BRCA1 half-life. Control cells have BRCA1 half-life about 20 hours, while NPRL2/TUSC4 knockdown cells have BRCA1 half-life about 5 hours.

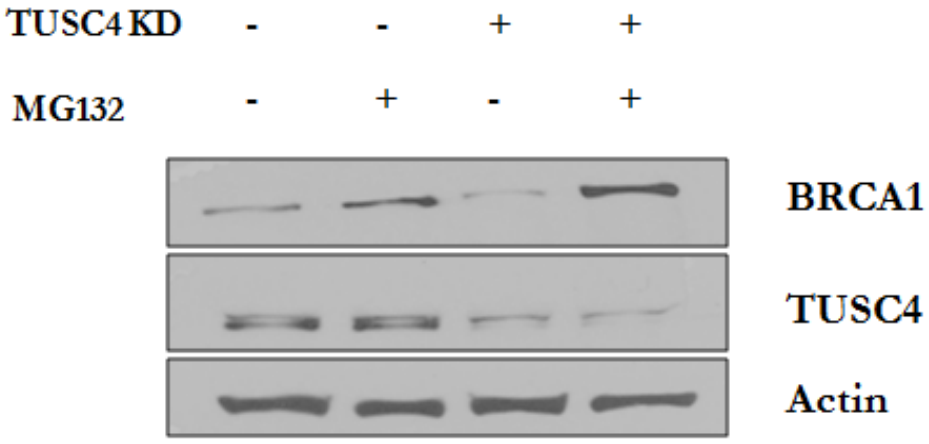



**Figure 17. NPRL2/TUSC4 regulates BRCA1 stability via proteasome degradation pathway**

NPRL2/TUSC4 knockdown U2OS cells showed shortened half-life of BRCA1 level compared to control cells. All cells were treated with 1μM of CHX for 0 to 24 hours.

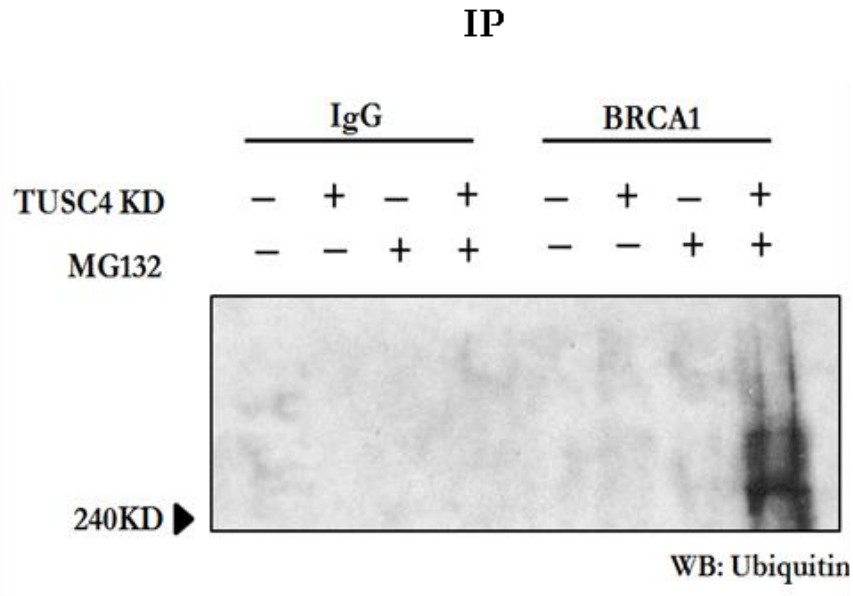
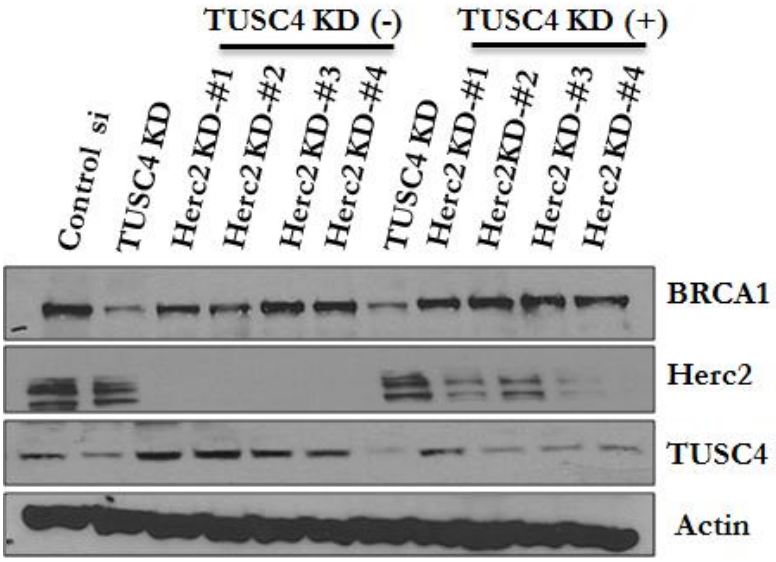



**Figure 18. NPRL2/TUSC4 regulates BRCA1 stability via ubiquitination pathway**

NPRL2/TUSC4 knockdown increased ubiquitination level of BRCA1 compared to control cells (After MG132 enrichment for ubiquitination).

**A.**


TUSC4 KD (-)
TUSC4 KD (+)
Control si
TUSC4 KD
Herc2 KD-#1
Herc2 KD-#2
Herc2 KD-#3
Herc2 KD-#4
TUSC4 KD
Herc2 KD-#1
Herc2KD-#2
Herc2 KD-#3
Herc2 KD-#4
BRCA1
Herc2
TUSC4
Actin

**B.**

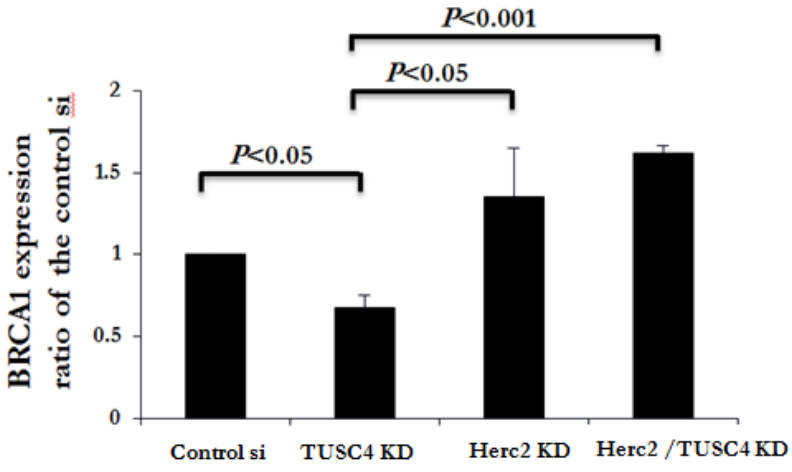

P<0.001
P<0.05
P<0.05
BRCA1 expression ratio of the control si
2
1.5
1
0.5
0
Control si
TUSC4 KD
Herc2 KD
Herc2 /TUSC4 KD

**Figure 19. HERC2 is E3 ligase to regulate BRCA1's stability**

A. HERC2 knockdown rescued BRCA1 expression level, with and without the presences of NPRL2/TUSC4.
B. Bar graph indicated a significant BRCA1 reduction after NPRL2/TUSC4 knockdown ($p$<0.05), BRCA1 increases after HERC2 knockdown with NPRL2/TUSC4 presence ($p$<0.05), or without NPRL2/TUSC4 ($p$<0.001). All values were compared to control cells.

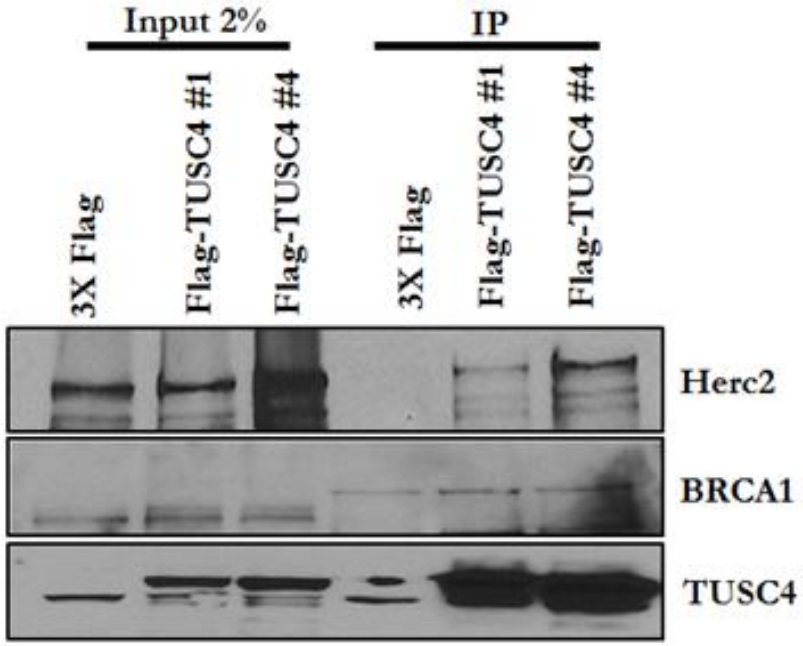



**Figure 20. NPRL2/TUSC4 physically interacts with HERC2 but not BRCA1**

3XFlag control and NPRL2/TUSC4-Flag tag U2OS cells were used to perform Immunopercipitation. Total of 4mg of protein were used in each IP conditions, and 2% input were loaded as control and indicate the overexpression of TUSC4.

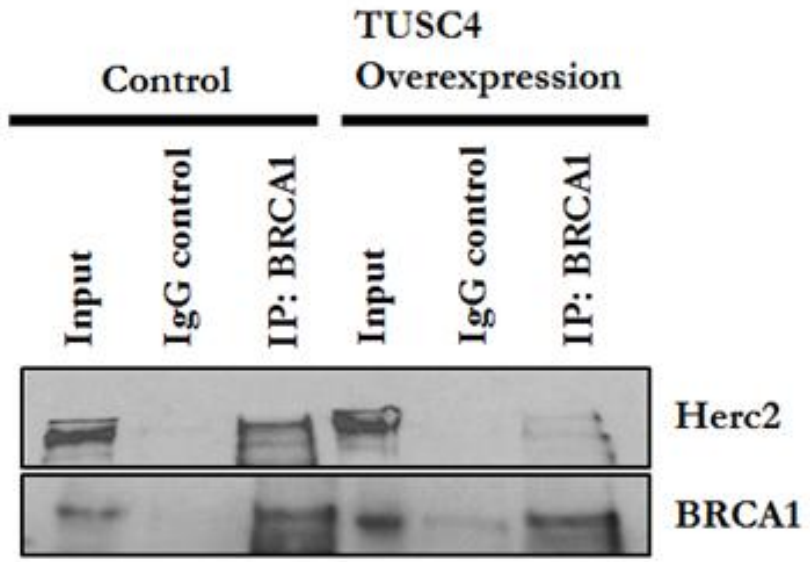
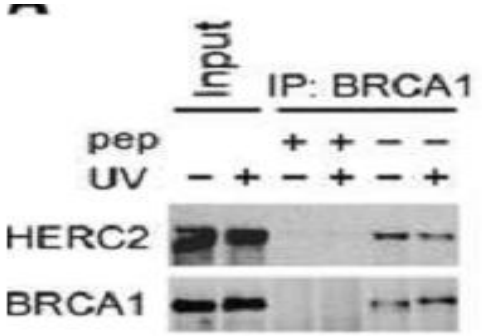



**Figure 21. NPRL2/TUSC4 overexpression reduced the binding between BRCA1 and HERC2**

NPRL2/TUSC4 overexpression reduced the binding between BRCA1 and HERC2 in Hela cell lines. 2% input were used to indicate the level of HERC2, IgG control antibody and BCRA1 antibody were used to perform IP in both control cells and NPRL2/TUSC4 overexpression cells. Total of 4mg of protein were used to perform in each condition.

**A.**




**B.**

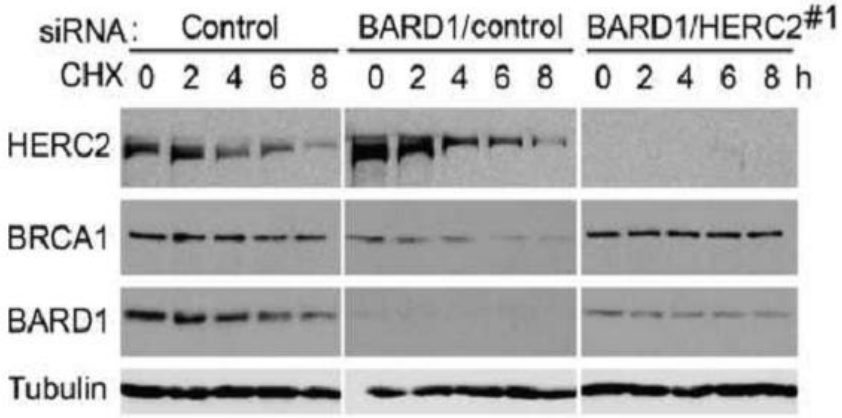



**Figure 22. BRCA1 interacts with HERC2 and HERC2 regulates BRCA1 depends on BRAD1**

A. BRCA1 physically interacts with HERC2 regardless of the irradiation.
B. HERC2 degrades BARD1-uncoupled BRCA1 in the CHX treatment, BRCA1 is destabilized while BARD1 is deficient; and BRCA1 is stabilized when HER2 is depleted. *Adapted from Wu et al., Cancer Research 70, 6384-6392 (2010) with permission from Elsevier.*

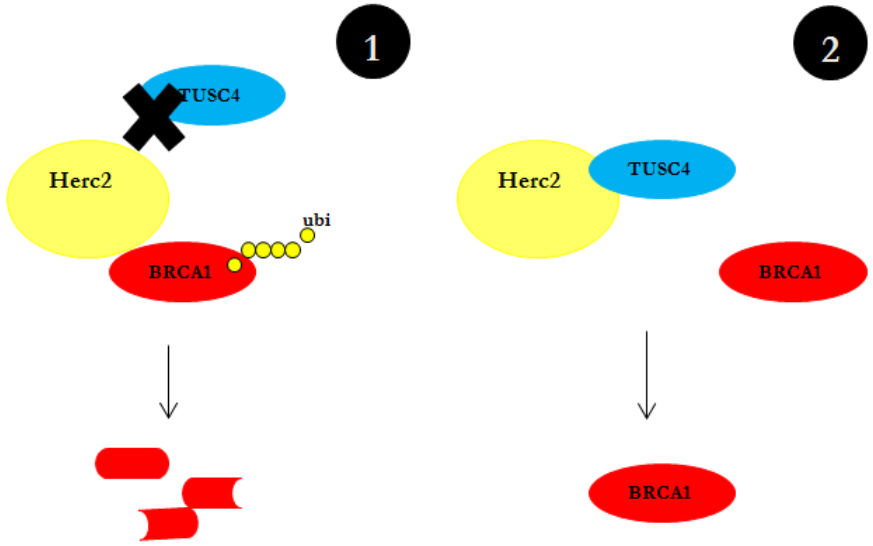



**Figure 23. Simplified model of the mechanism NPRL2/TUSC4 stabilizes BRCA1**

When NPRL2/TUSC4 is depleted in breast cancer, HERC2 has increased binding to BRCA1, thus ubiquitinates BRCA1 and degrades in proteasome-depedent pathway; while TUSC4 is overexpressed, TUSC4 prevents the binding between HERC2 and BRCA1, thus stabilize BRCA1.

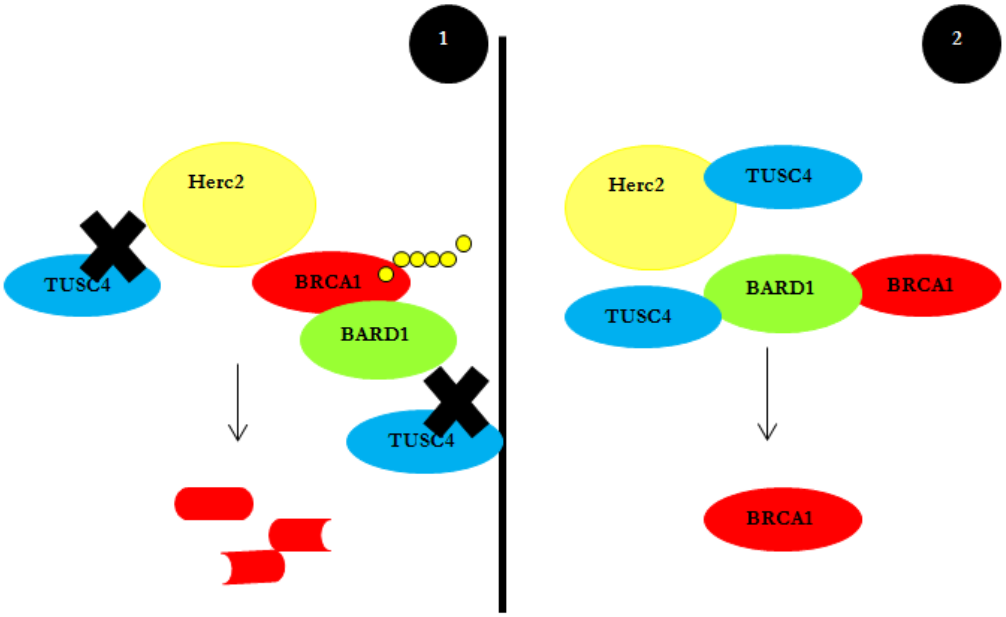
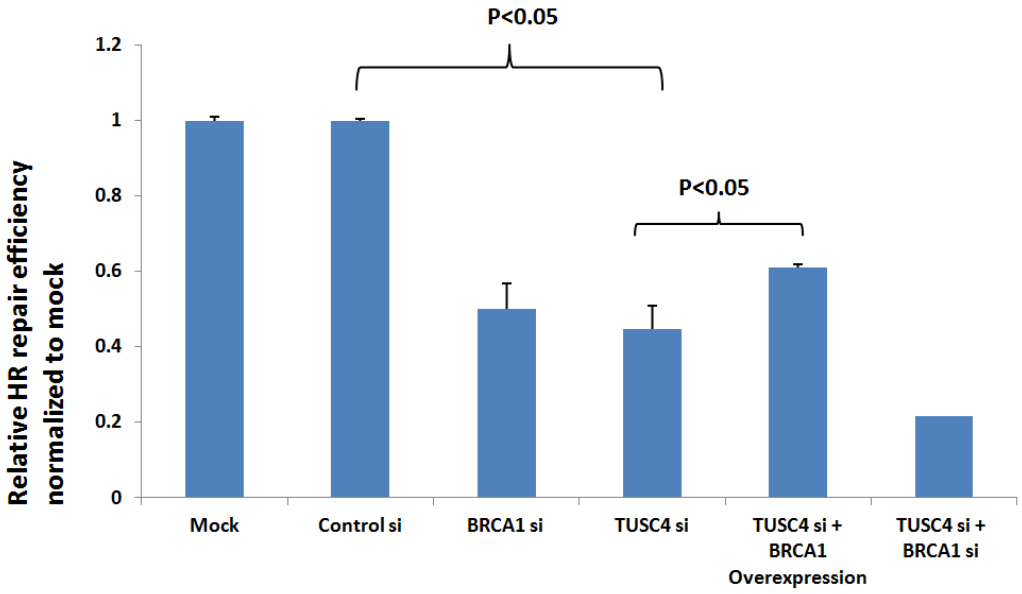



**Figure 24. Predicted model of the mechanism NPRL2/TUSC4 stabilizes BRCA1 through BARD1**

When NPRL2/TUSC4 is depleted in breast cancer, HERC2 has increased binding to BRCA1/BARD1 complex, thus ubiquitinates BRCA1 and degrades in proteasome-depedent pathway; while NPRL2/TUSC4 is overexpressed, TUSC4 prevents the binding between HERC2 and BRCA1, and enhances the BRCA1/BARD1 binding. thus stabilize BRCA1.

**A.**


P<0.05
P<0.05
Relative HR repair efficiency normalized to mock
1.2
1
0.8
0.6
0.4
0.2
0
Mock
Control si
BRCA1 si
TUSC4 si
TUSC4 si + BRCA1 Overexpression
TUSC4 si + BRCA1 si

**B.**

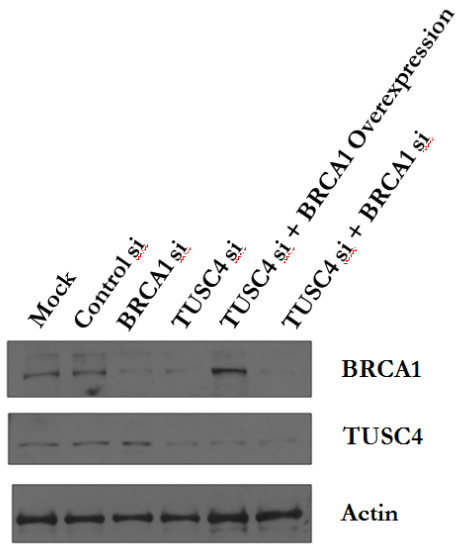

Mock
Control si
BRCA1 si
TUSC4 si
TUSC4 si + BRCA1 Overexpression
TUSC4 si + BRCA1 si
BRCA1
TUSC4
Actin

**Figure 25. NPRL2/TUSC4 and BRCA1 double knockdown further reduced HR repair efficiency.**

A. BRCA1 and NPRL2/TUSC4 knockdown significantly reduced the HR repair efficiency compare to control, while NPRL2/TUSC4 knockdown with BRCA1 overexpression restored the HR repair, double knockdown of both genes further reduced HR repair efficiency.
B. Western blot confirmed the NPRL2/TUSC4 and BRCA1 knockdown efficiency and overexpression level of BCRA1.

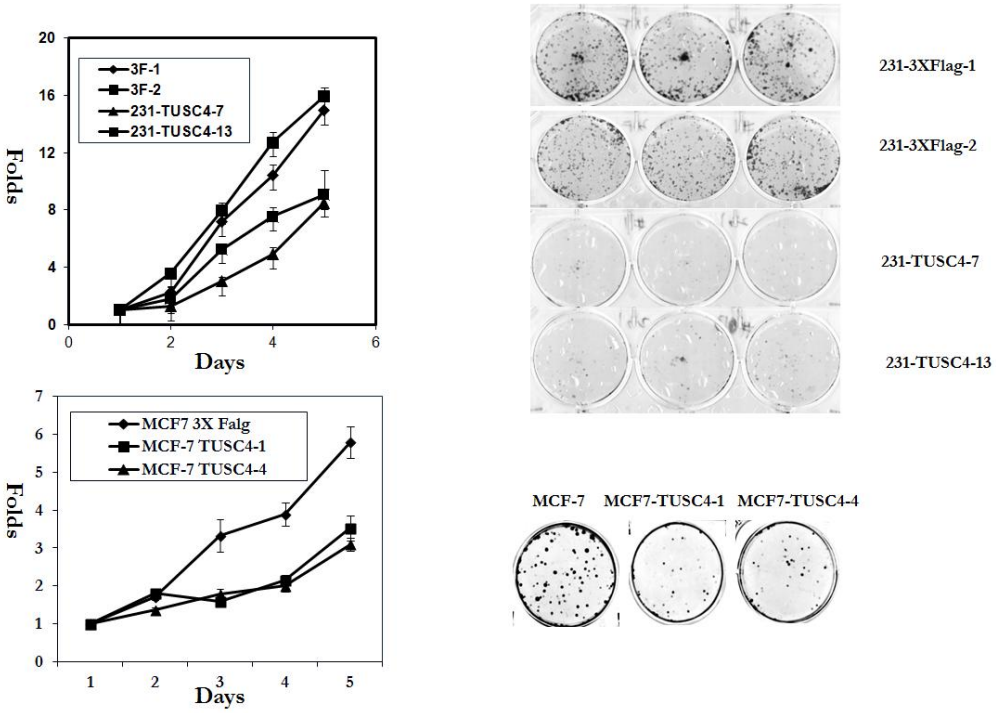



**Figure 26. NPRL2/TUSC overexpression is associated lower breast cancer proliferation rates**

NPRL2/TUSC4-flag tag overexpression exhibit significant slower proliferation rates in both MDA-AB-231 cell line and MCF-7 cell line. In 231 cells, clone #7 and #13 were used to compare with 3xF flag control in both MTT and colony formation assay(upper panel); In MCF7 cells, clone #1 and #4 were used to compare with 3xF flag control in both MTT and colony formation assay(lower panel)

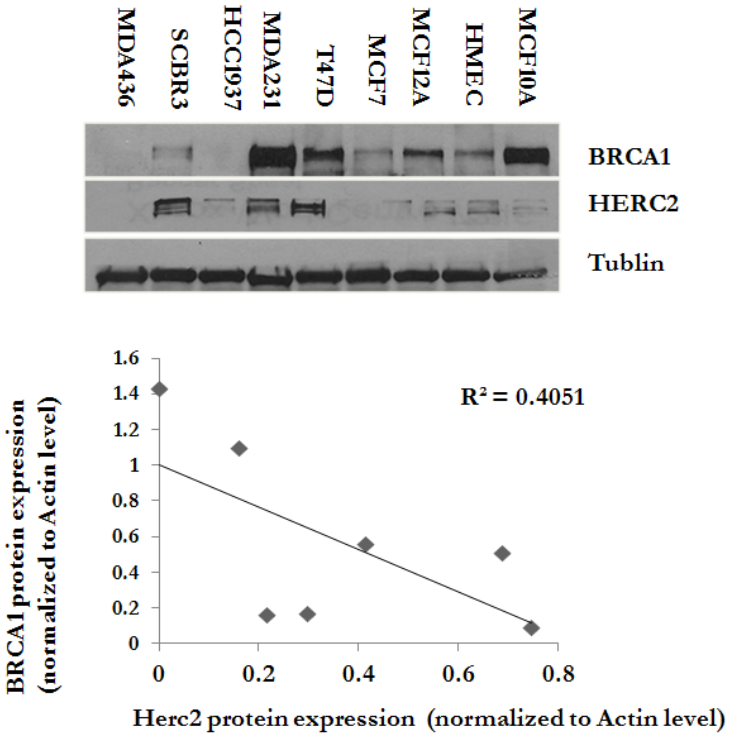



**Figure 27. BRCA1 expression level is negatively associated with HERC2 expression level in breast cancer cell lines.**

MDA436, SCBR3, HCC1937,MDA231,T47D, MCF7, MCF12A, HMEC and MCF10A were used to conduct the western blot**;** expression level of BRCA, HERC2 were evaluated, and tublin were used as loading control.

# CHAPTER 4

## NPRL2/TUSC4 FUNCTIONS AS TUMOR SUPPRESSOR PROTEIN IN BREAST CANCER

## RESULTS

### NPRL2/TUSC4 suppresses the tumorigenicity of human breast cancer cells

We have identified NPRL2/TUSC4 functions as tumor suppressor gene in breast cancer, possibly through positively regulating BRCA1. Next, we postulated that overexpression of NPRL2/TUSC4 also suppresses breast tumor proliferation *in vitro* and in *vivo*. To validate this, we compared the proliferation rate of the breast cancer cell line MDA-MB-231 with or without NPRL2/TUSC4 overexpression. Colony formation assays indicated markedly reduced proliferation of the NPRL2/TUSC4-overexpressing cells (Figure 26, 30). Because NPRL2/TUSC4 effectively inhibits breast cancer cell growth *in vitro*, we further examined the effect of NPRL2/TUSC4 overexpression in a xenograft mouse model of breast cancer. We injected female mice with control or NPRL2/TUSC4-overexpressing MDA-MB-231cells into the mammary fatpads. We then monitored and measured tumor growth weekly. By week 6 after injection, 5 of 10 mice injected with NPRL2/TUSC4-overexpressing cells remained tumor-free, whereas all 5 mice injected with control 231 cells had large tumors (Figure 28 A, B; Table 1).

### NPRL2/TUSC4 knockdown transforms normal mammary epithelial cells

Previous reports indicated that HR repair defect sensitizes cancer cells to DNA damaging drug (Foray et al., 1999; Deng and Wang 2003) and the poly(ADP-ribose) polymerase (PARP) inhibitor (Alli et al, 2009; Powell et al., 2003). Thus, NPRL2/TUSC4 deficiency cells with HR repair defect are highly likely to be more sensitive to the treatment of PARP inhibitor, which can effectively inhibit the repair of single strand DNA break. To confirm this hypothesis, we performed colony formation assay in U2OS cell with NPRL2/TUSC4 knockdown after PARP inhibitor Olaparib and Rucaparib treatment, as well as control cells. As we expected, both drug significantly reduced the colony formation in NPRL2/TUSC4 knockdown cells compared the control cells (Figure 29 A, B). Additionally, we examined NPRL2/TUSC4 depletion to determine whether it initiates breast tumor development in a xenograft mouse model. We injected MCF-10A cells with stable knockdown of NPRL2/TUSC4 expression and the control cells into the mammary fatpads of female nude mice. Similarly to the procedure described above, we closely monitored tumor formation in the mice. Notably, tumors began to form in 3 of 10 mice injected with NPRL2/TUSC4-knockdown cells after 3 weeks, whereas no tumors formed in the control groups (Table 2). These results demonstrated that loss of NPRL2/TUSC4 expression alone is sufficient to initiate malignant transformation of immortalized nontransformed mammary epithelial cells, which is consistent with our hypothesis that NPRL2/TUSC4 functions as a bona fide tumor suppressor in breast cancer.

**NPRL2/TUSC4 also regulate the foci formation of Rad51**

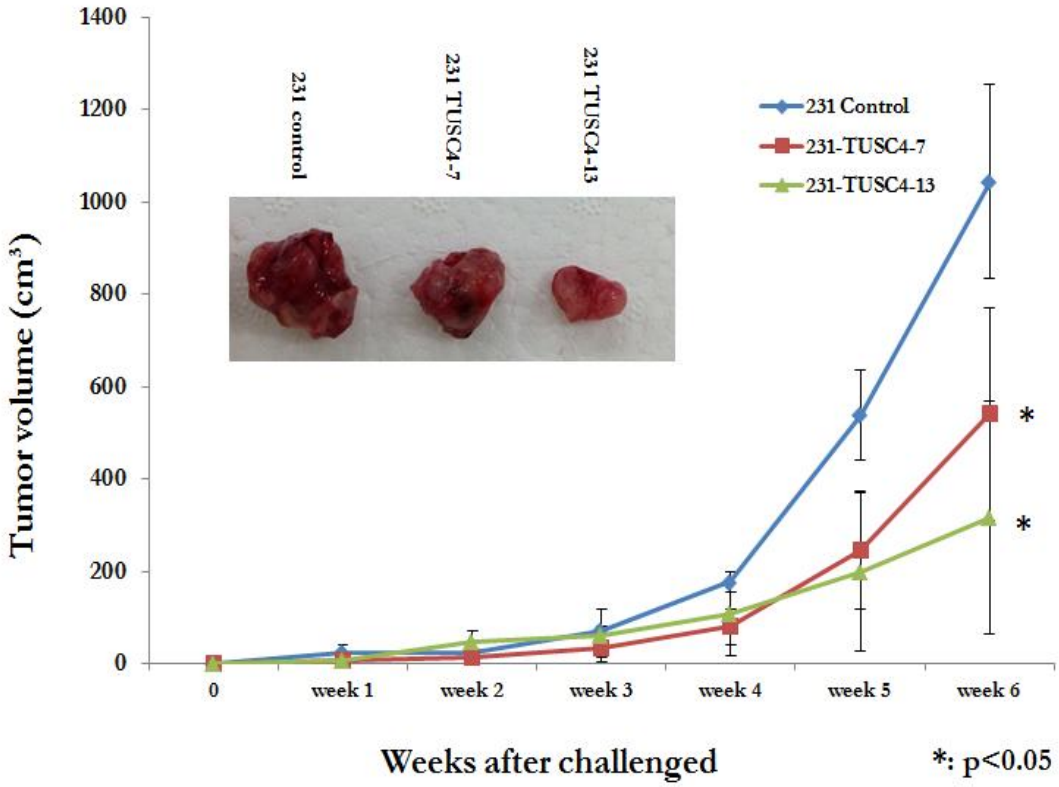
We also performed Rad51 foci staining followed by IR irradiation. NPRL2/TUSC4 knockdown significantly demolished the BRCA1 foci formation after irradiation, whereas control small interfering RNA (siRNA) did not affect the formation of Rad51 foci. Previous reports has suggested BRCA1 promote the recruitment of Rad51 and Rad51 forms complex with BRCA1 in the HR repair, it's highly likely the knockdown of NPRL2/TUSC4 reduced BRCA1 expression and further lead in the Rad51 foci formation decrease (Figure 31 A, B).

**NPRL2/TUSC4 knockdown delays cell cycle after irradiation**

We have proven that knockdown of NPRL2/TUSC4 under normal conditions, next we sought to investigate if NPRL2/TUSC4 is associated with cell cycle arrest under stress conditions. So, we added hydroxyurea (HU) into medium cultured with both control cells and NPRL2/TUSC4 knockdown cells, we observed that NPRL2/TUSC4-deficient cells exhibited noticeable slower cell cycle recover from G1 arrest compare to control cells and NPRL2/TUSC4 deficient cells with reintroduction of NPRL2/TUSC4 plasmid transfected cells. All the samples were also treated with Taxol which is widely used in cell biology to cause G2/M phase arrest. After 8 hours of Taxol treatment, control cells were already arrested in G2 while both NPRL2/TUSC4 deficient and NPRL2/TUSC4 transfected cells were still blocked in G1, but after 16 hours of treatment, NPRL2/TUSC4 deficient cells were shifted into G2 arrest, while both control cells and NPRL2/TUSC4 transfected cells were able to break the G2 arrest (Figure 32).

As an alternative way to evaluate the if cells can recover from stress, phosphorylated histone 3 is used to indicate the mitosis activity, so we also performed p-H3 staining to evaluate if proliferation rate of NPRL2/TUSC4 deficient cells were affected. As shown in figures, after 8 hours and 16 hours of Taxol treatment followed by HU removal, control cells had 1.28% and 12.8% p-H3 positive population while NPRL2/TUSC4 deficient cells had only 0.82% and 5.86% p-H3 positive population respectively, that was about 50% decrease of mitotic level compare to control cells. NPRL2/TUSC4 transfected sample didn't display high p-H3 as the control cell, but after 8 hours and 16 hours of Taxol treatment followed by HU removal, the p-H3 positive population was 1.54% and 1.5% compare to 0.025% and 0.040% in NPRL2/TUSC4 deficient cells, similar results supported the role of NPRL2/TUSC4 in replication stress recovery were described previously. Together, these results suggested NPRL2/TUSC4 plays an important role in maintain genomic stability and inhibit the proliferations of breast tumor cells both *in vitro* and *in vivo*, and this function is dependent on NPRL2/TUSC4's capacity to stabilize tumor suppressor BRCA1 and regulate cell cycle checkpoint, HR repair (Figure 33 A, B).

**A.**


231 control
231 TUSC4-7
231 TUSC4-13
231 Control
231-TUSC4-7
231-TUSC4-13
Tumor volume (cm³)
1400
1200
1000
800
600
400
200
0
0
week 1
week 2
week 3
week 4
week 5
week 6
*
*
Weeks after challenged
*: p<0.05

**B.**

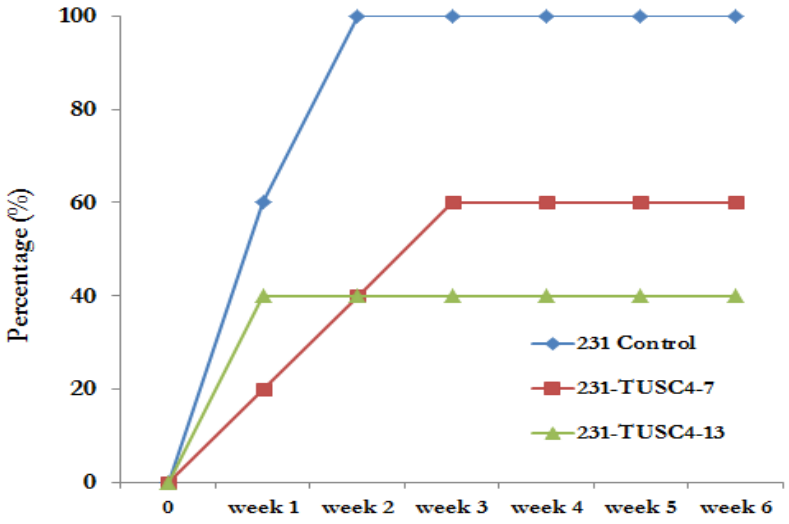
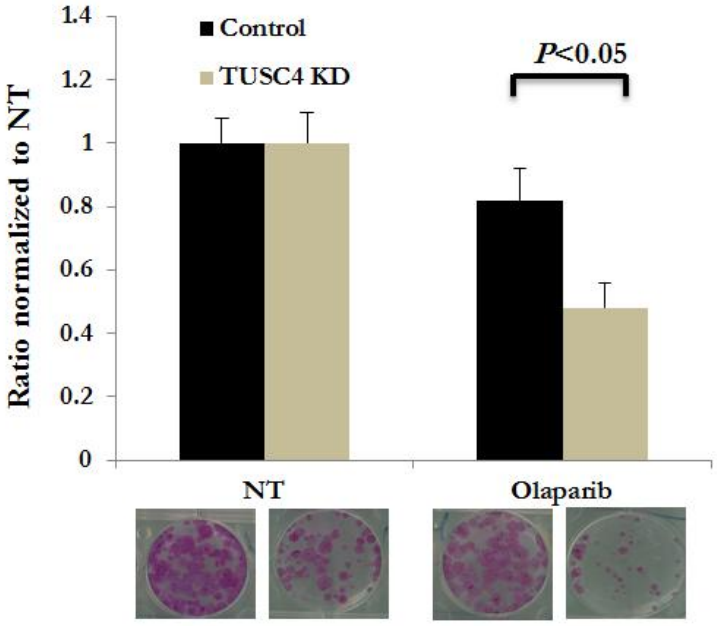



**Figure 28. NPRL2/TUSC4 plays an important role as a tumor suppressor in breast cancer**

A. NPRL2/TUSC4 overexpression in MDA-MB-231cells (stable clones NPRL2/TUSC4 #7 and #13) significantly reduced the breast tumor growth in nude mice ($p$<0.05) compared to control 231 cells, the relative tumor size were indicated and compared as shown.

B. Tumor growth percentage of total injected mice by week after the injection of the MDA-MB-231 cells as well as two NPRL2/TUSC4 overexpression cell lines (#7 and #13).

**A.**


Control
TUSC4 KD
P<0.05
Ratio normalized to NT
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NT
Olaparib

**B.**

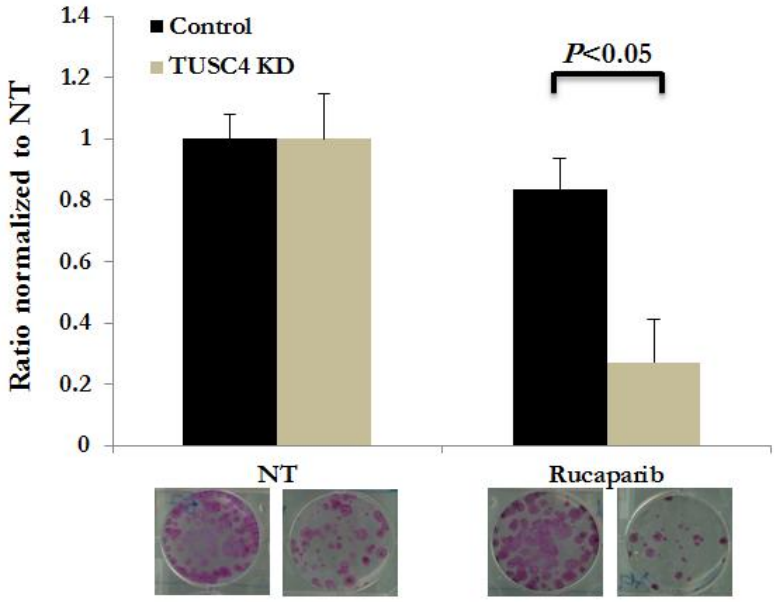



**Figure 29. NPRL2/TUSC4 deficiency sensitized U2OS cells to PARP inhibitor**

A. Colony formation assay indicated that normal U2OS cells are not sensitive to PARP inhibitor Olaparib (1μm) but NPRL2/TUSC4-knockdown cells exhibited increased sensitivity to Olaparib, the colonies were significantly reduced after the treatment ($p$<0.05);

B. Colony formation assay indicated that normal U2OS cells are not sensitive to PARP inhibitor Rucaparib (1μm) but NPRL2/TUSC4-knockdown cells exhibited increased sensitivity to Rucaparib, the colonies were significantly reduced after the treatment ($p$<0.05).

Tumorigenicity of Orthotopically Implanted Control and TUSC4-overexpression MDA-MB-231 cells

Number of Mice with Tumors (%)

| | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
|---|---|---|---|---|---|---|
| 231 Control | 3/5 (60) | 5/5 (100) | 5/5 (100) | 5/5 (100) | 5/5 (100) | 5/5 (100) |
| TUSC4-7 | 1/5 (20) | 2/5 (40) | 3/5 (60) | 3/5 (60) | 3/5 (60) | 3/5 (60) |
| TUSC4-13 | 2/5 (40) | 2/5 (40) | 2/5 (40) | 2/5 (40) | 2/5 (40) | 2/5 (40) |

**Table 1. Tumorigenecity of MDA-MB231 cells with NPRL2/TUSC4 overexpression**

$5\times10^6$ cells from MDA-AB-231 control and two independent NPRL2/TUSC4-overexpressing MDA-AB-231 cell lines (NPRL2/TUSC4 #7 and NPRL2/TUSC4 #13) were injected per mouse into mammary tumor sizes were analyzed.

Tumorigenicity of Orthotopically Implanted Control and TUSC4-Knockdown MCF10A cells

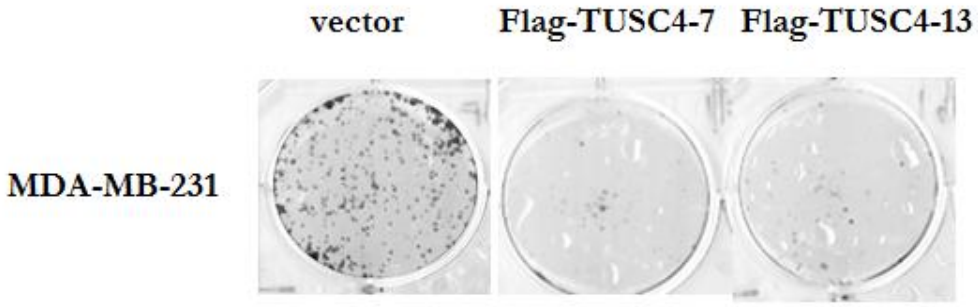
Number of Mice with Tumors (%)

| | Week 1 | Week 3 | Week 5 | Week 7 | Week 9 |
|---|---|---|---|---|---|
| MCF10A Control | 0/5 (0) | 0/5 (0) | 0/5 (0) | 0/5 (0) | 0/5 (0) |
| TUSC4-1 | 0/5 (0) | 2/5 (40) | 2/5 (40) | 2/5 (40) | 2/5 (40) |
| TUSC4-4 | 0/5 (0) | 0/5 (0) | 0/5 (0) | 1/5 (20) | 1/5 (20) |

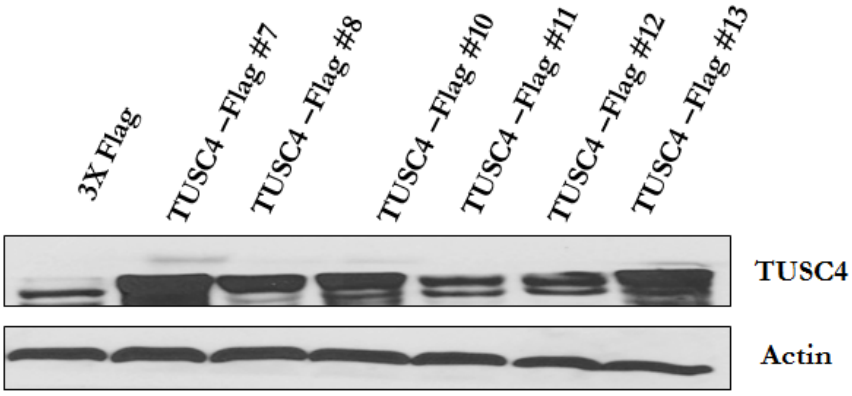
**Table 2. Tumorigenecity of MCF10A cells with NPRL2/TUSC4 depletion**

$1\times10^7$ cells from MCF-10A control and two independent NPRL2/TUSC4-knockdown MCF-10A cell lines (NPRL2/TUSC4 #1 and NPRL2/TUSC4 #4) were injected per mouse into mammary fat pads glands of 6-week-old female nude mice. Each cell line was injected in five different mice, and tumor sizes were analyzed.

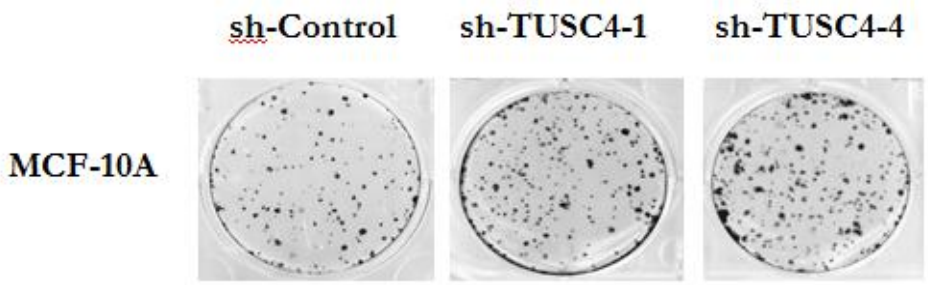
**A.**


vector
Flag-TUSC4-7
Flag-TUSC4-13
MDA-MB-231

**B.**


3X Flag
TUSC4 –Flag #7
TUSC4 –Flag #8
TUSC4 –Flag #10
TUSC4 –Flag #11
TUSC4 –Flag #12
TUSC4 –Flag #13
TUSC4
Actin

**C.**




**D.**

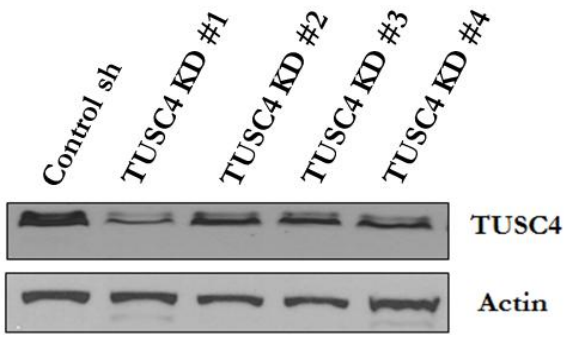
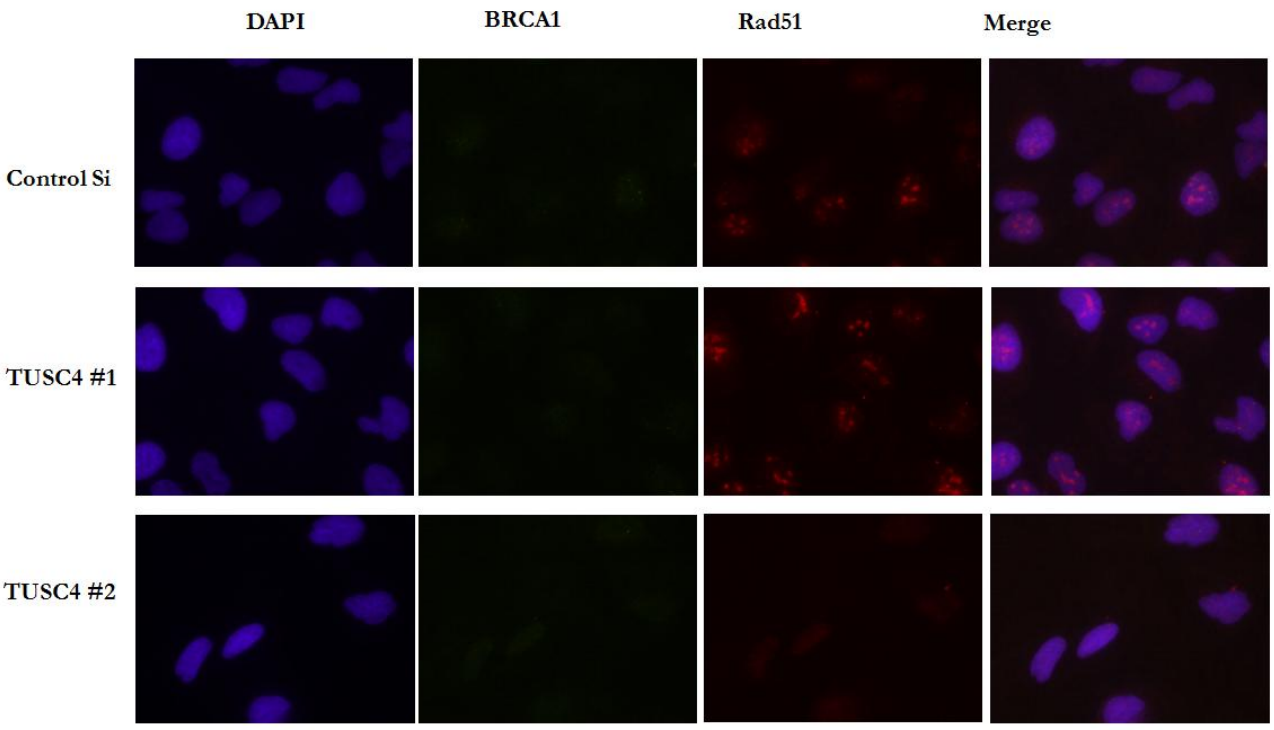



**Figure 30. Colony formation assay of NPRL2/TUSC4 overexpression and NPRL2/TUSC4 knockdown cells**

A. Colony formation of MDA-MB-231 cells, as well as 2 stable NPRL2/TUSC4 overexpression 231 cells (#7 and #13) before injection was displayed. B. Western Blot confirmed the NPRL2/TUSC4 overexpression in 231 cells, including #7 and #13. C. Colony formation of MCF-10A cells, as well as 2 stable 10A knockdown cell lines (#1 and #4) before injection was displayed. D. Western Blot confirmed the NPRL2/TUSC4 knockdown in 10A cells, including clone #1 and #4.

**A.**



**B.**

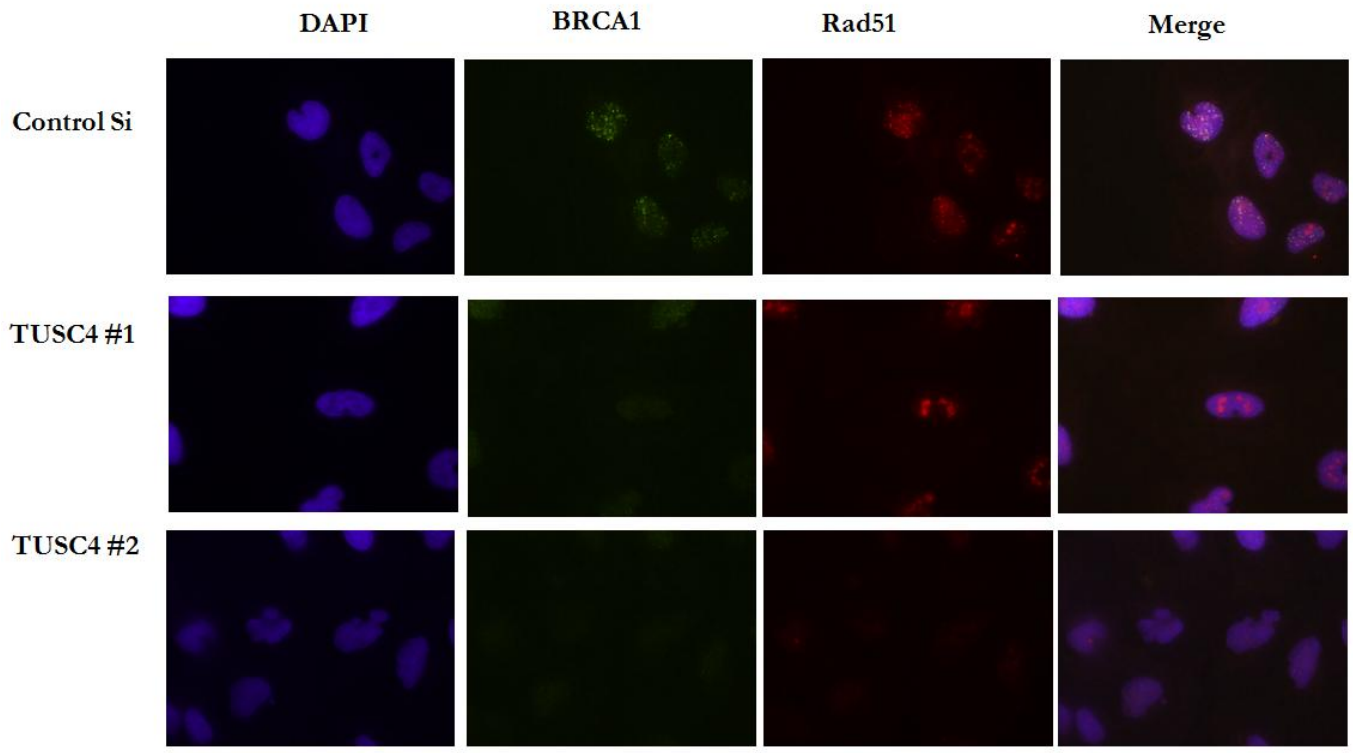



**Figure 31. NPRL2/TUSC4 knockdown reduced Rad51 protein foci after UV**

NPRL2/TUSC4 knockdown reduced Rad51 foci formation after UV, while control cells (NT) didn't have such effect. BRCA1 indicated the efficiency of irradiation.

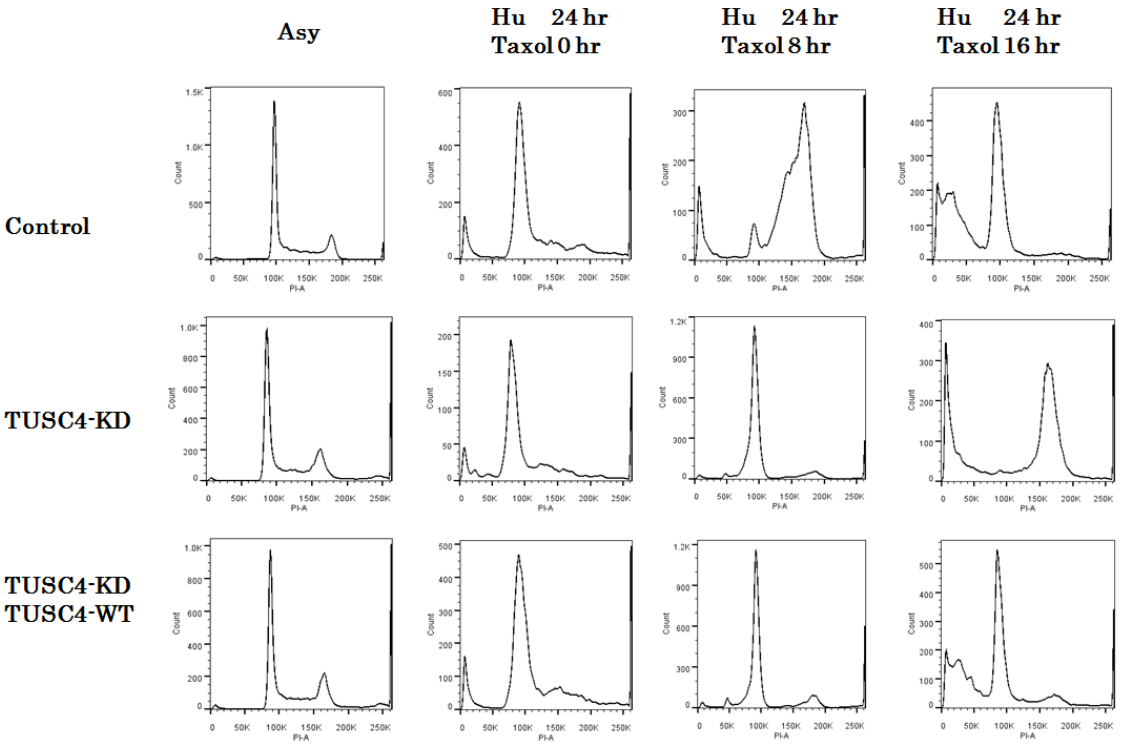
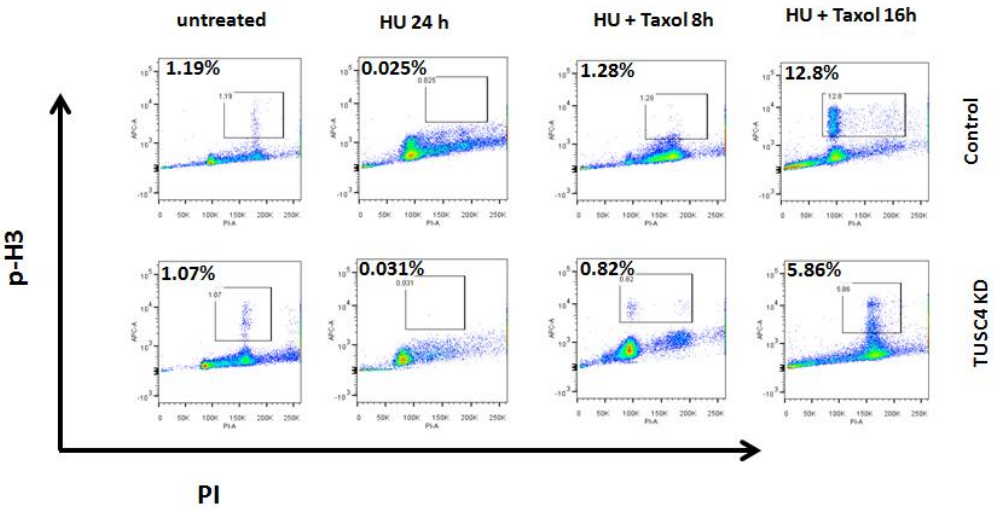
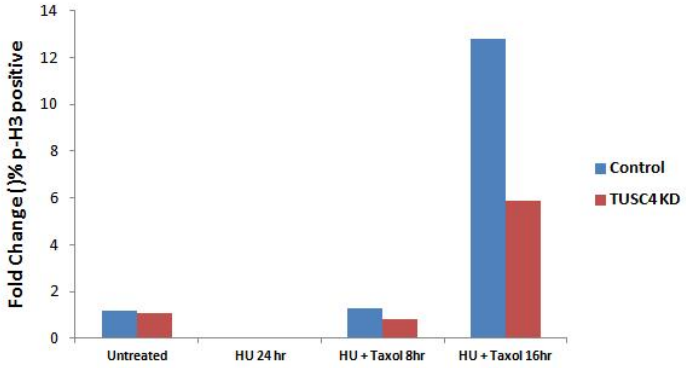



**Figure 32. NPRL2/TUSC4 depletion slowed down the cell cycle progression under stress conditions**

Control, NPRL2/TUSC4 knockdown and NPRL2/TUSC4 knockdown/reintroduction cells were treated with HU for 24 hours, then HU were removed and Taxol were added to arrest cells in G2 phase, 0 h, 8 h, 16 h after Taxol treatment were collected for cell cycle analysis

**A.**


untreated
HU 24 h
HU + Taxol 8h
HU + Taxol 16h
1.19%
0.025%
1.28%
12.8%
1.07%
0.031%
0.82%
5.86%
Control
TUSC4 KD
p-H3
PI


Fold Change ()% p-H3 positive
14
12
10
8
6
4
2
0
Untreated
HU 24 hr
HU + Taxol 8hr
HU + Taxol 16hr
Control
TUSC4 KD

**B.**

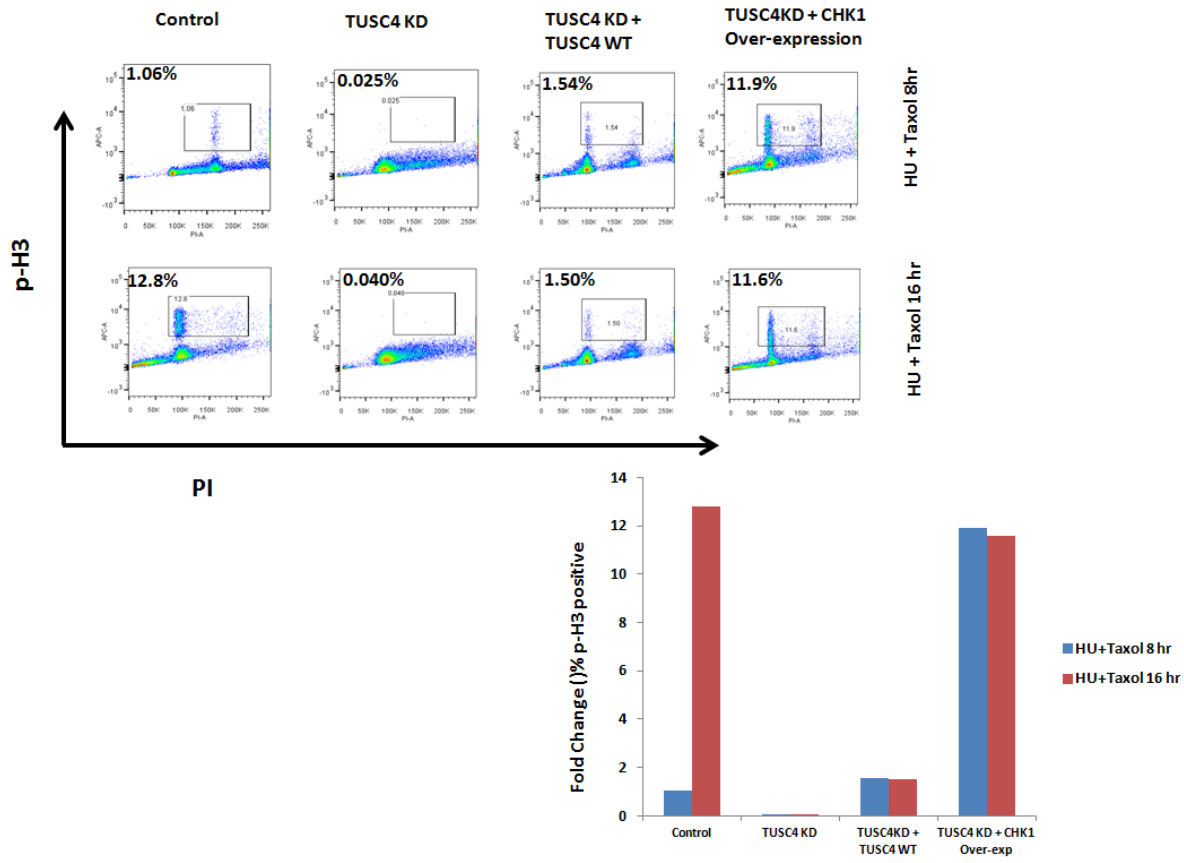



**Figure 33. NPRL2/TUSC4 depletion is associated with reduced proliferation rate**

A. NPRL2/TUSC4 depleted cells showed significantly reduced p-H3 staining compare to control cells after Taxol treatment in 8 hours and 16 hours;

B. Reintroduction of NPRL2/TUSC4 and CHK1 in NPRL2/TUSC4 depleted cells rescued p-H3 staining compare to NPRL2/TUSC4 depleted cells alone. All cells were treated with HU for 24 hours and Taxol for 8 or 16 hours.

## Chapter 5

## DISCUSSION

DNA damage response is critical to maintain genomic integrity and it safeguards the fidelity of genetic information to pass to the next generation of cells. The deficiency of DNA damage response especially in the early stage increased the incidence of cancer lesions. As one of the most important protein in DNA damage response, BRCA1 mediates the error-free homologous recombination repair and suppress the occurrence of early cancer lesions. Here we provided evidence that tumor suppressor protein NPRL2/TUSC4 can stabilize the expression levels of BRCA1 by preventing BRCA1's E3 ligase HERC2 from binding it. Interestingly, inactivation of NPRL2/TUSC4 has also been shown to associate with poor 5-year clinical survival rates in breast cancer patients, indicating NPRL2/TUSC4 plays a role in inhibiting tumor growth and maintaining genome integrity against the multiple types of stress.

BRCA1's recruitment to damage sites is considered one of the critical events in HR repair. Inactivation of NPRL2/TUSC4 abolished the DNA damage foci of BRCA1 under both IR and UV treatments, suggested the regulation BRCA1's stability in DNA damage response may not only go through the process followed by the CHK2 phosphorylation, due to the lack of kinase activity of NPRL2/TUSC4. In fact, a previous report described that NPRL2/TUSC4 inhibits AKT pathway by physically interacting with the upstream PDK1 protein, and it can block the phosphorylation sites on PDK1 and prevents co-activator Src from binding. Thus, it's not surprising that NPRL2/TUSC4's binding domain is responsible for multiple

proteins' interaction and functions as tumor suppressor. Indeed, NPRL2/TUSC4 does bind to BRCA1's E3 ligase HERC2 but not with BRCA1 itself.

Our initial observation that NPRL2/TUSC4 can form damage foci after UV and IR suggested that NPRL2/TUSC4's potential function in nuclear. This result was confirmed by nuclear fractionation experiments showing that NPRL2/TUSC4 is diversely expressed from cytoplasm to nuclear. Although it's not clear whether NPRL2/TUSC4 co-localizes with BRCA1 after irradiation, we noticed that after NPRL2/TUSC4 was knocked down, BRCA1 foci was significantly reduced. This leads to two possibilities; either BRCA1's stability was impaired thus foci couldn't be formed or the BRCA1's recruitment to damaged sites was blocked. Considering BRCA1's important role in homologous recombination repair, we suspected that if BRCA1's expression level is reduced, the HR repair efficiency would also get affected. So, we used previously established U2OS cell systems to evaluate the HR repair efficiency by the levels of released GFP. The graphs clearly show that knockdown of NPRL2/TUSC4 has similar effects compared to BRCA1 knockdown samples, which served as positive control. This results was consistent with our hypothesis that inactivation of NPRL2/TUSC4 leads to the reduced expression levels of BRCA1. More interestingly, after the analysis of Western blots, we also identified that BRCA1's expression level was significantly reduced in the lane of NPRL2/TUSC4 knockdown sample, indicating the NPRL2/TUSC4 inactivation leads to the decreased stability of BRCA1 protein itself. These data support the idea that reduced BRCA1 damage-induced foci is due to reduced BRCA1 stability. However, we still can't rule out the possibility that NPRL2/TUSC4 inactivation also

impairs the cell's capacity to recruit BRCA1 to the foci sites. Furthermore, to confirm that the defective HR repair efficiency was not caused by transfection efficiency or inaccurate efficiency from I-*Sce*I, we reintroduced BRCA1 plasmids into the NPRL2/TUSC4-knockdown cells, and as expected, we observed a significant increase in HR repair efficiency compare to that in NPRL2/TUSC4-knockdown-only cells. The introduction of BRCA1 expression caused partial restoration of HR repair efficiency, the main reason is that NPRL2/TUSC4 also interacts with other DNA damage response and repair proteins involved in HR repair (data not shown).

For a better understanding of the relationship between NPRL2/TUSC4 and BRCA1, we first conducted microarray analysis by using the NPRL2/TUSC4 stable U2OS cell lines with the appropriate controls. We then isolated the significantly different genes with both up- and down-regulation and input into Ingenuity Pathway Analysis (IPA) system for pathway enrichment analysis. The results indicated NPRL2/TUSC4 knockdown leads to the critical pathway changes including cancer, cell cycle, cell death, etc. This is consistent with the previous studies that NPRL2/TUSC4 has been widely accepted as a tumor suppressor gene. Top molecular pathways also indicated NPRL2/TUSC4 knockdown cells is associated with role of BRCA1 in DNA damage response, which further supported our statement that NPRL2/TUSC4 knockdown is negatively associated BRCA1's function.

We previously established HR repair-deficiency gene signatures and identified 230 genes that predict HR repair deficiency across tumor types. BRCA1

deficiency had been utilized as one of the phenotypes to generate the signature. We surprisingly identified similar HRD gene signature patterns in NPRL2/TUSC4- and BRCA1-deficient cells, suggesting an association between these two proteins and shedding light for the exploration of regulation networks for NPRL2/TUSC4 and BRCA1. To further validate this hypothesis, we also performed RPPA with BRCA1 and NPRL2/TUSC4 deficient cells as well as control cells, at the protein level, NPRL2/TUSC4 and BRCA1 knockdown exhibited similar protein expression patterns in the clusters, indicating similar protein and pathway changes were observed and this result is highly consistent with the microarray analysis at mRNA level. Multiple research groups published that BRCA1-deficicency is correlated with high sensitivity of tumor cells and animal models to PARP inhibitor. The underlying mechanism is that inhibition of PARP1 will accumulate unrepaired single strand break and ultimately leads to double strand break. Our data also showed the TSUC4 deficient cells are more sensitive to the treatment of multiple PARP inhibitors, which is consistent with the rationales of our work. It also suggested the potential clinical usage of PARP inhibitors to treat NPRL2/TUSC4-defieicent breast cancer.

To determine whether the regulation of BRCA1 by NPRL2/TUSC4 is caused by the altered cell-cycle distribution because BRCA1 expression is known to be cell-cycle regulated, we first performed cell cycle analysis and found no significant difference between the normal U2OS cells and NPRL2/TUSC4 stable knockdown cells. This ruled out the possibility the reduced BRCA1 expression level is due to the different stages of cell cycle. To test whether BRCA1 is regulated by

NPRL2/TUSC4 at the mRNA level, RT-PCR was performed. We observed that BRCA1 mRNA levels had no significant differences between control cells and two NPRL2/TUSC4 deficient cells. This result is also consistent with the previous microarray analysis, which showed no changes at the BRCA1 mRNA level, even clear molecular pathway changes were identified.

Another possibility could be that NPRL2/TUSC4 regulates BRCA1's stability at the protein level. To determine if this hypothesis is true, we treated control and NPRL2/TUSC4-knockdown U2OS cells with CHX**,** for the purpose of blocking protein synthesis, and proteasome inhibitor MG132. Surprisingly, we found that NPRL2/TUSC4 knockdown reduced the half-life of BRCA1 from about 20 h to less than 6 h. MG132-based treatment restored BRCA1 expression in cells with NPRL2/TUSC4 knockdown but such phenotype was not observed in control cells. These two results combined together suggested that NPRL2/TUSC4 plays an essential role in stabilizing BRCA1 at the protein level and NPRL2/TUSC4 regulates BRCA1 protein stability via the proteasome-dependent pathway. Multiple groups have reported HERC2 serves as the E3 ligase of BRCA1. To determine whether NPRL2/TUSC4 regulates BRCA1 stability via HERC2, we performed ubiquitination assay by transfecting (HA)-tagged ubiquitin plasmids into cells with or without NPRL2/TUSC4 knockdown, followed by immunoprecipitation with control IgG or an anti-BRCA1 antibody. After treatment with MG132, Western blotting for HA-tagged ubiquitin showed that NPRL2/TUSC4-knockdown cells underwent heavy ubiquitination, whereas control cells exhibited only a light polyubiquitination ladder indicating that knockdown of NPRL2/TUSC4 expression caused a robust

increase in BRCA1 protein polyubiquitination. We also confirmed that depletion the expression of HERC2 led to increased expression of BRCA1 regardless of the presence of NPRL2/TUSC4. These data further suggested the role of NPRL2/TUSC4 in regulation of BRCA1 stability via ubiqutination pathway via HERC2.

Thus, the next question to be answered was how NPRL2/TUSC4 affects the ubiquitination of BRCA1 via HERC2. By performing immunoprecipitation, we found that NPRL2/TUSC4 physically interacts with HERC2 but not with BRCA1, which strongly suggested that NPRL2/TUSC4 regulates BRCA1 stability via interaction with HERC2. Considering the previously reported binding functions of NPRL2/TUSC4, we suspected that NPRL2/TUSC4 may prevent physical interaction between BRCA1 and HERC2. The binding between these two proteins has been previously described, so we performed further immunoprecipitation to determine whether overexpression of NPRL2/TUSC4 weakens this binding. Intriguingly, overexpression of NPRL2/TUSC4 interrupted the binding between HERC2 and BRCA1, indicating that NPRL2/TUSC4 may regulate BRCA1 stability by preventing physical interaction between BRCA1 and HERC2.

In the present study, we demonstrated for the first time that expression of NPRL2/TUSC4 can positively regulate the stability of BRCA1 by preventing physical interaction between BRCA1 and its identified E3 ligase HERC2, which in turn protects BRCA1 from ubiquitination and degradation. Because NPRL2/TUSC4 knockdown is negatively correlated with BRCA1 protein expression and considering the previously reported protein-binding and -blocking capacity of

NPRL2/TUSC4 between PDK1 and its co-activator Src, we suspected that NPRL2/TUSC4 plays a similar role in disrupting the interaction between BRCA1 and HERC2. Indeed, we found that NPRL2/TUSC4 physically interacts with BRCA1's E3 ligase HERC2 but not with BRCA1 itself. Overexpression of NPRL2/TUSC4 markedly weakened their interaction in immunoprecipitation experiments. This observation explained how NPRL2/TUSC4 overexpression can protect BRCA1 from degradation and greatly reduce the proliferation of breast cancer. Thus, the regulatory mechanisms of NPRL2/TUSC4 function in blocking the AKT pathway and stabilizing BRCA1 protein expression may be similar. NPRL2/TUSC4 does not have phosphorylation or E3 ligase activity according to functional domain analysis, so we speculated that its potential functions in the DNA damage response network and as a tumor suppressor are common mechanisms by the physical interaction, stabilizing or preventing activation of target proteins.

Regardless of the effect of HERC2 on BRCA1, our data has demonstrated that NPRL2/TUSC4 inactivation significantly impaired the stability of BRCA1, and in turn negatively regulate the efficiency of HR repair and cells' capacity to response to PRAP inhibitor. We demonstrated further that the reduced stability of BRCA1 is owing to its E3 ligase HERC2 binding, and NPRL2/TUSC4 can physically interact with HERC2 and weaken the binding between BRCA1 and HERC2. When NPRL2/TUSC4 is inactivated, HERC2 is able to reform the binding complex with BRCA1 and thus decrease BRCA1's expression via protein degradation pathway. Our data strongly supported our hypothesis that NPRL2/TUSC4 regulates BRCA1's stability.

BRCA1 expression level was reported to be negatively regulated by apoptosis pathway under the condition of UV. More specifically, BRCA1 is cleaved by caspase-3 after UV treatment, while mutated BRCA1 with non-cleavable capacity can negatively regulate cell death and block GADD5/JNK signaling (Zhan et al., 2002). Although BRCA1 plays an important role in DNA damage response and repair, the mechanism of when BRCA1 participates into the DNA double strand break response is still largely unknown. Thus, to understand the physiological condition of when BRCA1 is regulated will promote our understanding when and why the regulation between NPRL2/TUSC4 and BRCA1 take place. In fact, our data (not shown) also indicated that NPRL2/TUSC4 knockdown altered the cell cycle distributions of U2OS cells compare to ATM or ATR knockdown, after IR treatments. Considering BRCA1 is cell cycle dependent, this result suggested the regulation of BRCA1 by NPRL2/TUSC4 may not only happen under normal conditions, but also play roles under the conditions of IR. However, further research need to be performed to confirm this hypothesis.

Upon DNA damage, BRCA1 was previously reported to aggregate and co-localize with γ-H2AX to the nuclear foci (Paull et al., 2000). Multiple factors can affect BRCA1's foci formation including UBC13/RNF8 ligase in ubiquitination pathway (Wang and Elledge, 2007). In our current study, although the knockdown of NPRL2/TUSC4 reduced the stability of BRCA1, but whether the reduced BRCA1 foci formation was owing to the reduced BRCA1 expression is still unknown. Considering the fact that NPRL2/TUSC4 regulates BRCA1's stability via the ubiquitination pathway, it's also likely that NPRL2/TUSC4 also involves the

BRCA1's recruitment to the foci. This hypothesis can be tested by checking if NPRL2/TUSC4 co-localize with BRCA1 after irradiation.

Another function of NPRL2/TUSC4 was reported in 2013 (Bar-Peled, 2013), explaining that NPRL2/TUSC4 forms complex with NPRL3 and DEPDC5 to negatively regulate mTORC1 pathway. Inactivation of this complex will lead hyperactive cell growth, due to the insensitivity to amino acid deprivation, and hypersensitive to mTORC1 target drug rapamycin. Because Gag guanosine triphosphatases (GTPases) involves in the translocation of mTORC1 to lysosomal surface, and the complex mentioned above suppress the GTPases activity, this result suggested that NPRL2/TUSC4's potential function in promoting or suppressing the protein trafficking, and might shuttle proteins among different cellular apparatuses. In fact, nuclear transport of BCRA1from cytoplasm to the nucleus by NLS receptor mediated system has been previously described (Chen et al, 1996). Therefore, the regulation location between NPRL2/TUSC4 and BRCA1 needs to be further investigated. Additionally, the difference between normal conditions and under irradiation will also need to be compared. In other words, upon the DNA damage response, will BRCA1 be shuttled from cytoplasm to nucleus, and whether NPRL2/TUSC4 stabilizes BRCA1 in nucleus? If this is true, the recruitment of NPRL2/TUSC4 to DNA damage foci will occur with BRCA1's transport simultaneously, even NPRL2/TUSC4 doesn't physically interact with BRCA1. In this case, future work still need to be done to identify the mechanism behind NPRL2/TUSC4 also positively regulates BRCA1's stability at normal physical conditions, is there any feedback loop involves in this negative regulation

of BRCA1 expression to maintain the minimal BRCA1 expression level (Baldassarre G et al, 2003; Mueller CR and Roskelley CD, 2003).

Other than HR repair, BCRA1 was also reported to associate with KU80 during NHEJ repair to maintain the fidelity end-joining and maintain the repair efficiency (Jiang et al, 2013). However, the regulation only happens in certain types of NHEJ (in-vivo precise NHEJ), thus, even NPRL2/TUSC4 might play roles in regulating NHEJ repair, such functions are most likely to limited into the framework of BRCA1 stability based on our current understanding. However, further research can be performed to identify if NPRL2/TUSC4 affect the activities of critical NHEJ repair effectors such as KU70-KU80 dimers and 53BP1.

**Clinical relevance of NPRL2/TUSC4 regulation of BRCA1**

We have identified NPRL2/TUSC4 functions as tumor suppressor gene in breast cancer, possibly through positively regulating BRCA1. Next, we postulated that overexpression of NPRL2/TUSC4 also suppresses breast tumor proliferation *in vitro* and in *vivo*. To validate this, we compared the proliferation rate of the breast cancer cell line MDA-MB-231 with or without NPRL2/TUSC4 overexpression. Colony formation assays indicated markedly reduced proliferation of the NPRL2/TUSC4-overexpressing cells (Figure 30A). Because NPRL2/TUSC4 effectively inhibits breast cancer cell growth *in vitro*, we further examined the effect of NPRL2/TUSC4 overexpression in a xenograft mouse model of breast cancer. We injected female mice with control or NPRL2/TUSC4-overexpressing MDA-MB-

231cells into the mammary fatpads. We then monitored and measured tumor growth weekly. By week 6 after injection, 5 of 10 mice injected with NPRL2/TUSC4-overexpressing cells remained tumor-free, whereas all 5 mice injected with control 231 cells had large tumors (Figure 28 A, B; Table 1).

Previous reports indicated that HR repair defect sensitizes cancer cells to DNA damaging drug (Foray et al 1999; Deng and Wang 2003) and the poly(ADP-ribose) polymerase (PARP) inhibitor (Alli et al., 2009; Powell and Kachnic, 2003). Thus, NPRL2/TUSC4 deficiency cells with HR repair defect are highly likely to be more sensitive to the treatment of PARP inhibitor, which can effectively inhibit the repair of single strand DNA break. To confirm this hypothesis, we performed colony formation assay in U2OS cell with NPRL2/TUSC4 knockdown after PARP inhibitor Olaparib and Rucaparib treatment, as well as control cells. As we expected, both drug significantly reduced the colony formation in NPRL2/TUSC4 knockdown cells compared the control cells (Figure 29 A,B). Additionally, we examined NPRL2/TUSC4 depletion to determine whether it initiates breast tumor development in a xenograft mouse model. We injected MCF-10A cells with stable knockdown of NPRL2/TUSC4 expression and the control cells into the mammary fatpads of female nude mice. Similar to the procedure described above, we closely monitored tumor formation in the mice. Notably, tumors began to form in 3 of 10 mice injected with NPRL2/TUSC4-knockdown cells after 3 weeks, whereas no tumors formed in the control groups (Table 2). These results demonstrated that loss of NPRL2/TUSC4 expression alone is sufficient to initiate malignant transformation of immortalized nontransformed mammary epithelial cells, which is

consistent with our hypothesis that NPRL2/TUSC4 functions as a bona fide tumor suppressor in breast cancer.

## Chapter 6

## FUTURE WORK

### Further explore the interaction between NPRL2/TUSC4 and HERC2

We have successfully identified that NPRL2/TUSC4 physically interacts with E3 ligase HERC2 but not BRCA1, thus blocking the binding between HERC2 and BRCA1, in turn stabilizing BRCA1's expression and maintaining its functions in HR repair and other DNA damage responses. Previous report indicated NPRL2/TUSC4's N-terminal 133 amino acid residues are responsible for the binding of NPRL2/TUSC4 to PDK1's phosphorylation sites (Kurata et al., 2008). Also, HERC2's C-terminal HECT domain is responsible for the binding of BRCA1's N-terminal degron domain (Wu et al, 2010). The unanswered question is whether NPRL2/TUSC4's N-terminal domain is also responsible for the binding of HERC2, and if so, is HERC2's C-terminal domain involved in the binding. We have generated deletion mutations of NPRL2/TUSC4-FLAG stable clones to test this hypothesis. If the results show that NPRL2/TUSC4's N-terminus physically interact with HERC2's c-terminus, it will not only confirm the functions of both NPRL2/TUSC4 and HERC2 's binding domain, but it will also provide clinical insights to target binding domains of HERC2 to treat BRCA1down-regulation patients in multiple types of cancers (Moskwa et al, 2010; Deutsch et al.,2003; Beger et al., 2004). Furthermore, it has been also shown BARD1 protects BRCA1 from HERC2-mediated ubiquitination and HERC2 degrades BRCA1 regardless of the existence of BARD1 (Wu et al., 2010). as an extensively studies BRCA1-Ring domain binding protein, if NPRL2/TUSC4 knockdown affect BARD1's binding with

BRCA1, if so, does this disassociation allow BRCA1's RING domain becomes more accessible for HERC2's targeting? An alternative model could be NPRL2/TUSC4 binds HERC2 and BARD1 (instead of BRCA1) to realize its function in mediating BRCA1's stability. Together these experiments could provide evidence to support our current model or to help establish revised model of NPRL2/TUSC4's function in BRCA1 and HERC2 interaction (Figure 23, 24).

**Determine the function of NPRL2/TUSC4 in replication stress response**

Besides the data shown in this dissertation, we have also discovered the association of NPRL2/TUSC4 in replication stress response. NPRL2/TUSC4 exhibited extensive binding capacity to proteins other than PDK1 and HERC2, as mentioned previously. Other major DNA damage response kinase such as ATM and ATR also physically interact with NPRL2/TSUC4. This would explain our observation in the HR repair assay that double knockdown of TSUC4 and BRCA1 further reduced the reporter protein GFP's level, and overexpression of BRCA1 in the cells with NPRL2/TUSC4 knockdown didn't completely restore the HR repair efficiency. If BRCA1 is not the only downstream targets of NPRL2/TUSC4 inactivation, the phenomenon we observed matched the hypothesis that NPRL2/TUSC4 play a more intensive role in DNA damage response network. Although NPRL2/TUSC4 knockdown cells shows no significant difference of cell cycles distribution compare to control, after both cells were treated with Hydroxyurea (HU), NPRL2/TUSC4-deficient cells exhibited extended G1 arrest while controls cells were able to escape from G1 arrest. This data suggested

NPRL2/TUSC4's function in mediating cell cycle checkpoints, which is consistent with our previous IPA analysis, which indicated that NPRL2/TUSC4 knockdown globally changed the gene expression profiles of cell cycle checkpoint regulation and DNA damage responses. The mechanisms under this regulation needs to be explored to test if these bindings are also regulated by the ubiquitination pathway. Based on the recent findings that NPRL2/TUSC4 mRNA levels are significantly reduced with the progression of cancer development, and low NPRL2/TUSC4 level correlates with multiple drugs' resistance so it is worth taking the possibility into consideration in which decreased NPRL2/TUSC4 expression levels are associated with the stability or functions of these major DNA damage response kinase. Understanding of the mechanism whereby NPRL2/TUSC4 negatively regulates these kinases is significant because of the effect it has on replication stress response and potential application in clinical drug design or selection. Most likely, these regulation will process through the binding or blocking due to the lack of functional domains of NPRL2/TUSC4 such as phosphoryation, so the direct regulation of NPRL2/TUSC4 to these kinase is not likely. But considering, the conserved SQ/TQ motif in NPRL2/TUSC4 sequences cross the species, it's highly possible NPRL2/TUSC4 can also serve as the binding substrates of ATM/ATR protein, and regulate their activities.

**Further demonstrate NPRL2/TUSC4-deficient cells' drug sensitivity**

We have identified the increased sensitivity of U2OS cells with NPRL2/TUSC4-deficiency, largely due to decreased BRCA1 stability and impaired

HR repair capacity by the cells. The signatures involves in HR repair deficiency has been established by our previous work (Peng et al., 2014). Although NPRL2/TUSC4 KD can also clearly form a cluster pattern based on the 230 identified gene signatures, it will be interesting to evaluate the changes in more details. For example, HR repair proteins other than BRCA1 could also be involved after NPRL2/TUSC4's expression is reduced. It's logical that these gene changes in NPRL2/TUSC4 deficient cells overlaps BRCA1 deficiency (and PTEN deficiency) gene signatures which the signature established from, it actually narrowed down the numbers of genes potentially involves in the pathways followed by NPRL2/TUSC4 inactivation. Additionally, NPRL2/TUSC4's low expression has also been identified as the cause of CPT resistance in lung cancer. The proposed mechanism is that NPRL2/TUSC4 deficiency failed to stabilize the DNA damage response signaling cascades (Jayachandran et al., 2010). It's still unclear what other proteins associated with drug responses after NPRL2/TUSC4's inactivation and it would be interesting to map out the pathways affected by NPRL2/TUSC4's deficiency, and to determine if BRCA1 is the only readout which affected the sensitivity of drug treatment such as PARP inhibitors and DNA damaging reagents. Annexin V staining can also be conducted to investigate if NPRL2/TUSC4 knockdown leads to the increase of apoptosis cell populations before and after treatment with different drugs. We propose that depending on the drugs' mechanism and target, the results will not provide same effect of TSUC4 deficiency cells, it also needs to take the cell or cancer types into consideration.

**Further investigate NPRL2/TUSC4's role as a tumor suppressor**

We and others provide evidence that both NPRL2/TUSC4 overexpression *in vitro* and *in vivo* inhibited breast cancer cell proliferation and tumor development. The same effect of NPRL2/TUSC4 was identified in non-small cell lung cancer (Li et al., 2004). Moreover, we showed for the first time that depletion of NPRL2/TUSC4 in non-transformed 10A cell lines promoted the genomic instability and lead to the occurrence of early breast cancer lesions in nude mice models. To further confirm this hypothesis, additional experiments are necessary to include into the future work. For example, comet assay can be done to determine the genomic instability after NPRL2/TUSC4 knockdown and invasion assay can be done to explore if NPRL2/TUSC4 deficiency increases cell's malignancy and mobility.

In our *in vivo* experiments, we injected MDA-MB-231 cells with NPRL2/TUSC4-flag overexpression into nude mice and observed much slower tumor growth rates compared to normal MDA-MB-231 cells. MDA-MB-231 cells are categorized as basal breast cancer cell lines, which share common characteristics as triple negative breast cancer types (Badev et al., 2011). We identified that NPRL2/TUSC4 knockdown cells exhibit a more basal-like gene expression profiles than luminal. This indicates that NPRL2/TUSC4 can serve as a biomarker to treat basal-like or triple negative breast cancer, or the marker to indicate therapy efficiency. Drug screening will also be extremely useful to distinguish if NPRL2/TUSC4 can be used as marker for that purpose. Together, these

experiments will lead us to a better understanding of the roles of NPRL2/TUSC4 as tumor suppressor.

Finally, since NPRL2/TUSC4 expression levels vary depending on organs and tissues, it would be interesting to investigate whether this protein functions only in maintaining genome stability, and whether its function across various cancers is tissue-specific.

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