UNDERSTANDING THE ROLE OF BRCA1 IN REDOX HOMEOSTASIS REGULATION

By

SUSAN ZWYEA

A dissertation submitted to the

Graduate School-New Brunswick

And

The Graduate School of Biomedical Sciences,

Rutgers, The State University of New Jersey,

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Microbiology and Molecular Genetics

Written under the direction of

Associate professor Bing Xia

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2017

ABSTRACT OF THE DISSERTATION

Understanding the Role of BRCA1 in Redox Homeostasis Regulation

By SUSAN ZWYEA

Dissertation Director:

Associate professor Bing Xia

The *BRCA1* (Breast Cancer 1, early onset) tumor suppressor gene is the most frequently mutated gene in familial breast cancer. Loss of BRCA1 function results in severe defects in DNA double-strand break repair and increased reactive oxygen species, both of which are threats to genomic stability. Although BRCA1 has been extensively studied, how it regulates redox homeostasis remains poorly understood and need to be further elucidated. Of note, a recent study suggested that BRCA1 promotes the transcription of *SOD2/MnSOD*, the mitochondrial superoxide dismutase.

In this study, we found that BRCA1 depletion showed limited effect on *SOD2* mRNA levels in multiple settings. Instead, we found an inverse between BRCA1 and SOD2 protein abundance, as breast cancer cells depleted of BRCA1 show increased SOD2 protein levels. In addition, deletion of *Brca1* in mice led to increased SOD2 amount in the liver. Our studies suggest that BRCA1 may

be involved in the regulation of SOD2 protein stability and/or that SOD2 is stabilized in response to the oxidative stress induced by BRCA1 loss.

Our gene expression analysis of human breast cancers showed that triple negative breast cancers have the highest *SOD2* mRNA levels among all subtypes. Interestingly, low *SOD2* mRNA level is associated with slightly better prognosis in all patients but substantially worse survival in patients with triple negative or HER2-positive tumors. This finding suggests that SOD2 may modulate sensitivity of breast cancer cells to chemotherapies. Indeed, triple negative breast cancer cell lines depleted of SOD2 show resistance to breast cancer chemotherapies. Taken together, our studies identify a novel mechanism of BRCA1 in regulating redox homeostasis and a novel role of SOD2 in modulating therapy response.

Acknowledgements

I am indebted to all of the following people for their support that let me reach a point where you could be reading this right now.

First and foremost, I am grateful to my thesis advisor, Dr. Bing Xia for everything. Dr. Xia enabled me to contribute to our understanding in a meaningful way by challenging my abilities. He is genuinely the most exceptional scientist I have ever had the pleasure of working with. More importantly, his impact on my scientific capacities will be invariably obvious to anyone lucky enough to know that man. Thank you for your support as without your support this would not be possible to any extent at all.

Also I would like to sincerely stress how grateful I am to all of the members of my thesis committee: Dr. Nancy Walworth, Dr. Victor Jin, and Dr. Michael Gatza. Thank you for the support as well as your critical assessment of my progress that has enriched my development. I could not have done this without the guidance of these truly fantastic scientists. There are no words that could ever convey how important they have been.

I would also like to thank all of Xia lab members: Yanying, Gabriele, Tzeh, Kevin, Amar, Bola, Hong, and Srilatha. They have been a source of strength that has helped me move through the PhD process smoothly. Special thanks to Dr. Yanying for training me, helping me and paving the way for me.

Finally, I would like to thank my family. I would like to thank my mother and father for making me well equipped to take this journey. Above all else, I owe my devoted husband and my kids the most appreciation. They make all of the sacrifices and sleepless nights worthwhile.

Table of Contents

List of tables

List of figures

1. INTRODUCTION

Section I – BRCA1 in Genomic Maintenance

BRCA1, breast cancer 1 early onset gene

BRCA1 is a breast cancer susceptibility protein that has a role in both hereditary and sporadic breast cancer. The *BRCA1* gene was localized to chromosome 17q12-q21 by linkage analysis of kindred's in 1990 and cloned in 1994. (Hall, Lee et al. 1990, Ford, Easton et al. 1994, Futreal, Liu et al. 1994, Miki, Swensen et al. 1994). Since that time, BRCA1 structure and molecular function have been the subject of intense study.

Germline heterozygous mutations in *BRCA1* increase the risk of developing early onset breast cancer and to a lesser extent ovarian, prostate and pancreatic cancer. Even though *BRCA1* mutations account only for about 3% of all breast cancer, due to the high incidence of the disease a large number of women are still affected by these mutations (Yi, Kang et al. 2014, Couch, Hart et al. 2015, Finn, Martin et al. 2016).

The *BRCA1* gene consists of 22 exons and encodes a large tumor suppressor protein composed of 1,863 amino acid residues. Its amino terminus contains a RING domain that associates with BRCA1 associated RING domain protein 1 (BARD1), two nuclear localization signal sequences (NLSs) and nuclear export signal (Fig. 1). BRCA1 carboxyl terminus contains a coiled-coil domain that mediates its association with partner and localizer of BRCA2 (PALB2). The carboxyl terminus also contains a SQ/TQ cluster domain (SCD) that has ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3-related (ATR) kinases phosphorylation sites, as well as two BRCT domains that binds to phosphorylated Abraxas, CtBP interacting protein (CtIP) and a BRCA1 interacting protein C terminal helicase 1 (BRIP1). The middle region of BRCA1 contains a CHK2

phosphorylation sites (Chapman and Verma 1996, Chen, Li et al. 1996, Monteiro, August et al. 1996, Thakur, Zhang et al. 1997, Rodriguez and Henderson 2000).

Moreover, functional studies have demonstrated that BRCA1 is a versatile multifunctional protein and its primary function is maintaining genome integrity (Savage and Harkin 2015). Evidences supporting a BRCA1 function in genomic stability maintenance has demonstrated that BRCA1 (1) promotes faithful DNA double strand breaks (DSBs) repair (Shinohara, Ogawa et al. 1992), (2) is essential for cell cycle checkpoint regulation (Aprelikova, Fang et al. 1999, Choudhury, Xu et al. 2004), (3) functions in oxidative stress regulation (Bae, Fan et al. 2004, Saha, Rih et al. 2009, Yi, Kang et al. 2014), (4) regulates transcription of several essential genes through its interaction with the RNA polymerase II (Gardini, Baillat et al. 2014), (5) is essential for proper replication and function of centromere (Di Paolo, Racca et al. 2014), and (6) induces energetic metabolism reprogramming (Privat, Radosevic-Robin et al. 2014).

Taken together, the established role of BRCA1 as a genome care taker has important implications for the prevention and treatment of BRCA1 associated cancers.

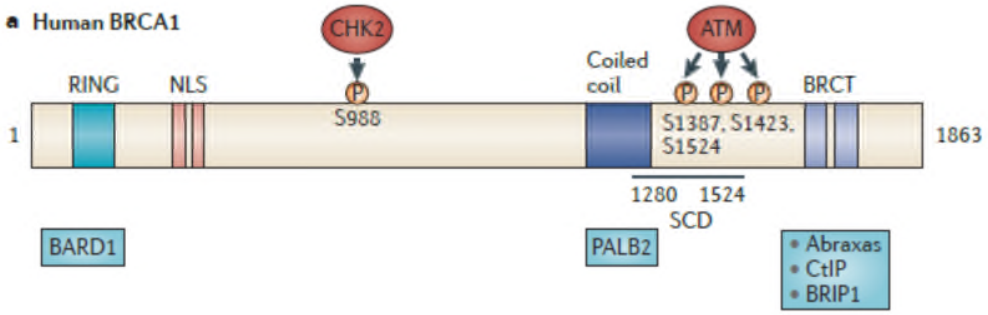

Figure 1. BRCA1 structure and functional domains (adapted from Roy et al., 2011, *Nat reviews* 12: 68-78). BRCA1 amino terminus contains a RING domains that associates with BRCA1 associated RING domain protein 1 (BARD1) and 2 nuclear localization signals (NLS). Its carboxyl terminus contains a coiled-coil domain that enables BRCA1 to associates with partner and localizer of BRCA2 (PALB2). The carboxyl terminus also contains a SQ/TQ cluster domain (SCD) that has more than ten ATM/ATR phosphorylation sites and a tandem BRCT domain that binds to phosphorylation Abraxas , CtBP interacting protein (CtIP) and a BRCA1 interacting protein C terminal helicase 1 (BRIP1).

Genomic Stability

Genomes are routinely challenged by diverse exogenous or endogenous insults. These insults can generate different types of lesions in the DNA that ultimately cause genetic instability, leading to abnormalities in cell functions and cancer initiation. The fidelity of the genome is highly protected at every stage of the cell cycle through faithful DNA replication and accurate repair of DNA damage. Of note, cells have many conserved mechanisms that repair damaged DNA and protect against alterations of the genome such as, Photolyase that can repair thymine dimers induced by UV light, O^6-alkylguanine alkyltransferase that repairs alkylated bases, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous

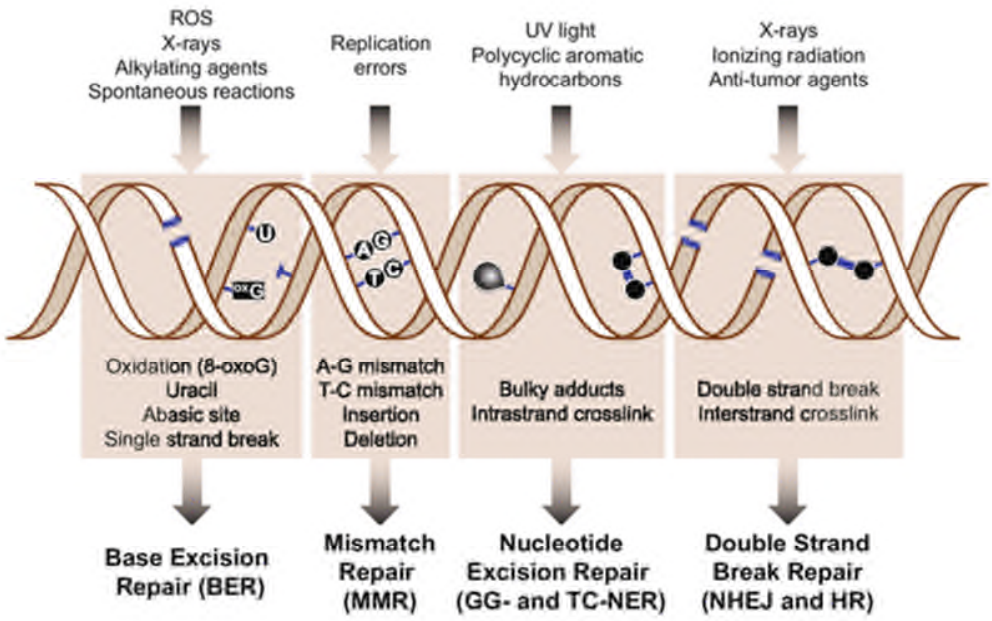

Figure 2. DNA damage and repair mechanisms (adapted from Thomas ,2012, *DNA Repair of Cancer Stem Cells (2)19-32*). DNA lesions caused by DNA damaging agents, and the relevant DNA repair mechanism responsible for damage removal.

recombination (HR) that restores genome to its original state, and non-homologous end joining (NHEJ) that prevents cell death by preserving genomic DNA (Fig. 2). All of these mechanisms have their own essential repair proteins which when lost or mutated cause accumulating of mutations and cancer initiation. Table 1-1 summarizes the essential proteins involve in the five major repair pathways. Mutations in key components of the DNA double strand repair and signaling pathway are a predisposing factor for hereditary breast and ovarian cancer, which suggest that DNA repair pathways suppress mammary tumorigenesis (Kennedy and D'Andrea 2006, Jalal, Earley et al. 2011).

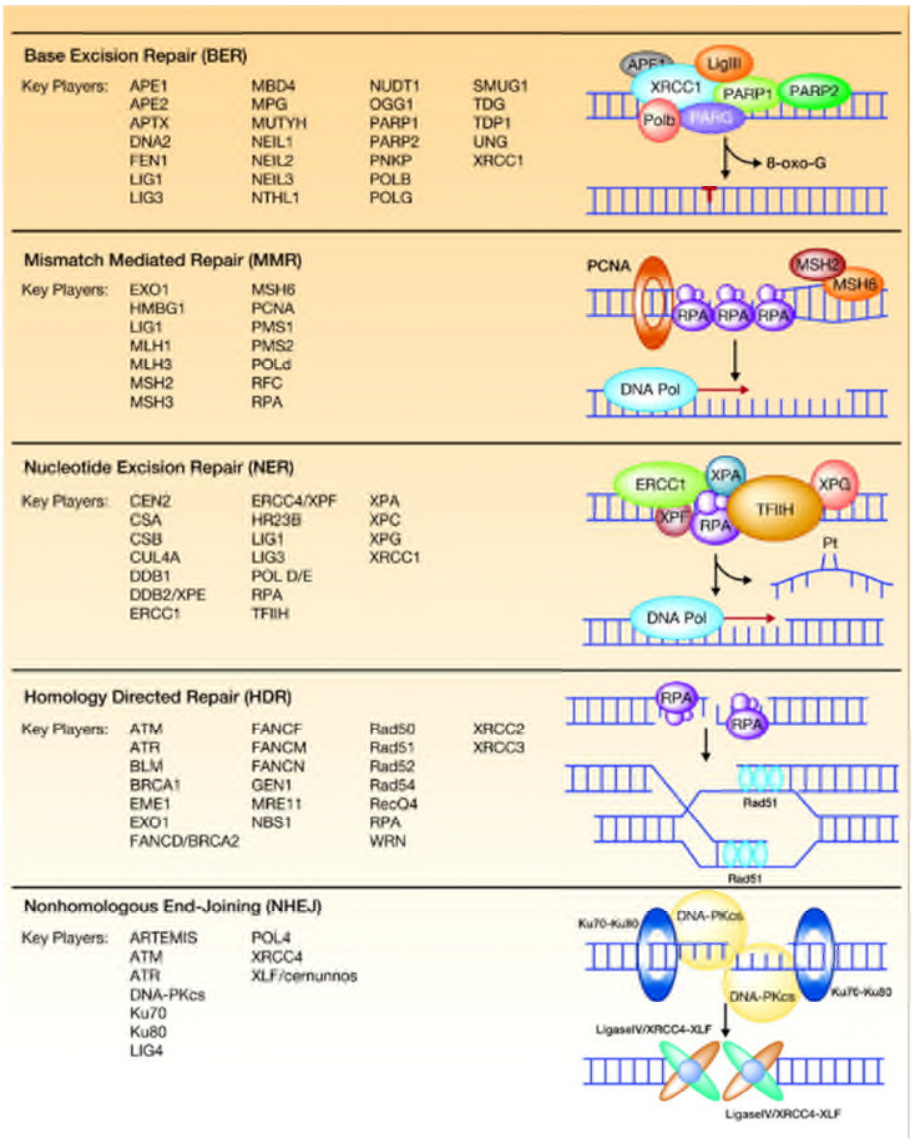

Table 1-1. Essential genes of the five major DNA repair mechanisms (adapted from Jalal. et al., 2011, *Clin Cancer Res;* 17(22) 6973-6984.

DNA Double-Strand Break Repair by Homologous Recombination

DNA double-strand breaks (DSBs) are the most hazardous lesions to the cells because it can lead to genomic instability. Cells have two major mechanisms to repair DSBs, HR and NHEJ. When DSBs form, the two major repair pathways compete to repair DSBs. HR requires a template and therefore operates mainly in the S and G2 phases when a sister chromatid is available and nearby, while NHEJ is active in all cell cycle phases. Due to its ability to repair DNA replication-associated complex DNA breaks and its high fidelity, HR plays an essential role in maintaining genome stability in proliferating cells. The protection of the genome by HR involves damage recognition by the MRE11-RAD50-NBS1 (MRN) complex, damage signaling by the ATM and ATR kinases, processing of DNA ends and the repair of the broken DNA (Shuen and Foulkes 2011).

BRCA1 appears to be necessary for HR to occur (Bunting, Callen et al. 2010, De Lorenzo, Patel et al. 2013, Zhang 2013). Upon DNA damage, BRCA1 together with the Abraxas-RAP80 macro complex can recognize and bind to DSBs. Next, BRCA1 interacts with MRN-CtIP complex to trigger DSBs processing (Fig. 3). The BRCA1- MRN-CtIP complex promotes 5'-end resection to generate single-strand regions necessary for HR to initiate (Reczek, Szabolcs et al. 2013, Cruz-Garcia, Lopez-Saavedra et al. 2014). In addition to promoting resection, BRCA1 plays an important role in RAD51 recruitment through its interactions with PALB2 and Breast cancer early onset 2 (BRCA2) (Buisson, Dion-Cote et al. 2010). Recruitment of RAD51 is critical to initiate HR through homologous template recognition and strand invasion. PALB2 plays important role in HR in part by serving as a linker of BRCA1 and BRCA2. The N-terminus of PALB2 contains a coiled-coil domain which binds BRCA1 while its C-terminus forms a beta propeller structure that binds BRCA2. Moreover, BRCA1 along with BRCA2 and PALB2 work together in a "BRCA" complex to promote homologous recombination (HR) (Xia, Sheng et al. 2006, Zhang, Ma

et al. 2009). All three proteins are important in maintaining genomic stability and all three respective genes are well-established tumor suppressors. Last and not the least, regulation of the HR process is tightly linked to cell cycle progression for many reasons. First, BRCA2 and Rad51 are expressed in S and G2 phases of the cell cycle meaning no HR in G1. Second, the cyclin dependent kinase CDK2, which catalyzes the phosphorylation of CtIP, is only active in S phase. Lastly, cells in G1 and G0 have no sister chromatids meaning they cannot provide homologous sequences as repair templates (Zhang, Ma et al. 2009, Buisson and Masson 2013, Trovesi, Manfrini et al. 2013).

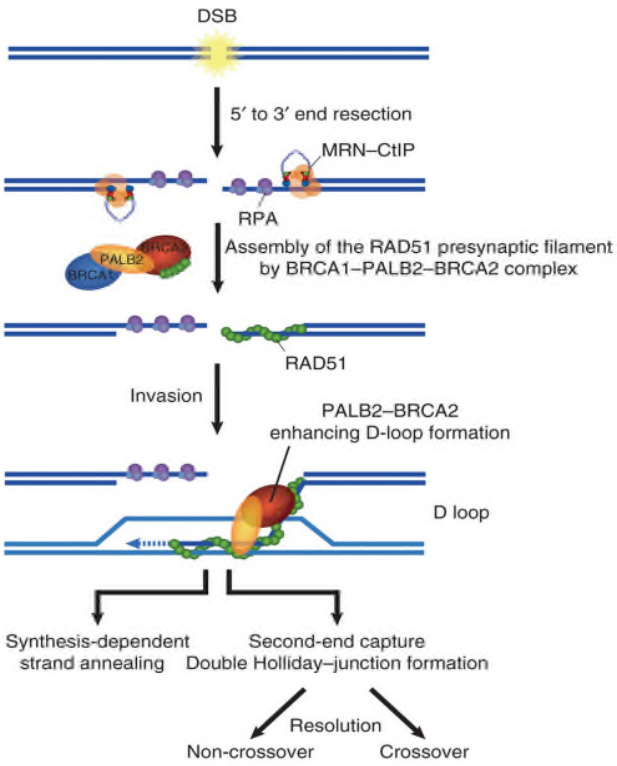

Figure 3. BRCA1 in homologous recombination (adapted from Buisson et al. *Nat Struc Mol Biol*. 17: 1247-1254). BRCA1 facilitates resection and the assembly of RAD51 filament to initiate HR.

BRCA1 in Oxidative Stress Response

Oxidative stress is the imbalance between the production of reactive oxygen species (ROS) and cell's antioxidant defenses. (Hanahan and Weinberg 2011). High levels of ROS can damage DNA, proteins and lipids that are vital for the cellular function, and therefore high ROS can induce apoptosis in the cells. However, moderately elevated levels of ROS can induce mutations within the genome, which may lead to transformation of normal cells into cancer cells. Cancer cells take advantage of the imbalance in redox system and proliferate (Acharya, Das et al. 2010). Cells developed an elaborate antioxidants system responsible for reducing ROS levels. The system

consists of antioxidants such as glutathione (GSH) and antioxidant enzymes such as superoxide dismutase (SOD2) (Naithani 2008).

Regulation of the antioxidant proteins and enzymes is not fully understood, yet but there are several lines of evidence that implicate tumor suppressors such as BRCA1 in the regulation of these antioxidants (Kang, Hong et al. 2011, Martinez-Outschoorn, Balliet et al. 2012, Gorrini, Baniasadi et al. 2013). It is well known that BRCA1 has multiple functions and one of its major functions is protecting cells against oxidative stress. BRCA1's effect on oxidative stress can be either direct or indirect. In this context, there are considerable mounting evidence suggesting that BRCA1 influence the transcription of the cytoprotective phase II proteins and antioxidant enzymes. First, deletion of exon 11 of *Brca1* in mouse cells downregulates the expression of multiple genes that are involved in the cytoprotective response, such as nuclear factor erythroid-derived 2 like 2 (NFE2L2 or NRF2) Table 1-2 lists some of the antioxidant response genes regulated by BRCA1 (Bae, Fan et al. 2004). This observation indicated that BRCA1 is directly involved in oxidative stress regulation.

Table 6 *Selected genes whose expression is decreased in* $Brca1^{\Delta ex11\Delta ex}$ *(versus* $Brca1^{+/+}$*) mouse embryo fibroblasts*

Accession no.	Gene name	Symbol	Ratio: Mean ± SE (range)	Ratio: Affymetrix Array ($N = 1$)
Transcription/Nuclear proteins				
AA208865	Nuclear receptor corepressor 1	Ncor1	0.47 ± 0.08 (2)	
AA276365	Myocyte enhancer factor 2c	Mef2c	0.47 ± 0.07 (2)	0.44
AA244944	CCAAT/enhancer binding protein α (C/EBP), related sequence 1	Cebpa-rs1	0.51 ± 0.02 (2)	
AA212695	*Trans*-acting transcription factor Sp1 (cell cycle-regulated)	Sp1	0.51 ± 0.09 (2)	
AA036347	Kruppel-like factor 9	Klf9	0.52 ± 0.11 (2)	
AA125037	Zinc finger protein 148	Zfp148	0.52 ± 0.01 (2)	
AA138529	Zinc finger protein 62	Zfp62	0.56 ± 0.15 (2)	
AA501045	Nuclear receptor subfamily 2, group E, member 2	Nr2e2	0.56 ± 0.11 (2)	0.061
AA155377	Zinc finger protein X linked	Zfx	0.57 ± 0.01 (2)	
W83524	Transcription factor 12	Tcf12	0.58 ± 0.06 (3)	
AA097341	Nuclear receptor coactivator 1	Ncoa1	0.59 ± 0.07 (3)	
Stress response: oxidative stress and xenobiotic detoxification				
AA200734	Ethanol decreased 2	Etohd2	0.47 ± 0.07 (2)	
AA445861	Activating transcription factor 2 (stress response transcription factor)	Atf2	0.50 ± 0.07 (2)	
AA139271	Glutathione peroxidase 3	Gpx3	0.51 ± 0.05 (3)	0.12
W54349	GST, α 1 (Ya)	Gsta1	0.51 (1)	
AA120574	Superoxide dismutase 1, soluble	Sod1	0.53 ± 0.07 (2)	
AA044475	Nuclear factor, erythroid-derived 2, like 2 (also called Nrf2)	Nfe2l2	0.53 ± 0.07 (2)	
AA033466	Myeloperoxidase	Mpo	0.53 ± 0.06 (2)	
AA060716	Early growth response 1 (stress-responsive transcription factor)	Egr1	0.54 ± 0.16 (2)	
AA276440	Selenoprotein P, plasma, 1	Sepp1	0.57 ± 0.02 (3)	
W82873	Aryl hydrocarbon receptor	Ahr	0.59 ± 0.15 (2)	
AA250120	GST, α 2 (Yc2)	Gsta2	0.61 ± 0.10 (2)	

Table 1-2 selected genes whose expression is decreased after deleting exon 11 of *Brca1* in mouse embryonic fibroblasts. Adapted from Bae, Fan et al. Cancer Res 64(21): 7893-7909.

Second, another group showed that overexpression of wt BRCA1 in MCF7 cells is reduces intracellular ROS levels and protection of cells against oxidative stress (Saha, Rih et al. 2009). Third, BRCA1 was also reported to indirectly protects against oxidative stress by regulating transcription-coupled repair of DNA lesions caused by oxidative stress such as 8-oxoguanine (8-oxoG) (Dziaman, Huzarski et al. 2009). Overexpression of BRCA1 in MCF7 breast cancer cells increased protein level of oxo-guanosine DNA glycosylase 1 (OGG-1), an enzyme responsible for cleavage in the area that surrounds the damaged nucleotides in BER. Subsequently it was found that downregulation of BRCA1 increases the level of 8-oxoG. This increase in 8-oxoG is linked to transcriptional regulation of BER pathway by BRCA1 and it further confirms the role of BRCA1 in oxidative stress regulation (Dziaman, Huzarski et al. 2009, Saha, Rih et al. 2009, Acharya, Das et al. 2010, Kang, Hong et al. 2012).

Fourth, recent studies revealed that BRCA1 contributes to oxidative stress through the tumor suppressor protein p53 (Fig. 4). P53 is known to function in regulation of oxidative stress in response to ROS (Vurusaner, Poli et al. 2012). These studies demonstrated that BRCA1 stabilizes p53 and induces p53-dependent gene expression in response to oxidative stress. Furthermore, stabilization of p53 by BRCA1 is dependent on ATM pathway (Liu and Xu 2011, Yi, Kang et al. 2014). Although the interplay of BRCA1 and p53 in oxidative stress is complex and still poorly understood, these studies further implicate BRCA1 in oxidative stress regulation.

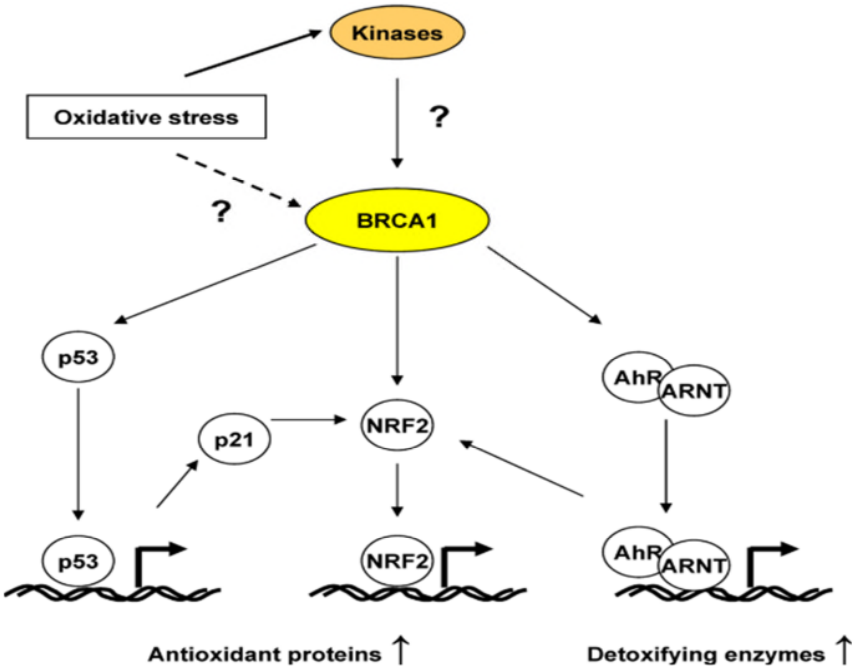

Figure 4. A model illustrating the role of BRCA1 in the regulation of oxidatives stress through p53 (adapted from Yi et al., 2014. *Cancers*, 6(2), 771-795. In response to oxidative stress BRCA1 activates p53 which has antioxidant activity. P53 is also able to induce NRF2 expression and its downstream target through p21(a cyclin dependent kinase inhibitor).

Fifth, it has been found BRCA1 induces p21 (a cyclin dependent kinase inhibitor) expression which itself exerts the antioxidant effects in response to oxidative stress most likely through its direct interaction with NRF2 (Zhang, Somasundaram et al. 1998, Chen, Sun et al. 2009).

NRF2 is a master transcription factor that drives the expression of many genes that function in detoxifying ROS (Miller, Gounder et al. 2012, Ma 2013). NRF2 availability is tightly regulated by Kelch ECH Associating protein 1 (KEAP1). Under unstressed conditions, KEAP1 binds to the "DLG" and "ETGE" motifs at the N-terminus of NRF2, and targets it for ubiquitination and proteasome-mediated degradation. Thus, KEAP1 is considered as a dominant negative regulator of NRF2 under non-stress conditions. However, under stress conditions, the interaction between NRF2 and KEAP1 is weakened, as high ROS levels oxidize some of the highly reactive cysteine

residues of KEAP1. Oxidation of the cysteine residues results in a conformation changed of KEAP1, which causes a partial dissociation of NRF2 from KEAP1. This conformation change prevents KEAP1 from mediating NRF2 degradation thereby leading to NRF2 stabilization and its accumulation in the nucleus (Fig. 5) (Kaspar, Niture et al. 2009, Taguchi, Motohashi et al. 2011, Kobayashi, Suzuki et al. 2013).

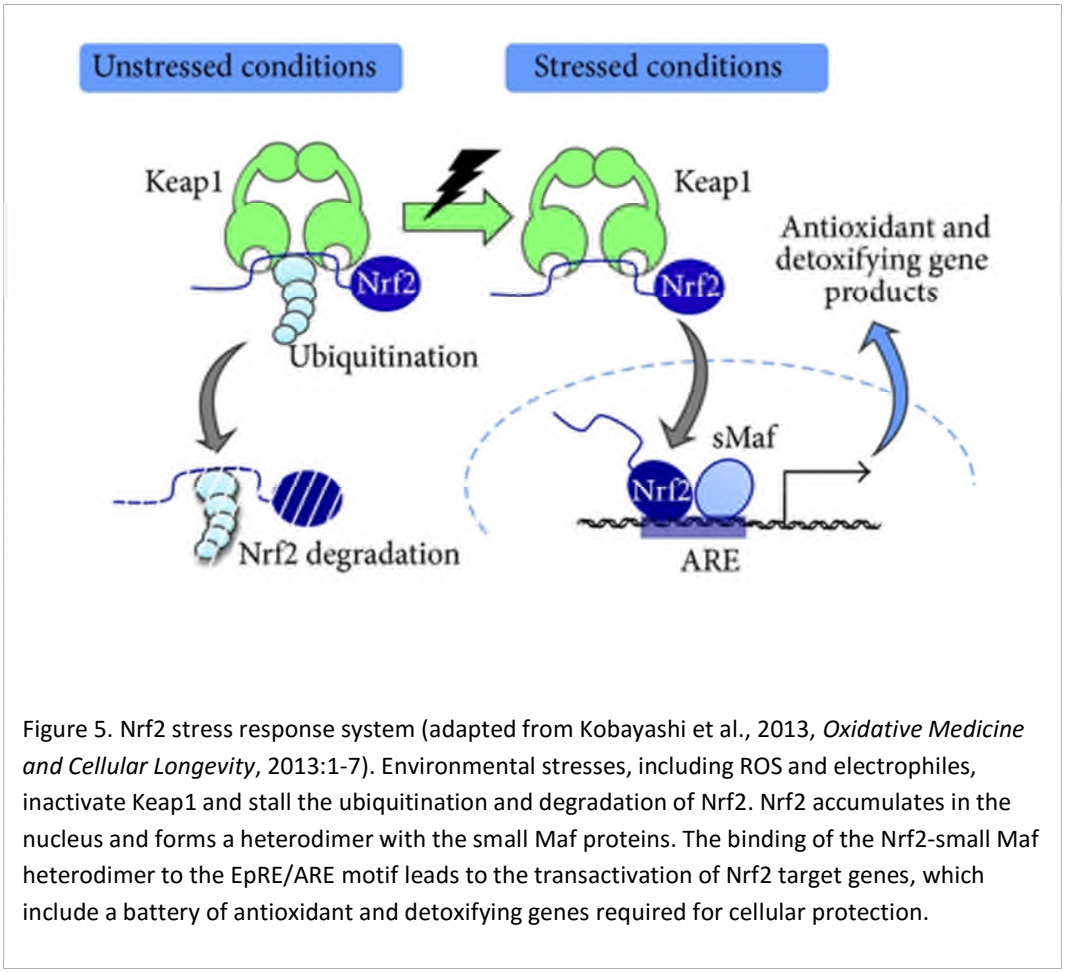

Figure 5. Nrf2 stress response system (adapted from Kobayashi et al., 2013, *Oxidative Medicine and Cellular Longevity*, 2013:1-7). Environmental stresses, including ROS and electrophiles, inactivate Keap1 and stall the ubiquitination and degradation of Nrf2. Nrf2 accumulates in the nucleus and forms a heterodimer with the small Maf proteins. The binding of the Nrf2-small Maf heterodimer to the EpRE/ARE motif leads to the transactivation of Nrf2 target genes, which include a battery of antioxidant and detoxifying genes required for cellular protection.

After NRF2 translocates into the nucleus, it binds to small Maf proteins to form a heterodimer. The heterodimer then binds to the anti-oxidant response elements (AREs) in the promoters of antioxidant genes such as NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate-cysteine

ligase, catalytic subunit (GCLC), glutamate-cysteine ligase, modifier (GCLM) hemeoxygenase 1 (HO-1), glutathione-s-transferase (GST),and thioredoxin (TXN) (Fig.6) (Gardini, Baillat et al. 2014, Ferguson, Chen et al. 2015).

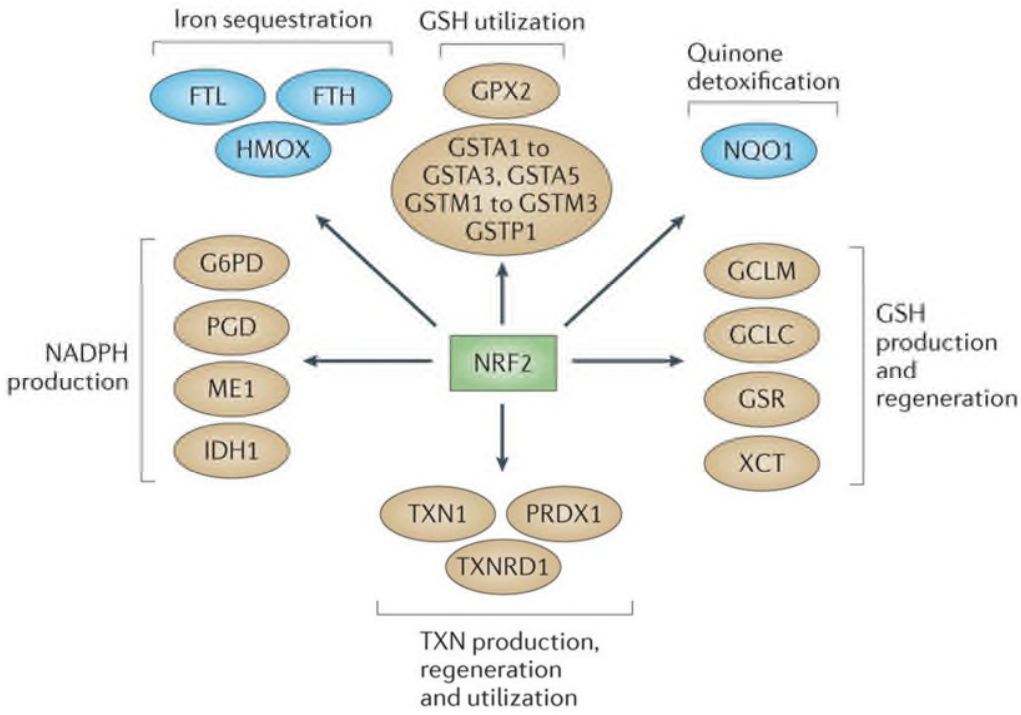

Figure 6 Regulation of antioxidant proteins by NRF2 (adapted from Gorini et al.,. *Nature reviews Drug discovery*. 12: 931-947). NRF2 regulates many different pathways. First, NADPH production. Second, glutathione (GSH) production. Third, thioredoxin (TXN). Fourth, GSH utilization. In addition, NRF2 regulates cytoprotective proteins including NQO1 and enzymes regulating iron sequestration.

The enhanced susceptibility of *Nrf2* knockout (*Nrf2−/−*) mice to the various toxins and environmental stresses clearly demonstrates the importance of this transcription factor in the protection against stress (Copple, Goldring et al. 2008). In addition, in breast cancer cells, NRF2 plays an important role in suppressing oxidative stress by upregulating phase II detoxifying enzymes. Also, overexpression of wtBRCA1 stimulates NRF2 expression thus indicating important function of NRF2 in BRCA1 associated breast cancer (Bae, Fan et al. 2004).

Sixth, it has recently been found that BRCA1 regulates another redox transcription factor NFkB and its downstream targets (Gardini, Baillat et al. 2014). Genome wide occupancy analysis revealed that BRCA1 occupies regulatory regions of hundreds of genes, among them NFkB and its downstream targets genes. It is well known that NFkB has multiple functions and one of its important functions is regulating oxidative stress through the major mitochondrial enzyme SOD2 (Harte, Gorski et al. 2014). Furthermore, BRCA1 knockdown appears to downregulates the expression of SOD2 in MCF7 breast cancer cells (Fig. 7) (Kamarajugadda, Cai et al. 2013, Gardini, Baillat et al. 2014). These studies further confirmed the role of BRCA1 in oxidative stress regulation.

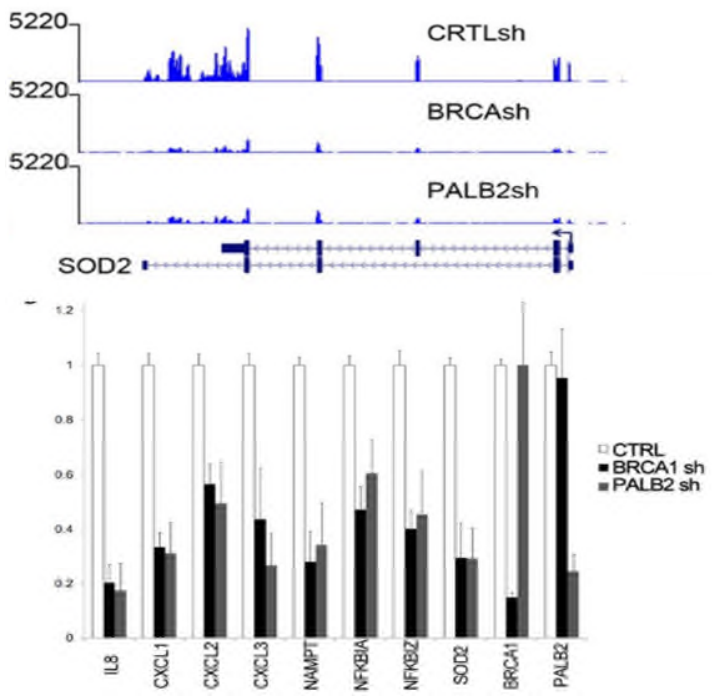

Figure 7. SOD2 regulation by BRCA1 (adapted from Gardini. *EMBO*. 15: 162-173). Top panel: BRCA1 occupies regions in SOD2 gene as revealed by CHIP seq analysis. Bottom panel: Knockdown of BRCA1 in MCF7 cells downregulates SOD2 RNA expression as analyzed by qRT-PCR.

BRCA1 Mouse Models

The role of BRCA1 in tumorgenesis has been well studied using different mouse models. Homozygous *Brca1* mutations or complete knockout of Brca1 results in early embryonic lethality, with the homozygous null embryos showing a severe defect in proliferation, hypersensitivity to radiation and genome instability (Evers and Jonkers 2006). However, different mouse models that can bypass this embryonic lethality have been generated to study BRCA1 functions.

It has been shown that conditional knockout of *Brca1* in the mammary gland induces breast cancer development only after a long latency (Brodie, Xu et al. 2001, Liu, Holstege et al. 2007, Shakya, Szabolcs et al. 2008), suggesting that neoplastic transformation may require additional mutations in other tumor suppressor genes. Simultaneous inactivation of p53 in combination with conditional inactivation of BRCA1 allows greatly accelerated mammary gland tumor development, indicating that p53 is a barrier to breast cancer development following *BRCA1* loss (Dine and Deng 2013). Table 1-3 summarizes known mouse models used in breast cancer research.

Table 1 Mutant mice carrying conventional mutations in Brca1 and their phenotypes

Brca1 alleles	Phenotypes
Replace exons 5 and 6 with a neo gene	Die between E6 and 7, proliferation defects
Replace 184 bp in 5′ portion of exon 11 with a neo gene	Similar to above
Replace exon 2 with a neo gene	Similar to above
Replace 330 bp of intron 10 and 1.5 kb of exon 11 with a neo gene	Die between E8.5 and 13.5, neuroepithelial abnormalities
Replace 330 bp of intron 10 and 440 bp of exon 11 with a neo gene	Die at E8.5, growth retardation, hypersensitive to γ-irradiation, chromosomal abnormalities
Insertion of a neo into intron 10 that blocks splicing between exons 10 and 11	Die at E9.5, growth retardation
Insert a neo into exon 22, which deletes the last 112 amino acids in the second BRCT domain	Die at E9.5–10.5 due to massive apoptosis
Insert 50 bp into exon 11, which truncates Brca1 after the first 924aa	Survive to adulthood in 129 background, develop various tumors
Delete short isoforms of Brca1 while keeping the full-length form intact	Mammary gland abnormalities and uterine hyperplasia after 1 year of age with spontaneous tumor formation. Abnormal G1/S transition, centrosome duplication
Delete full-length form while keeping short isoforms intact (Brca1Δ11/Δ11)	Die between E12.5 and 18.5, widespread apoptosis, genetic instability p53+/− or p53−/− mutation suppress apoptosis and embryonic lethality
Brca1Δ11/Δ11 in p53+/− or p53 −/− mice	Brca1Δ11/Δ11; p53−/−: develop lymphoma before 3 months of age Brca1Δ11/Δ11; p53+/−: 80 % undergo premature aging at 8 months of age; 90 % developed tumor at 1 year of age
Brca1Δ11/Δ11 in Chk2+/− or Chk2 −/− mice	Brca1Δ11/Δ11; Chk2−/−: 30 % developed premature aging and 35 % developed tumor at 1.5 years Brca1Δ11/Δ11; Chk2+/−: appeared normal during the studying period of time
Brca1Δ11/Δ11 in ATM+/− or −/− mice	Brca1Δ11/Δ11; ATM−/−: developed lymphoma before 6 months of age Brca1Δ11/Δ11; ATM+/−: appeared normal during the studying period of time
Brca1Δ11/Δ11 in 53BP1+/− or −/− mice	Brca1Δ11/Δ11; 53BP1+/−: no rescue Brca1Δ11/Δ11; 53BP1 −/−: developmentally normal although manifest constitutively high levels of genomic instability. Normal lifespan, with a surprisingly low incidence of overall tumor formation
Deletion of exon 11 in 53BP1 −/− mice	53BP1 deficiency restores an ATM-dependent error-free DNA repair by homologous recombination

Table 1-3. Mouse models generated for studying *brca1* associated breast cancer (adapted from Dine and Deng, 2013).

Section II –*SOD2 and Breast Cancer*

Breast Cancer and Antioxidant Capacity

Breast cancer is the second leading cause of cancer death in women with more than of 373,000 deaths in women every year (Conte, Guarneri et al. 2007). Despite all the progress in its diagnosis and treatment, a large number of women, diagnosed at late stages, still die from the cancer (Becuwe, Ennen et al. 2014).

Breast cancer can occur randomly in population (sporadic) or occur due to inherited gene mutations (familial). Clinically breast cancer can be classified into different subtypes depending on the presence of certain markers. These markers are estrogen receptor (ER), progesterone receptor (PR), and HER-2/Neu amplification. Basically, the markers allows stratification of breast cancer tumors into hormone receptor positive tumors (ER and PR positive subtypes or luminal A and luminal B subtypes), HER-2/Neu-amplified tumors, and triple negative tumors or basal-like tumors (ER, PR, and HER2/Neu negative tumors). The last subtype (Basal-like triple negative) in general lacks targeted therapies and often results in recurrent and lethal disease (Cheang, Voduc et al. 2008, Chavez, Garimella et al. 2010, Hudis and Gianni 2011, Pogoda, Niwinska et al. 2013).

Mechanisms leading to development of breast tumor are not clear. However, a number of risk factors have been identified for breast cancer. Increasing production of ROS inside cells has been proposed to be a common effect of most carcinogens (or risk factors) (Acharya, Das et al. 2010). Recent evidence suggests that increased ROS may play several roles such as effecting tumor initiation, growth and metastasis (Forristal, Wright et al. 2010, Brouckaert, Wildiers et al. 2012). Also, it was reported that cancerous cells generally produce higher level of ROS

comparing with normal cells. To cope with the increased production of ROS, cancer cells upregulate the cellular antioxidant system. One component of these antioxidant systems is the mitochondrial antioxidant enzyme SOD2, a target of NFkB. According to recent analysis of SOD2 in breast cancer patient, SOD2 was found often expressed at high levels in aggressive advanced grade cancers with negative status of hormone receptor and p53 mutations (Fig.8). This observation suggests potential impact of SOD2 on cancer development is likely through its product, hydrogen peroxide (H2O2) (Cobbs, Levi et al. 1996, Kahlos, Anttila et al. 1998, Quiros, Sainz et al. 2009, Sgambato, Camerini et al. 2009).

Interestingly, in chemotherapy and radiotherapy, which induce considerable levels of ROS, induction of ROS can selectively kill cancer cells by increasing ROS levels over their threshold that induce apoptosis (Dang 2012). However, in late stage cancers become resistant to these treatments due to acquired ability to resist ROS. ROS resistance can also be attributed to the manipulation of antioxidant defense gene expression. In addition to enhancing antioxidant capacity, most cancer cells alter their glucose metabolisms as they metabolize glucose via pyruvate to lactate through mitochondrial oxidative metabolism even in the presence of oxygen. (Kamarajugadda, Cai et al. 2013, Hart, Mao et al. 2015). A recent study shown that SOD2 may be central to the enhancement of H_2O_2-dependent signaling that enhance glycolytic metabolism, thus bolstering tumor progression and aggressiveness (Hart, Mao et al. 2015).

Recently, we found that depletion of SOD2 in some ER-negative breast cancer cell lines promotes resistance to chemotherapeutic drugs. Therefore, elucidation the role of SOD2 is an active area of research to find potential markers or targets for making chemotherapy more effective in breast cancer.

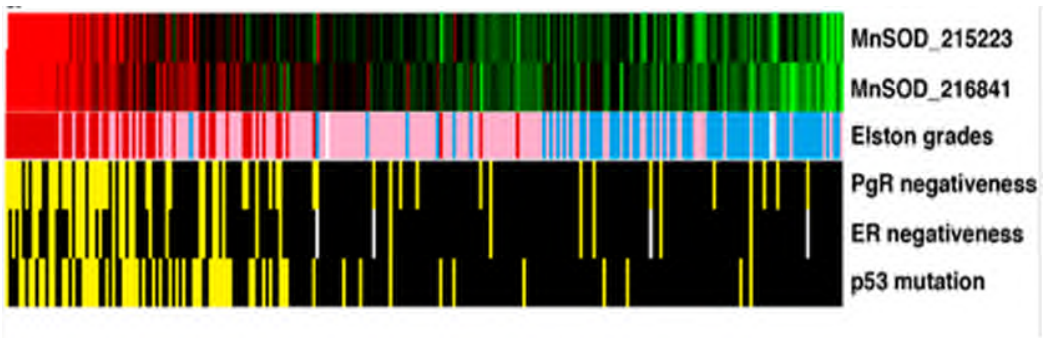

Figure 8. Expression of SOD2 mRNA in human breast cancer (adapted from Kamarajugadda et al., 2013.*cell death and disease*, 4:1-10). elevated expression of SOD2 is associated with high grades of tumors, negative status of estrogen receptor (ER) and progestron recptors (PgR). Elston grades of the tumor: high, red; medium, medium; low, blue. ER status: negative, yellow. p53 mutation: mutation, yellow.

SOD2 Function, Localization, Regulation and Roles in Breast Cancer

Several superoxide dismutase enzymes exist in the cell, including the cytosolic SOD (SOD1), the mitochondrial SOD (SOD2), and the extracellular matrix SOD (SOD3). SOD2 is considered to be the essential mitochondrial antioxidant enzyme for cell survival compared with the other two SODs (Zelko, Mariani et al. 2002). SOD2 knockout mice died shortly after birth with dilated cardiomyopathy and neurodegeneration (Weisiger and Fridovich 1973, Salin, Day et al. 1978, Carlioz and Touati 1986, Li, Oberley et al. 1995, Zelko, Mariani et al. 2002). Table 2-1 summarizes the characteristics of the three superoxide dismutases.

Summarized characteristics of three superoxide dismutases in eukaryotic cells

Isoform	Location	MW/kDa	Assembly of subunits	Metal ion
Cu/ZnSOD	cytoplasm, nucleus, mitochondrial membrane	88	homodimer	Cu^{2+} (catalytic active) Zn^{2+} (maintain enzyme stability)
MnSOD	mitochondria matrix	32	homotetramer	Mn^{2+} (catalytic active)
ECSOD	plasma membrane, extracellular fluids	135	hornotetrarneric glycoprotein	Cu^{2}+ (catalytic active) Zn^{2+} (maintain enzyme stability)

Table 1-4. Charecteristic features of three SODs in eukaryotic cells

SOD2 is a key mitochondrial metalloenzyme (manganese dependent) involved in balancing the mitochondrial redox state (Wheeler, Nakagami et al. 2001). SOD2 catalyzes the dismutation of matrix superoxide radicals ($O2^{\cdot}$), which form as a byproduct of the oxidative phosphorylation process, into hydrogen peroxide (H_2O_2) and molecular oxygen (Fig.9) (Weisiger and Fridovich 1973, Salin, Day et al. 1978, Sevilla, Lopez-Gorge et al. 1980, Papa, Hahn et al. 2014). H_2O_2 is then further eliminated in the mitochondria by the activity of glutathione peroxidases (Mills 1957) and in the peroxisome by catalase (Chelikani, Fita et al. 2004). Interestingly, the balance between SOD2 expression and the expression of peroxidases and catalase is largely lost in progressing tumors. Ratios of peroxidases and catalase has been shown to increase in cancer tissue (Miar, Hevia et al. 2015), indicating a loss of compensation in addition to the overexpression of SOD2 (Fig 10) that both facilitates tumor progression.

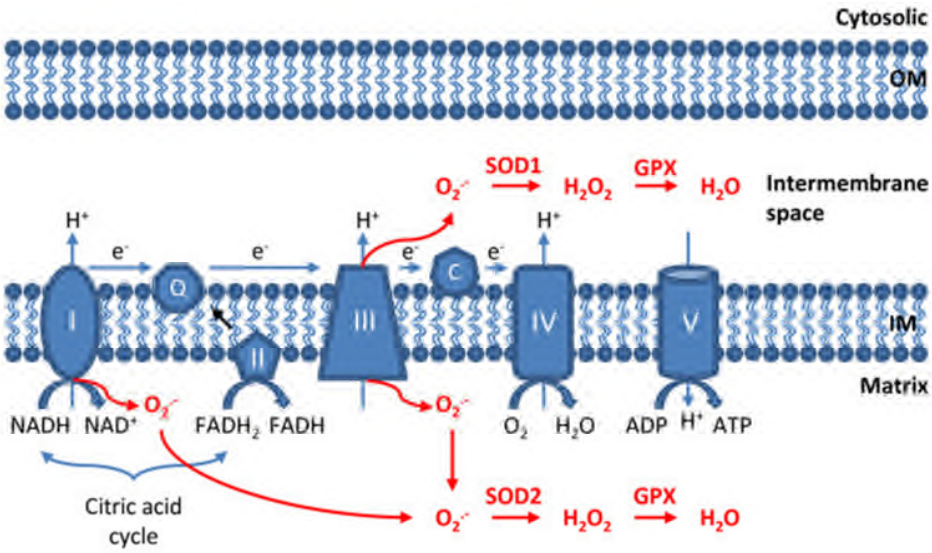

Figure 9. A model for production and disposal of mtROS. (adapted from Li X. *J Hematol Oncol*. 2013; 6: 19.). Electrons (e^-) donated from NADH and $FADH_2$ pass through the electron transport chain and ultimately reduce O_2 to form H_2O at complex IV. MtROS are produced from the leakage of e^- to form superoxide ($O_2^{\cdot-}$) at complex I and complex III. $O_2^{\cdot-}$ is produced within matrix at complex I, whereas at complex III $O_2^{\cdot-}$ is released towards both the matrix and the intermembrane space. Once generated, $O_2^{\cdot-}$ is dismutated to H_2O_2 by superoxide dismutase 1 (SOD1) in the intermembrane space and by SOD2 in the matrix. Afterwards, H_2O_2 is fully reduced to water by glutathione peroxidase (GPX). OM: outer membrane; IM: inner membrane.

The *SOD2* gene is located on chromosome 6q25.3. It has five exons interrupted by four introns (Fig. 10). The basal promoter of the gene lacks TATA and CAAT boxes but contains GC rich motifs and numerous Sp1 as well as several AP-2 consensus sequences in its proximal promoter region (Wan, Devalaraja et al. 1994, Miao and St Clair 2009).

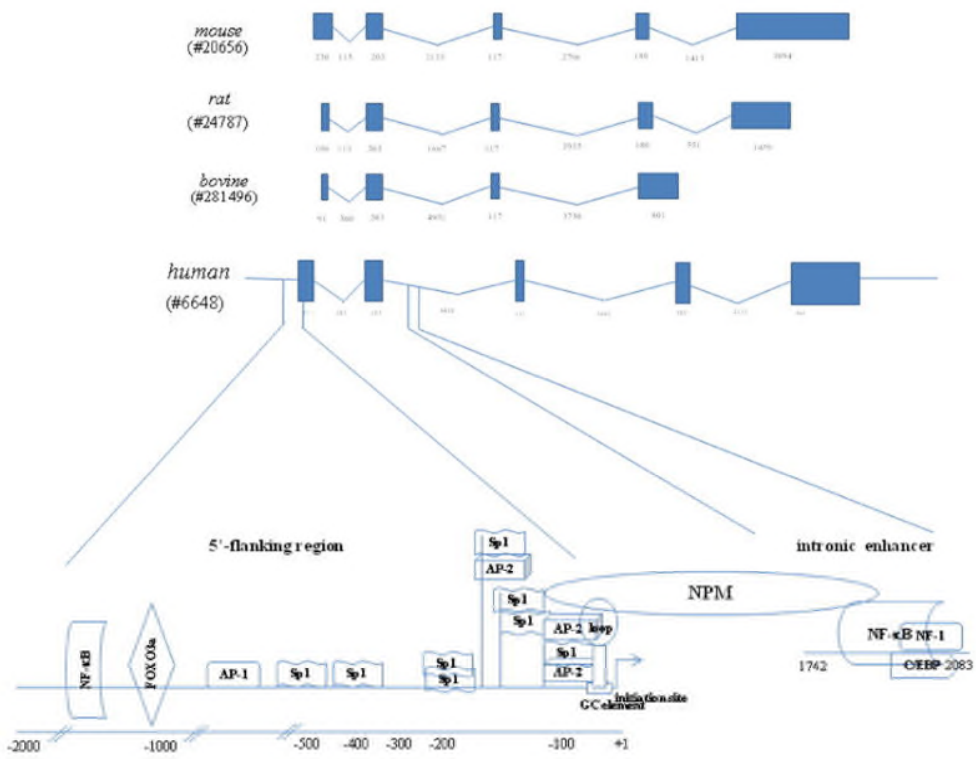

Figure 10. Genetic structure and regulatory factors of *SOD2* gene (adapted from Miao and St. Clair., 2009. Free Radic Biol Med. 2009; 47(4): 344–356).

Furthermore, multiple transcription factors can bind in the regulatory region of *SOD2* gene (Fig. 11). On one hand, some of these transcription factors such as NFkB and AP-1 (activator protein 2) upregulate its gene expression in response to numerous stimuli. On the other hand, other transcription factors such as DDB2 (damaged DNA binding 2) and AP-2 (activator protein 2) downregulate SOD2 expression (Becuwe, Ennen et al. 2014).

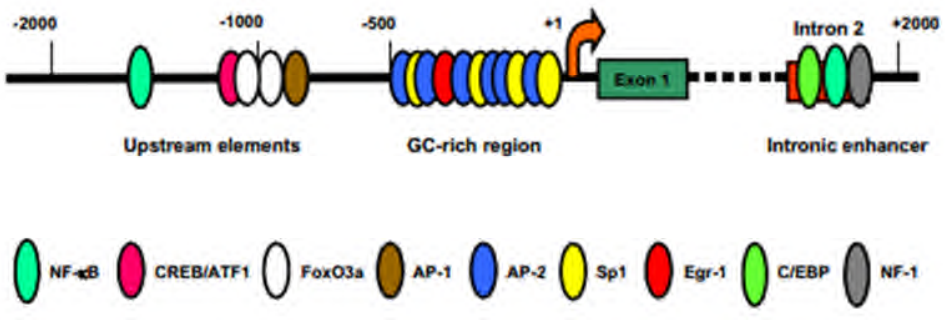

Figure 11. Regulatory factors of *SOD2* gene and their binding sites (adapted from Becuwe et al., *Free Radical Biology and Medicine* 77 (2014) 139–151).

Interestingly, up- or downregulation of SOD2 expression has been reported in tumor cells (Becuwe, Ennen et al. 2014). Analysis of human breast cancer revealed that elevated level of SOD2 is associated with poor prognosis while low expression is associated with better survival (Fig. 12).

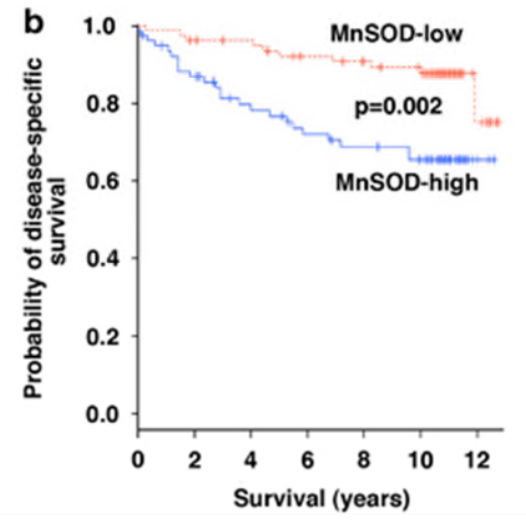

Figure 12. Expression of SOD2 affect survival in human cancer (adapted from Kamarajugadda et al., 2013.*cell death and disease*, 4:1-10). Elevated expression of SOD2 is inversely correlated with ten years patient survival.

Recently, through analysis of breast cancer gene expression datasets we found SOD2 expression is lower in tumors compared to adjacent normal tissues. Further analysis, found SOD2 mRNA

expression varies among different breast cancer tumor subtypes. The different *SOD2* gene expression among subtypes of breast cancer could serve as a useful marker for prognosis.

SOD2 in Breast Cancer Progression and Metastasis

Angiogenesis, a process in which new blood and lymph vessels form, is essential for the growth of solid tumors. Angiogenic activity can alter cell programmed through reactive oxygen species (ROS) (Hanahan and Weinberg 2011). The role of SOD2 in breast tumoral angiogenesis is not clear. Although enzymatic activity of SOD2 indicates it is a tumor suppressor, SOD2 is also shown to be able to promote breast tumor progression (Kattan, Minig et al. 2008). SOD2 expression was found to be elevated in advanced stages of breast cancer which support its role in breast angiogenesis (Kamarajugadda, Cai et al. 2013, Hart, Mao et al. 2015).

Interestingly, SOD2 has historically been considered as an anti-oxidant enzyme responsible for the conversion of potentially harmful $O_2^{\cdot}$ to H_2O_2 under unstressed condition. It has been found that low SOD2 expression accompanied by an increase in mitochondrial superoxide results in stabilization of HIF-1 and its target vascular endothelial growth factor (VEGF). Activation of these factors can increase cellular proliferation and angiogenesis in breast cancers. Also, it has been suggested that high SOD2 activity increase H_2O_2 levels which in turn result in angiogenesis, invasive and metastatic breast cancer cells through increased accumulation of HIF-1 and VEGF factors (Chandel, McClintock et al. 2000, Wang, Niu et al. 2004, Kaewpila, Venkataraman et al. 2008).

In line with the above, SOD2 by its production of mitochondrial H_2O_2 (mH_2O_2) may also control a key cellular process, epithelial mesenchymal transition (EMT), process that increases the

migration and invasiveness potentials of cancer cells (Micalizzi, Farabaugh et al. 2010). Furthermore, it has been shown that SOD2 contributes to the activation of matrix metalloproteases (MMPs) by means of increased H_2O_2. MMPs allow aggressive tumor cells with a mesenchymal phenotype to degrade surrounding extracellular matrix to facilitate invasion and metastasis (Vizoso, Gonzalez et al. 2007, Rezaei, Friedrich et al. 2012, Bae, Choi et al. 2013). In addition to the ability of SOD2 to promote EMT through mH_2O_2, it has been found that H_2O_2 can also activate the metabolic master switch AMPK. AMPK directly enhances aerobic glycolysis and herefore the survival of breast cancer cells through providing them with essential building blocks and energy (Hart, Mao et al. 2015). For all these reasons, it is important to investigate the correlation between SOD2 expression and tumor progression and survival of breast cancer patients.

2. RATIONALE

Preliminary studies of our lab and published reports from others have shown that BRCA1 is directly or indirectly involved in oxidative stress response (Bae, Fan et al. 2004, Acharya, Das et al. 2010). Multiple lines of evidence has suggested that, BRCA1 plays an important role in regulating NRF2, a master transcription factor for genes involved in antioxidant response, either by promoting its protein stability or its activity at the AREs (Copple, Goldring et al. 2008, Chen, Sun et al. 2009, Hayes, McMahon et al. 2010, Ma 2013). Still the exact mechanism of BRCA1 in oxidative stress, as well as the role of reactive oxygen species (ROS) in BRCA1 associated breast tumor remain unclear and need to be further elucidated.

We hypothesized that BRCA1 is important for maintaining low level of ROS. Any defect or loss in BRCA1 may increase ROS and/or disrupt the cellular capacity and the altered redox status impacts tumor development and response to chemotherapy. To test the hypothesis we pursued the following:

A. Analysis of BRCA1 Regulation of Antioxidant Response in Vitro

To determine the importance of BRCA1 in cellular redox homeostasis, we used siRNAs to knockdown BRCA1 in the MCF-10A immortalized human mammary epithelial cells and the U2Os osteosarcoma cells and measured cellular levels of ROS.

To identify the antioxidant genes that are subject to regulation by BRCA1, we used qRT-PCR and western blotting to analyze mRNA and protein levels of NRF2 and its downstream target genes such as NQO1, GCLC and GCLM, etc.

B-*Study BRCA1 Regulation of SOD2 Expression in Vivo*

In order to further study the role and mechanism of BRCA1 function in oxidative stress regulation in vivo, we deleted exons 5-13 of *Brca1* in adult *Brca1*$^{f/f}$ Ubc-CreERT2/+ mice and analyzed the levels of 8-oxo-dG, a marker of oxidative stress in vivo, and SOD2, a newly identified target of BRCA1.

C. Study SOD2 Role in Breast Cancer Patient Survival and Cellular Sensitivity to Chemotherapeutic Agents

Since different expression levels of SOD2 was found among breast tumors subtypes and between normal breast tissues and breast cancer tumors, we hypothesized SOD2 could be used as a prognostic marker and could play a role in breast cancer progression and metastasis. To test that hypothesis, we analyzed the correlation of different expression levels of SOD2 (high or low) on breast cancer patient survival.

To test the importance of this correlation in the context of cellular resistant to chemotherapy, we depleted SOD2 in ER negative breast cancer cell lines expressing high level of SOD2 and measured cellular sensitivity to commonly breast cancer chemotherapeutic agents. Similarly, we also knocked down SOD2 in MCF7 cells that express low level of SOD2 to test the effect on cellular sensitivity to breast cancer chemotherapies in ER positive cells.

3. MATERIALS AND METHODS

A. Cell Lines and Cell Culture

Human normal mammary epithelial cell line (MCF-10A) was used for preliminary oxidative stress studies and Mitosox red staining. Triple negative MDA-MB-231 breast cancer cells were used for oxidative stress studies, Mitosox red staining, cytotoxicity and western blot studies. ER-positive MCF-7 breast cancer cells were used for oxidative stress response studies, cytotoxicity and western blot. U2OS osteosarcoma cells were used for preliminary oxidative stress studies. T47D and ZR-75-30 cells (generously provided by Dr. Gatza), SUM149PT, HCC1954, MDA-MB-468, BT-549, SK-BR-3 and Hs578T breast cancer cells were used for protein expression analysis by western blot and for cellular sensitivity assay. Triple negative, *BRCA1*-mutant MDA-MB-436 cells were used for most of the assays to study the mechanisms behind breast cancer chemotherapeutics resistance.

All cells except MCF-10A were cultured in Dulbecco's Modified Eagle Medium F12 (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). MCF-10A cells were grown in DMEM F12 with 10% FBS, insulin, hydrocortisone, cholera toxin, epidermal growth factor and 1% Pen-Strep. All cells were grown in a humidified incubator at 37° C with 5% CO_2.

B. Plasmid Transfection and Western Blotting

cDNA constructs were transiently transfected into MDA-MB-436 cells for BRCA1 reexpression studies. MDA-MB-436 cells were plated in 6-well plates at 200,000 cells per well and allowed to attach for 16 hours (O/N). 3 ug plasmid was transfected using either XtremeGENE 9 (Promega) or XtremeGENE HP (Roche) in Opti-MEM Reduced Serum Medium (Invitrogen) according to

manufacturers' protocols. Approximately 48 hrs after transfection, cells were collected and lysed in NETNG-250 (250 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.5% Nonidet P, 10% glycerol) with Complete Protease Inhibitor Cocktail (Roche). Whole cell lysates were probed for Western blotting.

Proteins were resolved by SDS-PAGE on 3-12% Tris-Glycine polyacrylamide gels and transferred onto nitrocellulose membranes. Protein size was determined by pre-stained protein marker SeeBlue Plus 2 (Invitrogen). Following incubation with primary and secondary antibodies, blots were washed with PBS-T and then developed using Immunoblot Western Chemiluminescent HRP Substrate (Millipore). The following primary antibodies were used: BRCA1 (#07-434, Millipore or Ab-4, Calbiochem), KEAP1 (E20, Santa Cruz), NQO1 (Santa Cruz), GCLC (Santa Cruz), SOD2 (Santa Cruz), p53 (DO-1, Santa Cruz), HER2 (origene), beta-actin (Santa Cruz), NRF2 (abcam) HO-1(Santa Cruz), GAPDH (Santa Cruz), and NRF2 (abcam).

C. siRNA Transfection

In general, cells were seeded to reach approximately 30% confluence at the time of siRNA treatment. Knockdown of targeted genes were performed using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. Transfection was for 72 hrs at a final siRNA concentration of 10 nM. siRNAs were synthesized by Sigma. The specific siRNA used listed in Appendix A.

D. Annexin V and 7AAD staining and Flow Cytometry

Cells were seeded in 6 well plates at $1.5x10^5$ cells per well. After 16-18 hours cells were transfected with control and SOD2 siRNAs. After 24hrs of transfection 5 μm camptothecin was added to control and SOD2 transfected cells. 24hrs after drug treatment, cells were harvested

by trypsinization, washed with PBS containing 0.5% BSA, 2 x10^5 cells were resuspended in 100μl medium without phenol red and incubated with 100μl of Annexin V and 7 AAD stain at room temperature for 20 minutes. Cells were then analyzed with Guava easyCyte instrument. 7 AAD stained nuclear DNA content and Annexin V staining phosphotydile serine (PS) in apoptotic cells.

Flow Cytometry analysis was conducted using a Guava easyCyte flow cytometer and Guava Suite Software. Annexin V signal was measured with laser excitation at 488 nm and emission between 515-550 nm. 7 AAD was measured with laser excitation at 550 nm and emission between 600-620 nm. 1,000 – 2,000 events were captured and 2,000 cells were analyzed after gating to eliminate cell doublets and aggregates.

E. Immunofluoresence (IF)

Cell were grown on coverslips to 60-80% confluence and fixed with PBS containing 3% paraformaldehyde and 2% sucrose for 5 minutes after appropriate experimental treatment. Fixed cells were then permeabilized in CSK buffer (0.5% Triton X-100, 20mM Hepes pH7.4, 50mM NaCl, 3mM $MgCl_2$, 300mM sucrose) for 5 minutes on ice. Primary and secondary antibodies were diluted in PBS with 5% normal goat serum. 70 μL of each antibody solution was added onto the coverslip and incubated for 20 min each at 37°C. All steps had PBS washes in between except the final wash before mounting. Prior to mounting coverslips were washed once with PBS, twice with PBS containing 0.5% Tween20, and a final wash with PBS. Coverslips were mounted onto glass slides using Vectashield with DAPI (Vector Labs) for nuclear staining and sealed with clear nail polish. Images were captured with Nikon TE2000 fluorescence microscope using Nikon NIS Elements software.

F. Reactive Oxygen Species (ROS) Assay

MCF-7 cells grown to 80-90% confluence in 6-well plates were washed with PBS and then incubated with fresh phenol red-free DMEM containing 10% FBS and 10 μM 2',7'-dichlorofluorescein diacetate (DCF, Sigma) at 37°C for 30min. Cells were washed with PBS, harvested by trypsinization, and resuspended in cold PBS. ROS levels were measured by flow cytometry with excitation at 488 nm and emission at 515 to 545 nm. ROS assays were repeated independently three times.

G. Drug Sensitivity

$2x10^5$ U2OS cells were plated into 6- well plates 18 hrs the day before siRNA transfection. Cells were transfected with 10nM of control and SOD2 siRNAs. After 24 hr the media was refreshed. Another 24 hr later, cells were trypsinized and seeded into 96- well plates at 2,000 cells per well. After another 24 hr, 50 μl of different drugs at different (or gradient) concentrations were added to achieve desired final concentrations. 96 hrs following drug treatment, cell viability was determined using the CellTiterGlo kit (Promega). All experiments were performed in quadruplets.

H. RNA Extraction and Real-Time PCR

For RNA extraction, samples were processed using an RNA mini kit (QIAGEN) for whole tissues, the RNA aqueous mini kit (Ambion/Life Technologies) for mouse islets, and the RNA aqueous micro kit (Ambion) for sorted cells. cDNAs were synthesized with SuperScript III First-Strand Synthesis kit (Invitrogen, Life Technologies). PCR primers were: RPLP0, GAPDH, BRCA1, SOD2, GCLC, GSTM, P21 and p53. Messenger RNA expression was calculated from the C_T of target genes and RPLP0 using standard methods.

I. Genotyping of Mice

Genotyping was performed with genomic extraction of DNA from tail of 3 weeks age of mice. Briefly, tails were incubated overnight in 500 ul of TENES buffer (20mM Tris, 200mM NaCl, 20mM EDTA, 0.8% SDS) containing 0.25 mg/ml proteinase K at 56C overnight. Next day, 200ul of 5M NaCl was added to the digested tail to precipitate proteins. Tails incubated with NaCl for 20 min on ice followed by spinning down at high speed to remove proteins. The genomic DNA was precipitated from the supernatant of the previous step by adding ethanol then collected by spinning down at high speed. Then TE buffer (10mM Tris, EDTA) pH 8.0 was added to dissolve the genomic DNA. Sequence conformation was carried out after amplifying the region surrounding exons 5 and 13. The sequences of primers used are listed in Appendix B. The size of PCR products are 394 nt for floxed (*Brca1f/f*) and 200 nt for wt *Brca1* alleles. The reaction mix for genotyping was used as the following:

10x Hot master Taq buffer 1ul

dNTP (400 µM) 1 µl

Hot master Taq DNA polymerase 1 µl

Forward primer 0.3 µl

Reverse primer 0.3 µl

Genomic DNA 2 µl

H_2O_2 to 10 µl

The PCR cycling conditions were:

94°C 5min; 94°C 30 sec; 68°C 5 min X 40 cycles; 68°C 5 min; 72°C 10 min.

J. Tamoxifen Injection

Tamoxifen TAM (T5648, Sigma) was solubilized at 20 mg/ml in a mixture of 98% and 2% ethanol and delivered into mice by intraperitoneal injection (4mg/25g body weight, once per day for 3 days).

K. Tissue collection

CO_2 asphyxiation and their tissues collected immediately. One set of tissues was fixed with phosphate buffered formalin overnight, changed to 70% ethanol and stored at 4°C. Fixed tissues were embedded in paraffin, cross sectioned at a thickness of 3µm, and stained hematoxylen and eosin for histopathology. Unstained slides used for Immunohistochemistry analysis.

L. Immunohistochemistry (IHC)

IHC staining was carried out by Dr. Yanying Huo, an instructor in our lab. Unstained sections of mammary gland and liver were dewaxed in xylene and hydrated in a graded series of ethanol in Coplin jar. For SOD2, γH2AX, and p21 staining, after a brief wash with PBS, antigen retrieval was carried out with 10 mM sodium citrate buffer, pH 6.0 at 95C for 30 min. After cooling down the slides to room temperature. Sections were incubated in 3% H_2O_2 for 10 min and then blocked with 10% goat serum in PBS for 1 hr at room temperature. Incubation with SOD2, γH2AX, and p21 antibodies was carried out overnight at 4°C in a humidified chamber at a dilution of 1:100, 1:200 and 1:500, respectively, in 5% goat serum. Secondary antibody incubations were carried out using Dako LSAB2 system HRP following the manufacturer's protocol. Signals were visualized with 3,3 diaminobenzidine followed by three washes with distilled water.

L. Statistical Analyses

All pooled data presented are averages of at least 3 independent experiments. The *P* values were calculated by two-tailed Student's *t*-test using Microsoft Excel 2010.

4. EXPERIMENTAL RESULTS

Section I: Analysis of BRCA1 *Function in Redox Homeostasis*

1.1 Downregulation of BRCA1 Increases ROS Levels in Breast Cancer Cells

To study the role of BRCA1 in oxidative stress response, we first chose to measure the ROS levels after BRCA1 knockdown in U2OS osteosarcoma cells and normal epithelial breast cells (MCF 10A) using 2',7'-Dichlorofluorescein (DCF) in conjunction with FACS. As shown in Fig. 1A and B, BRCA1 knockdown was sufficient by itself to induce ROS in the cells.

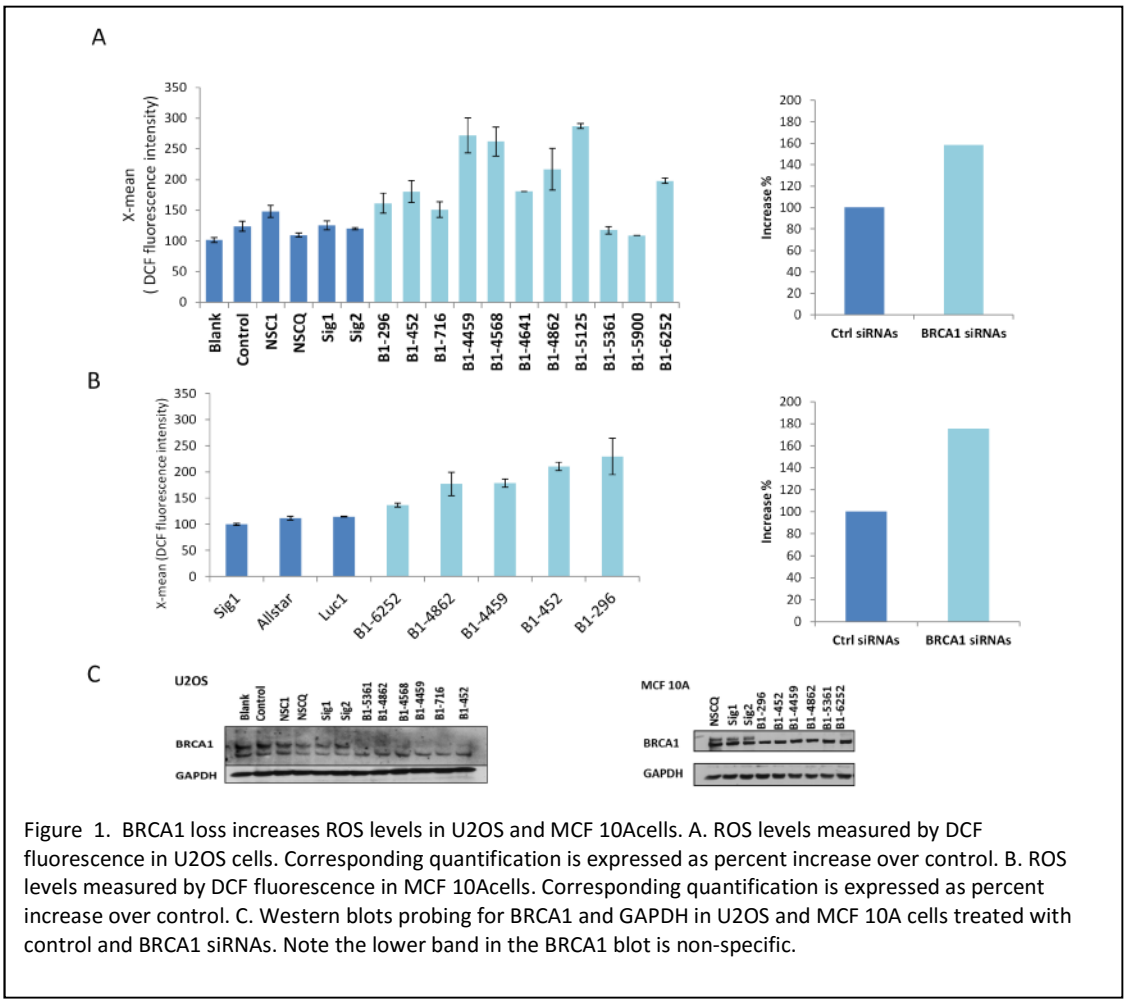

Figure 1. BRCA1 loss increases ROS levels in U2OS and MCF 10Acells. A. ROS levels measured by DCF fluorescence in U2OS cells. Corresponding quantification is expressed as percent increase over control. B. ROS levels measured by DCF fluorescence in MCF 10Acells. Corresponding quantification is expressed as percent increase over control. C. Western blots probing for BRCA1 and GAPDH in U2OS and MCF 10A cells treated with control and BRCA1 siRNAs. Note the lower band in the BRCA1 blot is non-specific.

Specifically, we found that ROS levels in BRCA1 depleted cells were 60-75% higher than ROS levels in cells transfected with non-targeting siRNAs (Fig. 1A and B). We also confirmed that the knockdown was efficient by western blot for endogenous BRCA1 (Fig. 1C).

Although U2OS and MCF 10A cells are sufficient for understanding potential role of BRCA1 in regulation oxidative stress in human cells, they may not be representative of cancers caused by *BRCA1* mutations. As there are a number of cell lines derived from patients lacking functional BRCA1, we chose to use two breast cancer cells (MCF7 and MDA-MB-231) in order to obtain more clinically relevant data on oxidative stress regulation. We found transient knockdown of BRCA1 (for 72hrs) in these cells also led to increase ROS, albeit to substantially lesser extents (Fig. 2A and B). Overall, ROS levels in BRCA1 depleted cells were 15-20% higher than ROS levels in cells transfected with non-targeting siRNAs (Fig. 2A and B). Western blot for endogenous BRCA1 showed that the knockdown was largely effective although incomplete (Fig. 2C). The increase in ROS level following BRCA1 knockdown confirmed a function of BRCA1 in oxidative stress homeostasis.

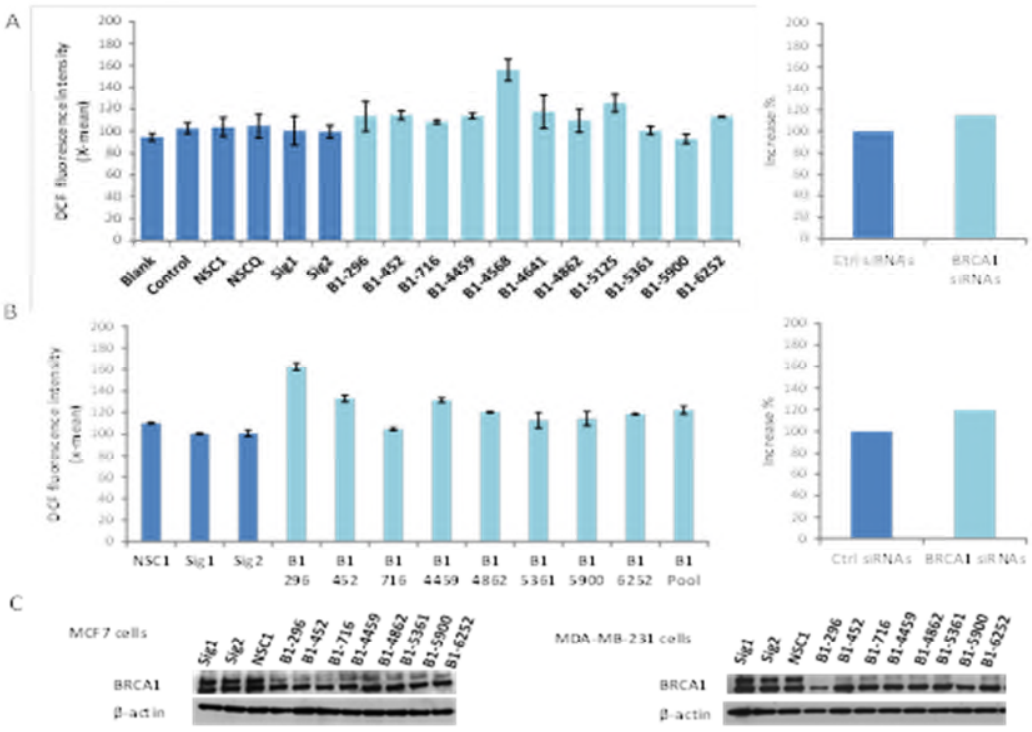

Figure 2. BRCA1 loss increases ROS levels in MCF7 and MDA-MB-231 cells. A. ROS levels measured by DCF fluorescence in MCF7 cells. Corresponding quantification is expressed as percent increase over control. B. ROS levels measured by DCF fluorescence in MDA-MB-231 cells. Corresponding quantification is expressed as percent increase over control. C. Western blots probing for BRCA1 and β-actin in MCF7 and MDA-MB-231 cells treated with control and BRCA1 siRNAs.

1.2 Downregulation of BRCA1 Increases Superoxide Level in Breast Cancer Cells

To further investigate the role of BRCA1 in oxidative stress response, we tested the effect of BRCA1 knockdown on superoxide (O_2^-) level in MCF 10A cells using Mitosox Red. We found that O_2^- levels in BRCA1 depleted cells were higher than O_2^-levels in cells transfected with non-targeting siRNAs (Fig. 3A). Additionally, we tested the basal level of O_2^- in MDA-MB-231 cells and MDA-MB-436 cells. MDA-MB-436 cells harbor a truncated nonfunctional form of BRCA1 while MDA-MB-231 cells have a wild type of BRCA1. We found that MDA-MB-436 cells have higher basal level O_2^- than MDA-MB-231 cells (Fig. 3C). These results further suggest that BRCA1 may regulate the production or elimination of mitochondria superoxide.

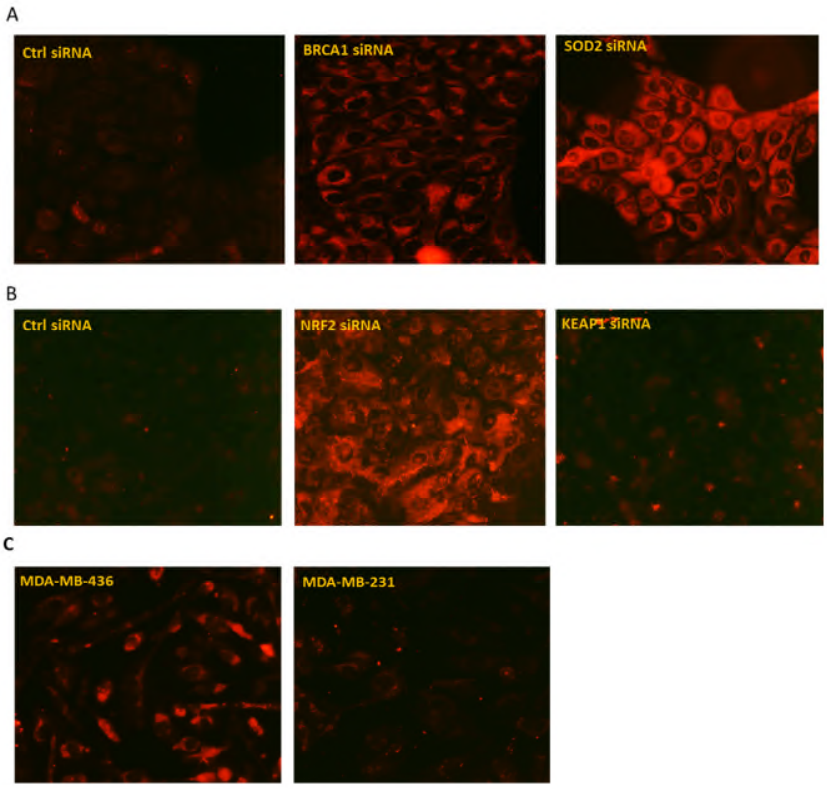

Figure 3. Superoxide levels in MCF 10A, MDA-MB-436 and MDA-MB-231 cells. A. Superoxide levels measured by mitosox Red in MCF 10A treated with Ctrl siRNA, BRCA1 siRNA and SOD2 siRNA. B. Superoxide levels measured by mitosox Red in MCF 10A treated with Ctrl siRNA, NRF2 siRNA and KEAP1 siRNA. C. Basal levels of superoxide in MDA-MB-436 and MDA-MB-231 cells by mitosox Red.

Moreover, we tested the effect of knocking down NRF2, a collaborator of BRCA1 in redox regulation and a master regulator of antioxidant genes involved in detoxifying ROS, on O_2^-levels in MCF-10A cells. We also tested the effect of knockdown of KEAP1, a negative regulator of NRF2. KEAP1 depleted cells also served as an additional control in this experiment. We found transient knockdown of NRF2 show similar effect on O_2^-levels as BRCA1 knockdown in the same cell line (Fig. 3B). These results are consistent with previous studies which suggested that BRCA1 and NRF2 function together in oxidative stress homeostasis (Bae, Fan et al. 2004).

The fact that BRCA1 and NRF2 single knockdowns result in increased superoxide levels does not necessarily mean that the BRCA1 regulates superoxide homeostasis directly through NRF2. Given the fact superoxide is mainly regulated by superoxide dismutase (SOD2). This begs the question whether SOD2 is a downstream target of NRF2 or BRCA1. Indeed, results from a recent study suggested that BRCA1 regulates SOD2 (Gardini, Baillat et al. 2014). Using genome wide sequence analysis, the study found that NFkB and its downstream targets including SOD2 were decreased after depletion of BRCA1 in MCF-10A cells. This indicates that SOD2 is a downstream target of BRCA1. Moreover, our analysis of SOD2 expression in the METABRIC and TCGA datasets showed a consistently positive correlation of SOD2 expression with NRF2 and NRF2 target genes in all tumors and all subtypes with the exception of the HER2-positive tumors (Fig. 4A and B). Although this does not necessary means SOD2 is a downstream target of NRF2, such as possibility is worthy of further investigation.

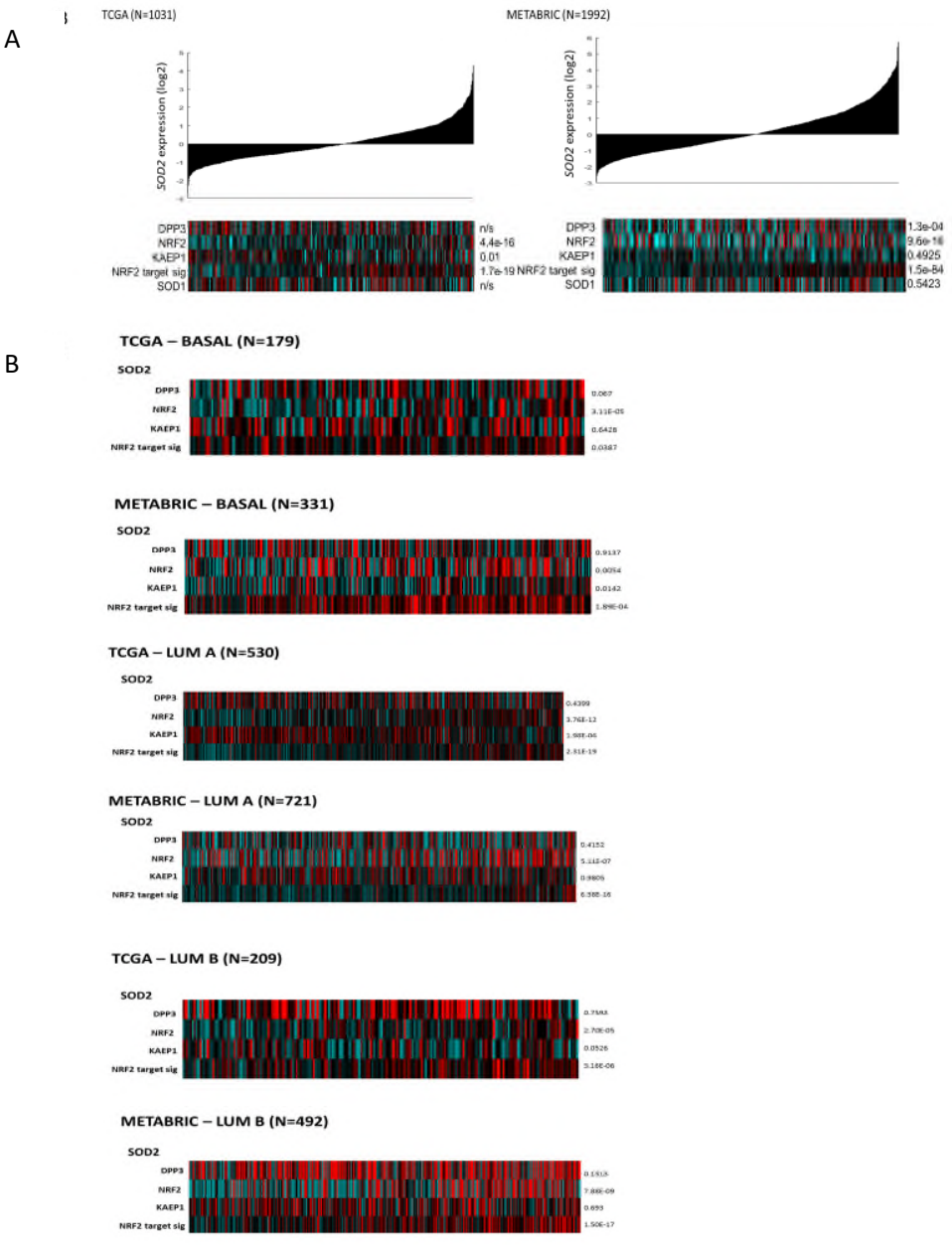

Figure 4 SOD2 correlates positively with NRF2 and the downstream target of NRF2 in human breast cancers. (**A**) Analysis of SOD2 correlation with NRF2 and NRF2 target genes in the METABRIC and TCGA datasets in all tumors. (**B**) Analysis of SOD2 correlation with NRF2 and NRF2 target genes in the METABRIC and TCGA datasets in different subtypes of breast cancer.

1.3 Downregulation of BRCA1 Decreased mRNA Expression of Some Antioxidant Genes in Breast Cancer Cells

In order to further confirm the role of BRCA1 in oxidative stress response, we analyzed the mRNA amounts of some antioxidant genes including *SOD2*, *GSTM*, *GCLC*, and *NQO1* by RT-qPCR after BRCA1 knockdown with 4 different siRNA sequences in MCF7 cells. As shown in Fig. 5, the knockdown efficiency of BRCA1 was approximately 80%. mRNA levels of *GCLC* and *NQO1* significantly decreased after BRCA1 knockdown (Fig. 5). In this analysis, *SOD2* mRNA amounts were also modestly reduced in cells treated with by 3 out of the 4 BRCA1 siRNAs used (Fig. 5). This more modest degree of decrease than previously reported could be due to the incomplete knockdown of BRCA1 or certain differences in the cell lines used. However, the overall results further confirmed the involvement of BRCA1 in regulating antioxidant response genes and oxidative stress response in general.

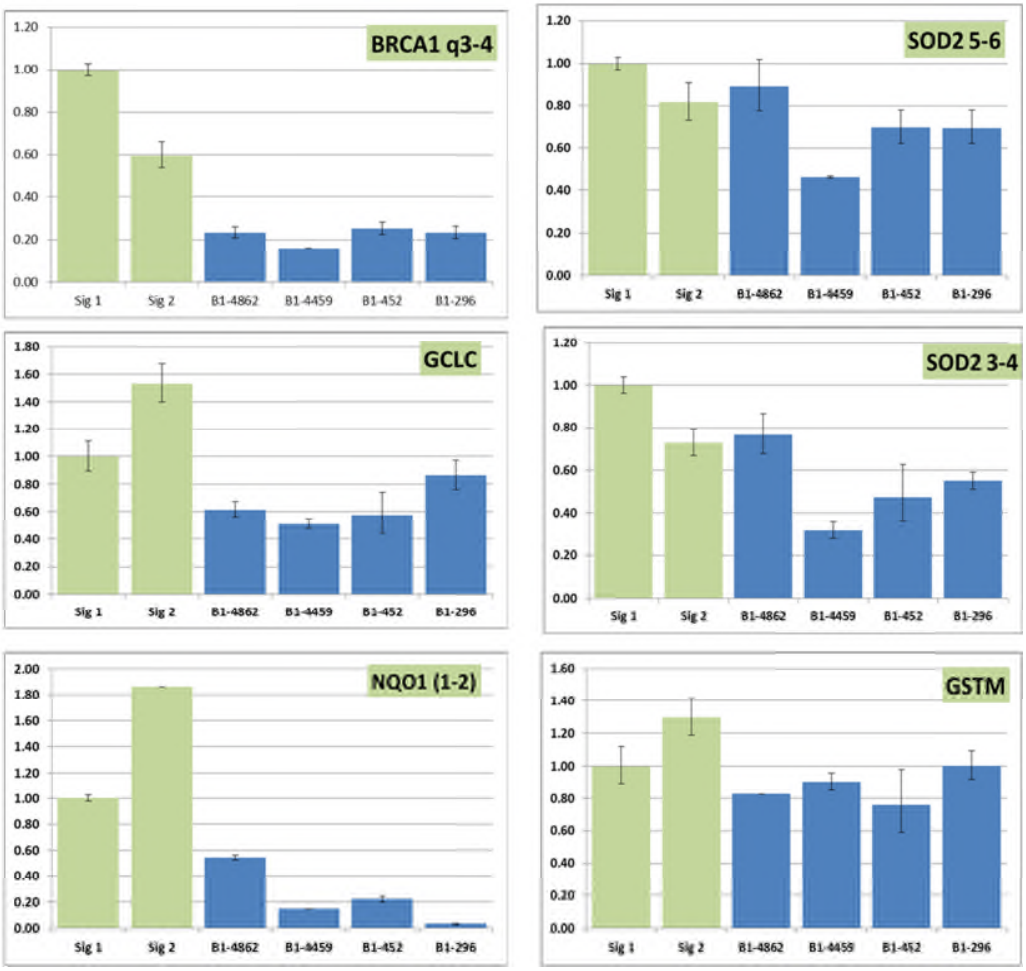

Figure 5. BRCA1 depletion decreases expression of some antioxidant genes. *BRCA1* mRNA amount is measured by qRT-PCR after its depletion in MCF7 cells using BRCA1 siRNA for 72 hrs. Then, *GCLC*, *NQO1*, *SOD2* and *GSTM* mRNA amounts were measured by qRT-PCR. Dramatic decrease was noticed in GCLC and NQO1 mRNA amounts after BRCA1 depletion. Slight decrease was noticed in *SOD2* and *GSTM* mRNA amounts after BRCA1 depletion in MCF7 cells.

1.4

Downregulation of BRCA1 Increases SOD2 Protein Expression in Breast Cancer Cells

Our finding that BRCA1 depletion slightly decreases SOD2 mRNA amount suggests that BRCA1 may regulates SOD2 in cancer cells. To investigate BRCA1 regulation of SOD2 protein expression, we first depleted MCF7 cells of BRCA1 using four different individual BRCA1 siRNAs targeting

different parts of BRCA1 as well as pool of BRCA1 siRNAs (pool of three different BRCA1 siRNAs). Additionally, we used three different non targeting siRNAs, pooled SOD2 siRNAs and individuals SOD2 siRNAs as an additional controls. We carried out a western blot and the results as shown in Fig. 6A, surprisingly, knockdown of BRCA1 increased the protein level of SOD2 in MCF7 cells.

Next, we used a larger set of eight different BRCA1 siRNAs to further confirm increased SOD2 protein level upon BRCA1 loss in MDA-MB-231 cells. Indeed, we found a pronounced increase in SOD2 expression after BRCA1 depletion with most of the siRNAs used (Fig. 6B). We also checked for NRF2 and KEAP1 expression after BRCA1 depletion. We did not find a significant change in their expression in response to BRCA1 depletion. Then, the same experiment was performed with MCF7, MDA-MB-231 and U2OS cells using a pool of BRCA1 siRNAs, with a pool of SOD2 siRNAs as an additional control. The results revealed that SOD2 protein level indeed increased after BRCA1 knockdown in all tested cell lines (Fig. 6C). Moreover, we found the basal level of SOD2 was higher in the BRCA1-mutant MDA-MB-436 cells (Fig. 6C) than in the above cancer cells with wt BRCA1. Additionally, SOD2 level also appeared to be high in another BRCA1-mutant breast cancer cell line, SUM149PT (Fig. 6D). These results demonstrate that BRCA1 negatively regulates SOD2 protein level.

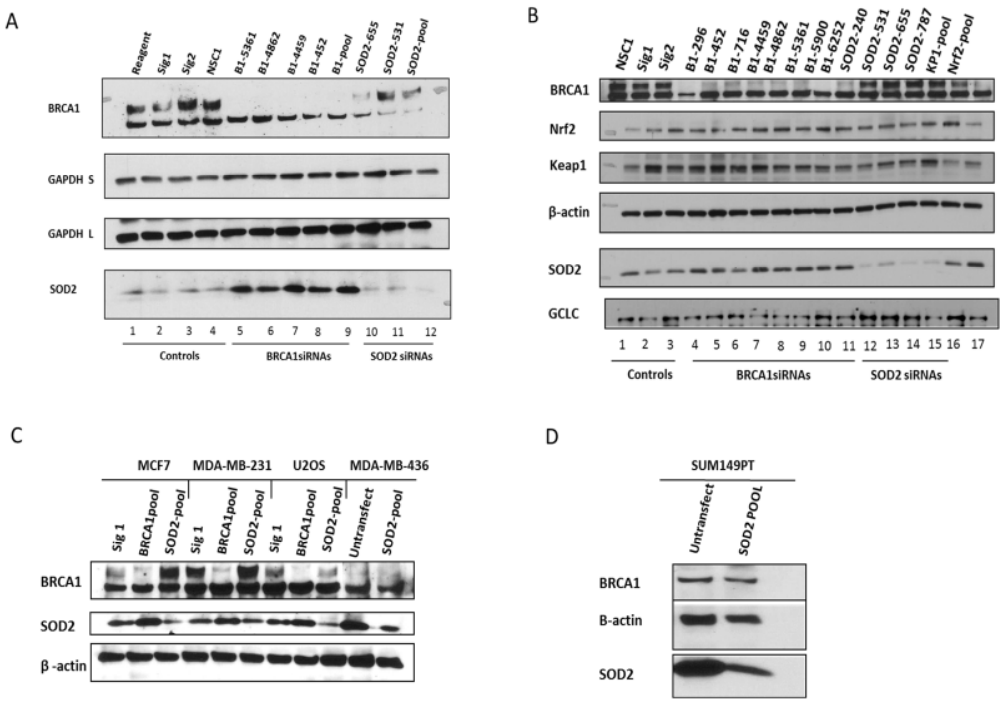

Figure 6. BRCA1 downregulation increases SOD2 expression in breast cancer cells (**A**) Western blots showing the depletion of BRCA1 in MCF7 cells using siRNAs increases SOD2. (**B**) Western blots showing the depletion of BRCA1 in MDA-MB-231 cells using siRNAs increases SOD2 (**C**) Western blots showing the depletion of BRCA1 in MCF7,MDA-MB-231, U2OS and MDA-MB-436 cells using siRNAs increases SOD2. (**D**) Western blots showing SUM149PT cells (BRCA1 deficient) have high SOD2 expression.

Additionally, we stained the cells with an anti-SOD2 antibody following BRCA1 knockdown to check for SOD2 expression by immunofluorescence (IF). As expected, SOD2 levels were increased after BRCA1 downregulation in all cells tested (Fig. 7A-E). These results further confirmed the role of BRCA1 in the regulation of SOD2. Also, the results suggest either BRCA1 directly regulates SOD2 stability (given the decrease in SOD2 mRNA amount) or SOD2 is responding to oxidative stress induction due to BRCA1 depletion.

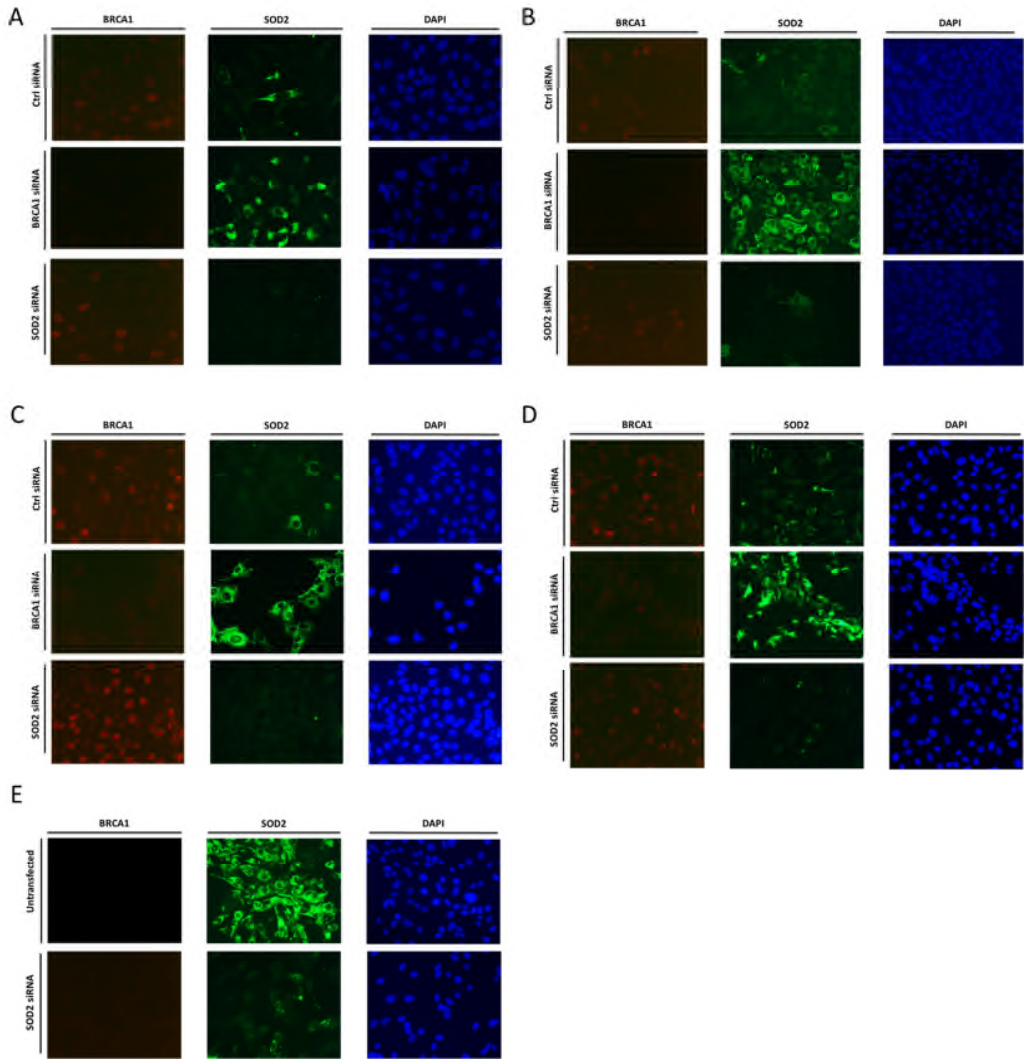

Figure 7. SOD2 upregulation in response to BRCA1 loss. (**A**) Representative U2OS cells stained for nuclear DNA content (DAPI), BRCA1 and SOD2 by IF. BRCA1-depleted cells showed increased SOD2 staining after 72 hrs of BRCA1 depletion. (**B**) Representative MCF-10A cells stained for nuclear DNA content (DAPI), BRCA1, and SOD2 by IF. BRCA1-depleted cells showed increased SOD2 staining after 72 hr of BRCA1 depletion. (**C**) Representative MCF7 cells stained for nuclear DNA content (DAPI), BRCA1, and SOD2 by IF. BRCA1-depleted cells showed increased SOD2 staining after 72 hr of BRCA1 depletion. (**D**) Representative MDA-MB-231 cells stained for nuclear DNA content (DAPI), BRCA1, and SOD2 by IF. BRCA1-depleted cells showed increased SOD2 staining after 72 hr of BRCA1 depletion. (**E**) Representative MDA-MB-436 cells stained for nuclear content (DAPI), BRCA1, and SOD2 by IF. Basal SOD2 level in MDA-MB-436 cells was much higher than in other BRCA1-wt_ cells SOD2. All cells were seeded on cover glasses and treated with indicated siRNAs (except MDA-MB-436 cells) for 72 hr. Cells were fixed then processed and stained with anti-BRCA1 and anti-SOD2.

1.5 Upregulation of SOD2 by BRCA1 Depletion Can Be Abrograted by NAC Treatment

BRCA1 functions in oxidative stress are closely linked to antioxidant response genes. Also, SOD2 protein is known to promote proper redox regulation and balance after oxidative stress (Wheeler, Nakagami et al. 2001). The fact that transfection of MCF7 cells with BRCA1 siRNAs for 72 hrs induces SOD2 protein accumulation (Fig. 6) suggests SOD2 could be responding to oxidative stress induction due to BRCA1 depletion. To test the interplay between BRCA1 and SOD2 in oxidative stress regulation and response, we seeded MCF7 cells in duplicate, transfected both sets with five individual BRCA1 siRNAs and then we treated one set with 2 mM N-acetylcysteine (NAC) for 2 hrs to determine if NAC treatment could SOD2 to its basal levels or the level in cells treated with a control siRNA. Cell lysates were collected and analyzed by western blot 72 hrs post transfection and 2 hrs of NAC treatment. As shown in Fig. 8A, compared with cells treated with the control siRNA, BRCA1-depleted cells showed a clear SOD2 accumulation. Not surprisingly, after 2mM NAC treatment for 2 hrs, BRCA1 depleted cells exhibited decreased SOD2. Thus, the above results indicate that the increase in SOD2 expression after BRCA1 knockdown was due to an induction of oxidative stress.

Several interesting inferences can be made from the upregulation of SOD2 expression after BRCA1 downregulation. First, BRCA1 does regulate SOD2 negatively. Second, this regulation is most likely through ROS (given the reduction in SOD2 expression following NAC treatment). Third, the SOD2 could be partially responsible for ROS regulation elicited by or following BRCA1 loss. Lastly, increased ROS levels after BRCA1 knockdown may activate genes products of which mediate antioxidant functions so that ROS levels are maintained under threshold levels that induce apoptosis.

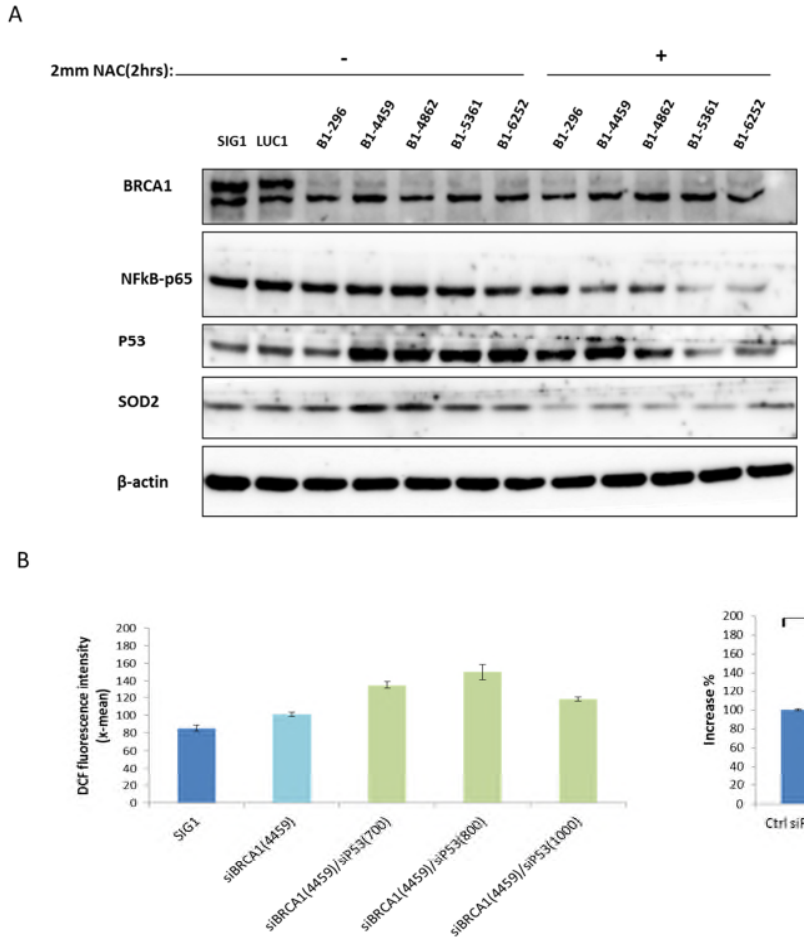

Figure 8. P53 limits ROS induction upon loss of BRCA1. (**A**) p53 induction following BRCA1 depletion and the effect of NAC. MCF7 cells were transfected with BRCA1 siRNAs for 72 hr followed by 2 mM NAC treatment for 2 hrs. Proteins levels were analyzed by western blotting. (**B**) ROS measurement by DCF fluorescence dye after knockdown of BRCA1 and co-knockdown of P53 and BRCA1 in MCF7 cells.

In order to test our hypothesis that BRCA1 knockdown can indirectly regulate or induce the expression of genes involved in antioxidant response through ROS, we performed western blot to cdetermine the expression of p53, a tumor suppressor and transcription factor that can directly regulate the expression of hundreds of genes, including genes involved in regulating oxidative stress (Liu and Xu 2011). MCF7 cells seeded in duplicate sets and both were

transfected with five individual BRCA1 siRNAs for 72 hrs. One set was treated with 2 mM N-acetylcysteine (NAC) for 2 hrs, while the other was left untreated and used as control. As shown in Figure 8A, P53 was induced in BRCA1-depleted cells that were not treated with NAC, whereas the induction of p53 was largely suppressed in cells treated with NAC. This suggests that p53 was responding to oxidative stress in addition to DNA damage after BRCA1 loss.

In order to further confirm the possible functional interplay between p53 and BRCA1 in stress response, we compared ROS levels in MCF7 cells treated with BRCA1 siRNA alone and cells treated with a combination of BRCA1 and p53 siRNAs. Although this approach is not sufficient to answer the question as to how P53 regulate ROS following BRCA1 loss, it will allow us to investigate the potential role of p53 upregulation in limiting ROS levels after BRCA1 downregulation. As shown in Figure 8B, 72 hrs after siRNA transfection, p53 and BRCA1 double knockdown cells showed higher ROS level than did BRCA1 single knockdown cells (25-35% vs 15% increase), as compared with control (NSC) siRNA treated cells. The more profound rise in ROS level in the double knockdown cells suggesting that p53 may act to reduce ROS accumulation after BRCA1 loss (Fig. 8B). Together, these data demonstrate that BRCA1 downregulation leads to p53 induction and further suggest that the induction of p53 may be important to limit ROS levels below a threshold that induces cell death. It is worth noting the possibility of BRCA1 and P53 having similar functions in oxidative response, particularly since many studies have shown that p53 also has a functional relevance in antioxidant response (Vurusaner, Poli et al. 2012, Gambino, De Michele et al. 2013). Our results also imply that BRCA1 regulation of SOD2 expression is indirect and is possibly through increased ROS levels and induction of p53, given that NAC treatment downregulates p53 and that downregulation was accompanied by SOD2 downregulation Figure 8 A. These data provided the impetus for further studies into the mechanism by which BRCA1 regulates SOD2 expression.

1.6 Re-expression of Wild Type BRCA1 in MDA-MB-436 Cells Decreases SOD2 Expression

In order to further confirm the role of BRCA1 in SOD2 regulation, we transfected MDA-MB-436 cells that are deficient in BRCA1 with two independent cDNA plasmids that overexpress wild type BRCA1 and used immunofluorescence staining to study the effect of re-expressing wild type BRCA1 on SOD2 protein amount. As shown in Fig. 9A, a fraction of transfected cells showed positive staining of BRCA1. Plasmid1 showed higher expression levels than plasmid2. Importantly, both plasmid1 and plasmid2 appeared to decrease the high basal SOD2 protein level in the cells (Fig. 9A and B), lending further support that BRCA1 negatively regulates SOD2 protein abundance. Additionally, our lab has generated three independent MDA-MB-436 cell lines that stably express wild type BRCA1 (referred to as WT3, WT6, and WT8). In order to confirm the above findings, we seeded the stable cells as well as parental cells in duplicate. One set was seeded on cover slips for IF and the other set was seeded in 6 wells plate for western blots. Not surprisingly, all of the stable cells showed decreased SOD2 expression (Fig. 10C). Specifically, we found that SOD2 expression in stable cells were 50-65% less than in parental cells (Fig. 9D). These results further confirmed the negative regulation of SOD2 protein expression by BRCA1.

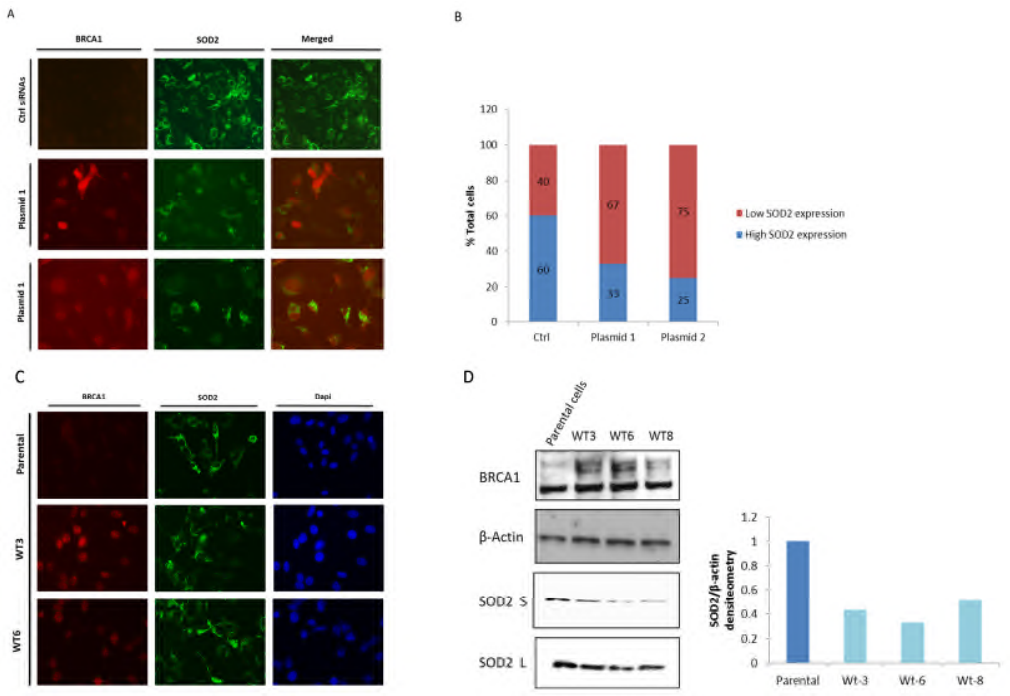

Figure 9. Re-expression of wild type BRCA1 in MDA-MB-436 cells. (**A**) Representative MDA-MB-436 cells stained for BRCA1 and SOD2 by IF. Cells transfected with plasmid 1 and plasmid 2 that express wild type BRCA1. SOD2 staining was performed after 48 hrs transfection with 3 µg DNA of plasmid 1 and plasmid 2 (**B**) Quantification of SOD2 expression after transfection of cells with wild type BRCA1 plasmids. Y axis means percent of total number cells. (**C**) Stable expression of BRCA1 in MDA-MB-436 cells causes a decrease in SOD2 staining. (**D**) Western blots showing the stable cell lines having SOD2 expression level less than of that in parental cells.

Section II: *Analysis of* BRCA1 *Function in Oxidative Stress Homeostasis in Vivo*

2.1 Generation of *Brca1$^{f/f}$;UBC-CreERT2/+* Mouse Strain

To circumvent embryonic lethality and determine the effect of BRCA1 loss on SOD2 in adult mice, we took advantage of a Cre-ERT2 transgenic mouse line that was generated using a lentivirus that express from human ubiquitin C promoter (UBC) (Lois, Hong et al. 2002). CreERT2 is a fusion protein of the Cre recombinase and a mutant form of estrogen receptor (ER) that is localized in the cytoplasm but will translocate into the nucleus upon binding to tamoxifen (TAM) (Feil, Wagner et al. 1997, Lois, Hong et al. 2002, Ruzankina, Pinzon-Guzman et al. 2007). In combination with a genetically engineered *Brca1* allele with its exons 5-13 flanked by loxP sites (floxed), the *Ubc-CreERT2* model provides a system to efficiently inactivate *Brca1* in the whole body of the mouse thereby allowing for studying BRCA1 function in different tissues at desired time.

To test the efficiency of Cre-mediated deletion of (the exons 5-13 of) *Brca1*, adult *Brca1$^{f/f}$;Ubc-CreERT2/+* mice (6-10 weeks of age) were treated once per day for three days with TAM by intraperitoneal injection (Fig. 10B). At 6, 12 and 20 days after the final treatment, mice were sacrificed and DNA from liver and spleen was analyzed by PCR to detect *Brca1* deletion. Partial but still significant deletion was observed in both tissues (Fig. 10C), indicating that CreERT2 was activated by TAM injection. Because the floxed allele remained intact in some cells in the two tissues and possibly in all other tissues, this system allowed production of mice that are mosaic for *Brca1* deletion.

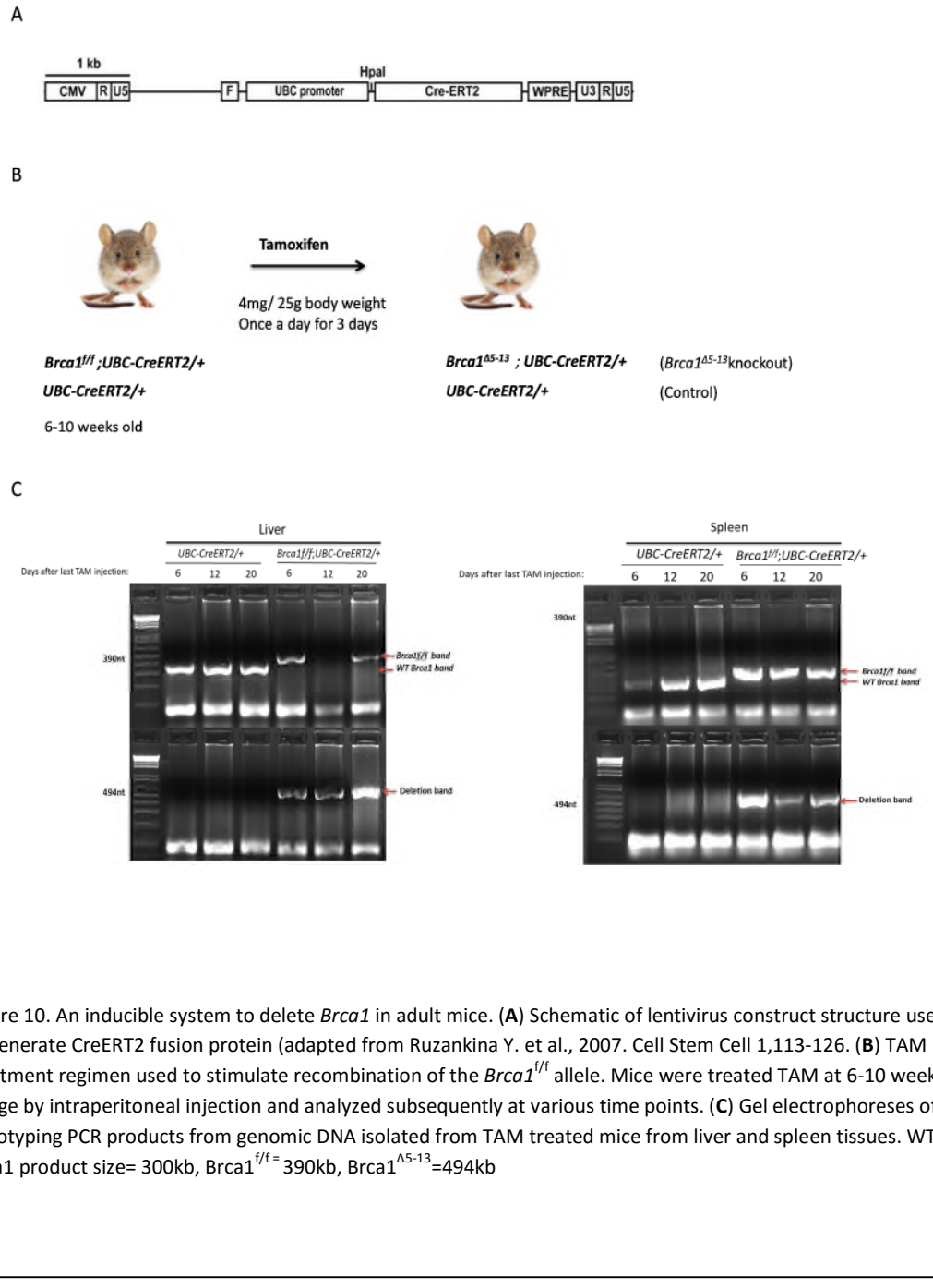

Figure 10. An inducible system to delete *Brca1* in adult mice. (**A**) Schematic of lentivirus construct structure used to generate CreERT2 fusion protein (adapted from Ruzankina Y. et al., 2007. Cell Stem Cell 1,113-126. (**B**) TAM treatment regimen used to stimulate recombination of the *Brca1*$^{f/f}$ allele. Mice were treated TAM at 6-10 weeks of age by intraperitoneal injection and analyzed subsequently at various time points. (**C**) Gel electrophoreses of genotyping PCR products from genomic DNA isolated from TAM treated mice from liver and spleen tissues. WT Brca1 product size= 300kb, Brca1$^{f/f}$ = 390kb, Brca1$^{\Delta 5\text{-}13}$=494kb

2.2 Upregulation of SOD2 expression in mouse liver after deletion of *Brca1*

To investigate the effect of *Brca1* deletion on SOD2 expression in vivo, we generated *Brca1$^{f/f}$;UBC-CreERT2/+* mice that have floxed *Brca1* exons 5-13. Deletion was induced by TAM treatment at 6-10 weeks of age (Fig. 10). *UBC-CreERT2/+* mice with wild type *Brca1* were used as controls. At 6, 12, and 20 days after TAM treatment, mice were sacrificed and tissues were collected. Total RNAs were isolated from the livers and qRT-PCR was carried out to determine the amount of *Sod2* mRNA. As shown in Fig. 11, *Sod2* mRNA expression appeared to be slightly downregulated after deletion of exon 5-13 in *Brca1$^{f/f}$;UBC-CreERT2/+* mouse liver. The reduction of *Sod2* mRNA amount was maintained up to 20 days after *Brca1* deletion, although there appeared to be moderate recovery at the later time points. This finding is consistent with

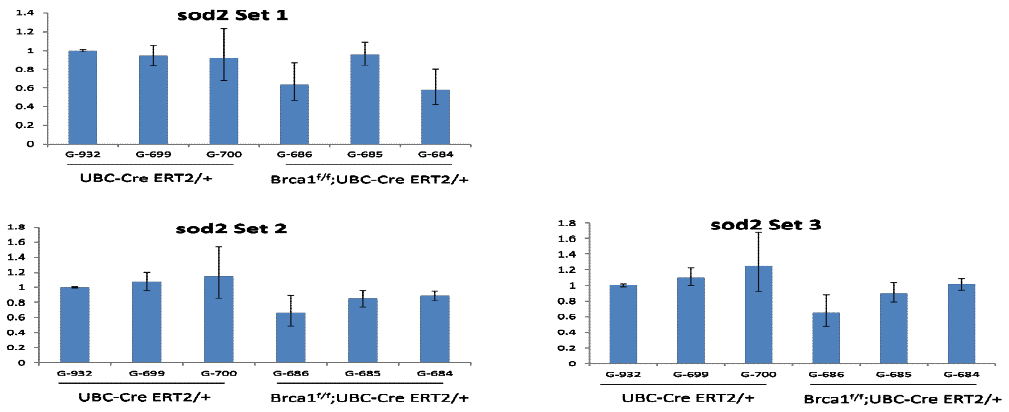

Figure 11. Brca1 deletion in Brca1$^{f/f}$;UBC-CreERT2/+mouse liver slightly decreases SOD2 mRNA . SOD2 mRNA amount was measured by qRT-PCR after BRCA1 deletion using two differents sets of primer for mRNA SOD2. G932 and G686 mice were sacrificed after 6 days of last TAM injection , G699 and G685 mice were sacrificed after 12 days of last TAM injection. G700 and G684 mice were sacrificed after 20 days of last TAM injection.

our previous observation that BRCA1 depletion in MCF7 cells led to a slight reduction of *SOD2* mRNA amount (Fig. 5C).

To further confirm the above results, we also performed an immunohistochemistry (IHC) staining to detect SOD2 protein level in the mammary gland and liver tissues collected from *Brca1$^{f/f}$;UBC-CreERT2/+mouse* after 6, 12 and 20 days of TAM injection. Unfortunately, we found no difference in SOD2 protein level between the mammary glands of control and *Brca1$^{f/f}$;UBC-CreERT2/+* mice after TAM treatment (Fig. 12). However, we found a robust increase in SOD2 protein level in the liver, which could be a common consequence of redox imbalance or increased ROS levels (Fig. 13). More specifically, at 6 days, we noticed that SOD2 protein level was already significantly increased in *Brca1*-deleted mice compared with control mice. SOD2 level in *Brca1*-deleted liver reached its peak at day 12 after TAM injection and then appeared to drop slightly by day 20, but it was still higher than in control mice. These results are especially interesting because we have uncovered a new mechanism for BRCA1 in the regulation of antioxidant response, which is through SOD2. Thus, there are two distinct mechanisms for oxidative stress regulation by BRCA1, one through NRF2 and one through SOD2. The two mechanisms likely cooperate with each other to ensure optimal redox homeostasis.

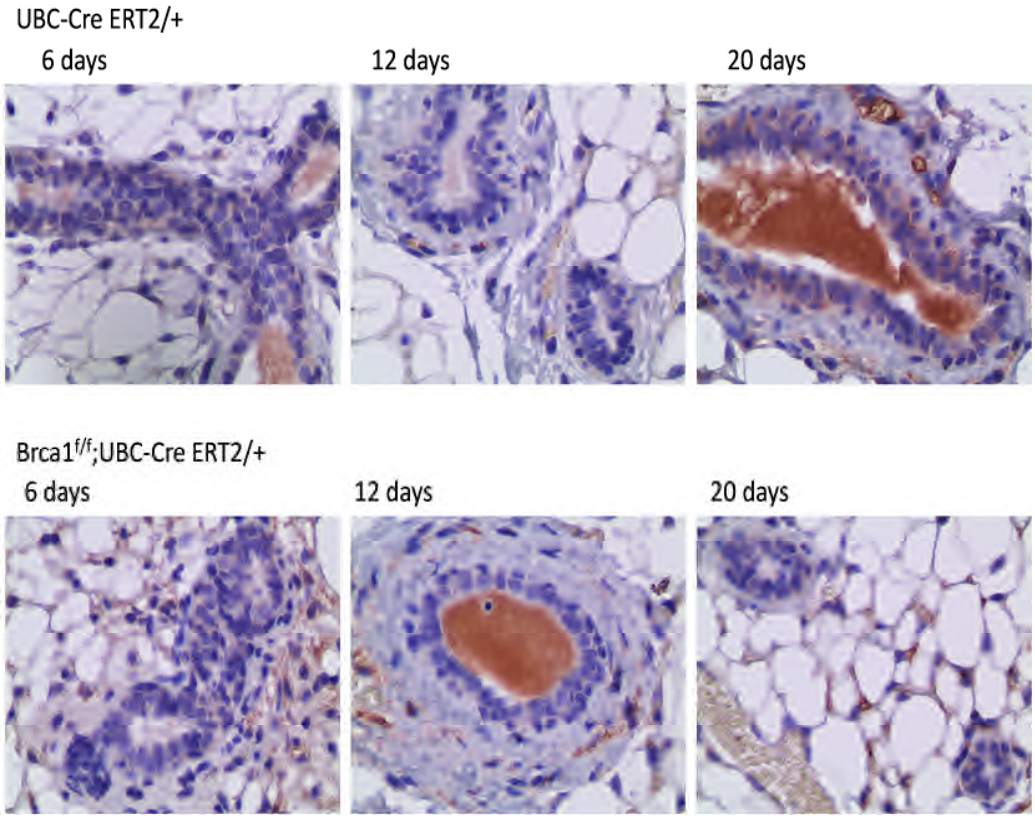

Figure 12. *Brca1* deletion has no effect on SOD2 protein level in the mamamry gland. Representive SOD2 immunohistochemistry for the mammary glands of UBC-CreERT2/+ (top panel) and Brca1$^{f/f}$;UBC-CreERT2/+ (bottom panel) mice. SOD2 protein level was not changed after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mouse.

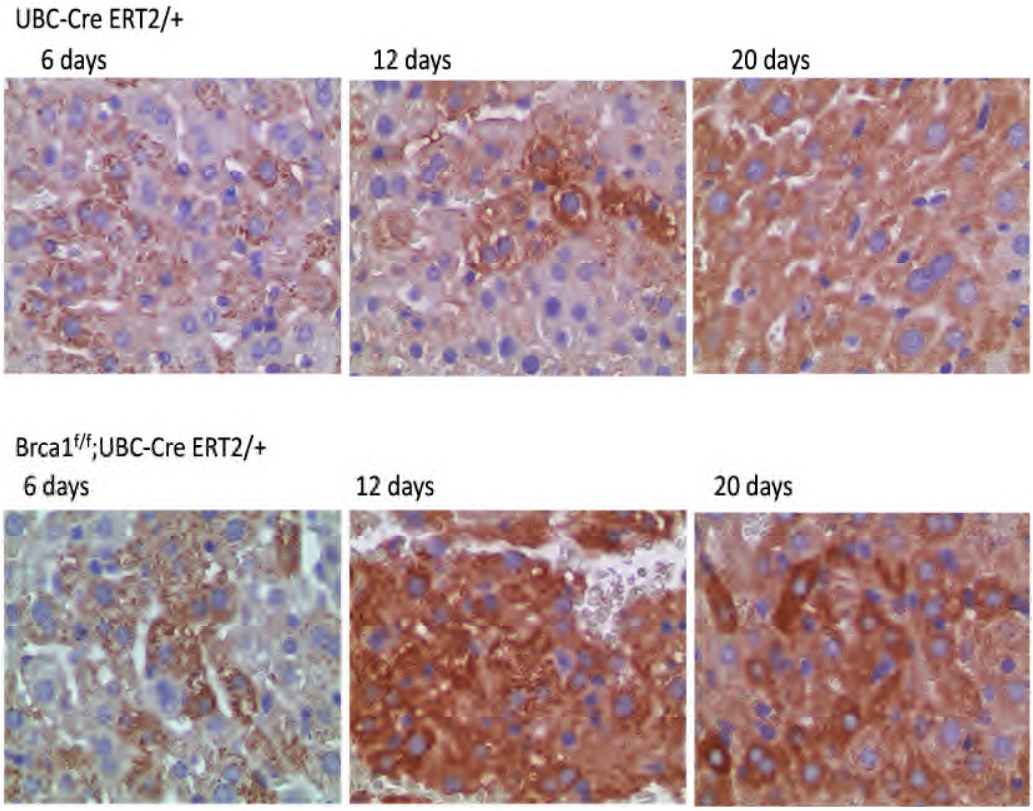

Figure 13. *Brca1* deletion caused increased SOD2 protein level in the liver. Representative SOD2 immunohistochemistry for the liver of UBC-CreERT2/+ (top panel) and Brca1$^{f/f}$;UBC-CreERT2/+ (bottom panel) mices. SOD2 protein level was increased after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mice.

2.3 Deletion of Brca1 Increases γH2AX *in Brca1$^{f/f}$;UBC-CreERT2/+ Mouse Liver*

After assessment of *Brca1* deletion in the liver and spleen (Fig. 10), we sought to determine the effect of the deletion on DNA damage to check whether its normal functions in maintaining genomic stability and repairing DNA damage were also compromised.

Accordingly, we tested the effect of deletion of *Brca1* in *Brca1$^{f/f}$;UBC-CreERT2/+* mice on γH2AX levels after treatment with tamoxifen (TAM) intraperitoneally. Tissues were collected at 6, 12, and 20 days after last injection. IHC was performed for mammary and liver tissues. Results from *Brca1$^{f/f}$;UBC-CreERT2/+* mice were compared to control (*UBC-CreERT2/+*) mice, which were also treated with TAM. We found *Brca1* deletion clearly increased γH2AX levels and foci in both tissues after TAM treatment (Fig. 14 and Fig. 15). This increased in γH2AX signal presumably reflects phosphorylation of γH2AX in response to DNA damage due to *Brca1* deletion. Moreover, we checked the γH2AX signal in lymph nodes of mammary glands. As expected, we found an increase in γH2AX signal in *Brca1$^{f/f}$;UBC-CreERT2/+* mice compared to control mice.

Taken together, these results are consistent with the established role of BRCA1 in genomic maintenance and damage repair and further confirm the efficiency of *Brca1* deletion in TAM-treated *Brca1$^{f/f}$;UBC-CreERT2/+* mice.

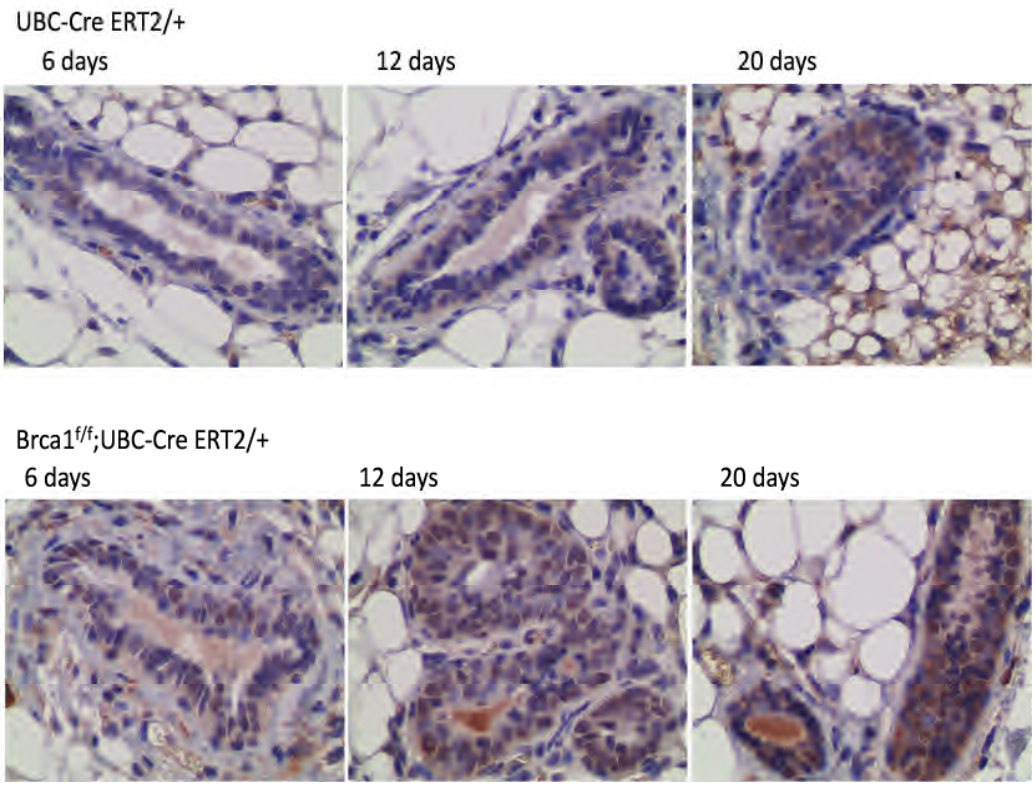

Figure 14. γH2AX levels in the mammary glands of TAM-treated Brca1$^{f/f}$;UBC-CreERT2/+ and control mice. Representive immunohistochemistry for mammary gland of UBC-CreERT2/+ (top panel) and Brca1$^{f/f}$;UBC-CreERT2/+ (bottom panel) stained with anti-γH2AX. γH2AX levels were increased after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mouse.

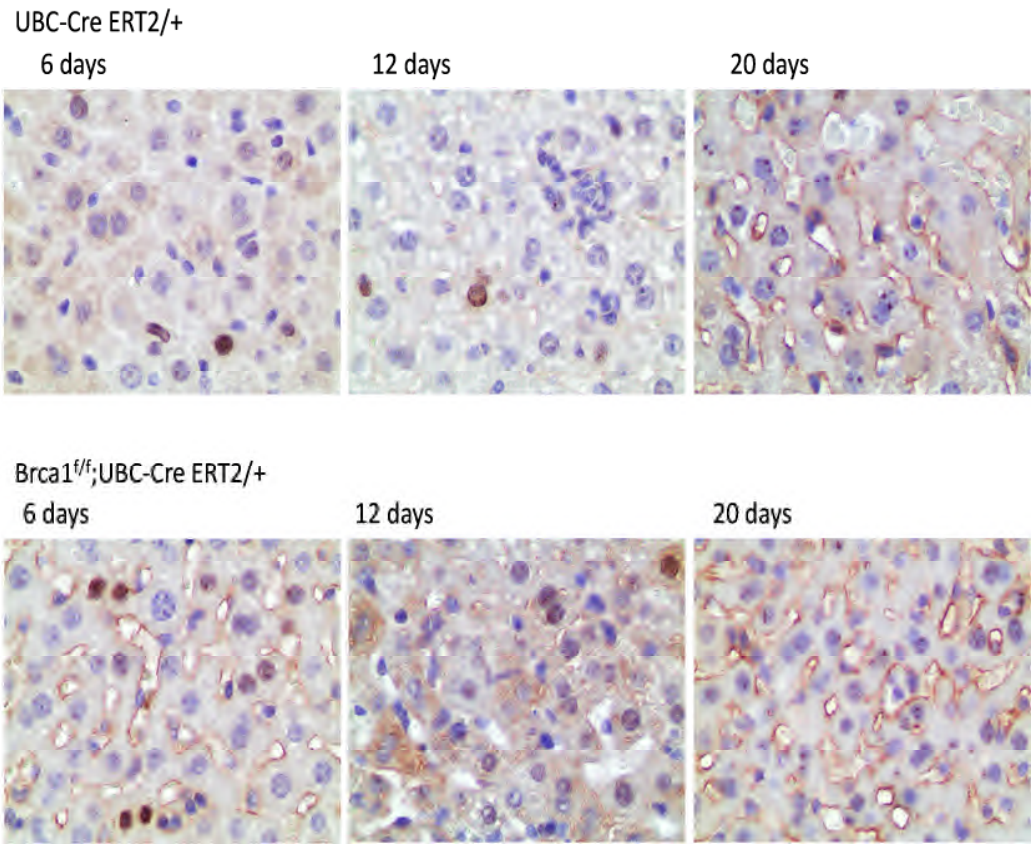

Figure 15. γH2AX levels in the livers of TAM-treated Brca1$^{f/f}$;UBC-CreERT2/+ and control mice. Representive immunohistochemistry for liver of UBC-CreERT2/+ (top panel) and Brca1$^{f/f}$;UBC-CreERT2/+ (bottom panel) stained with anti-γH2AX. γH2AX levels were increased after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mouse.

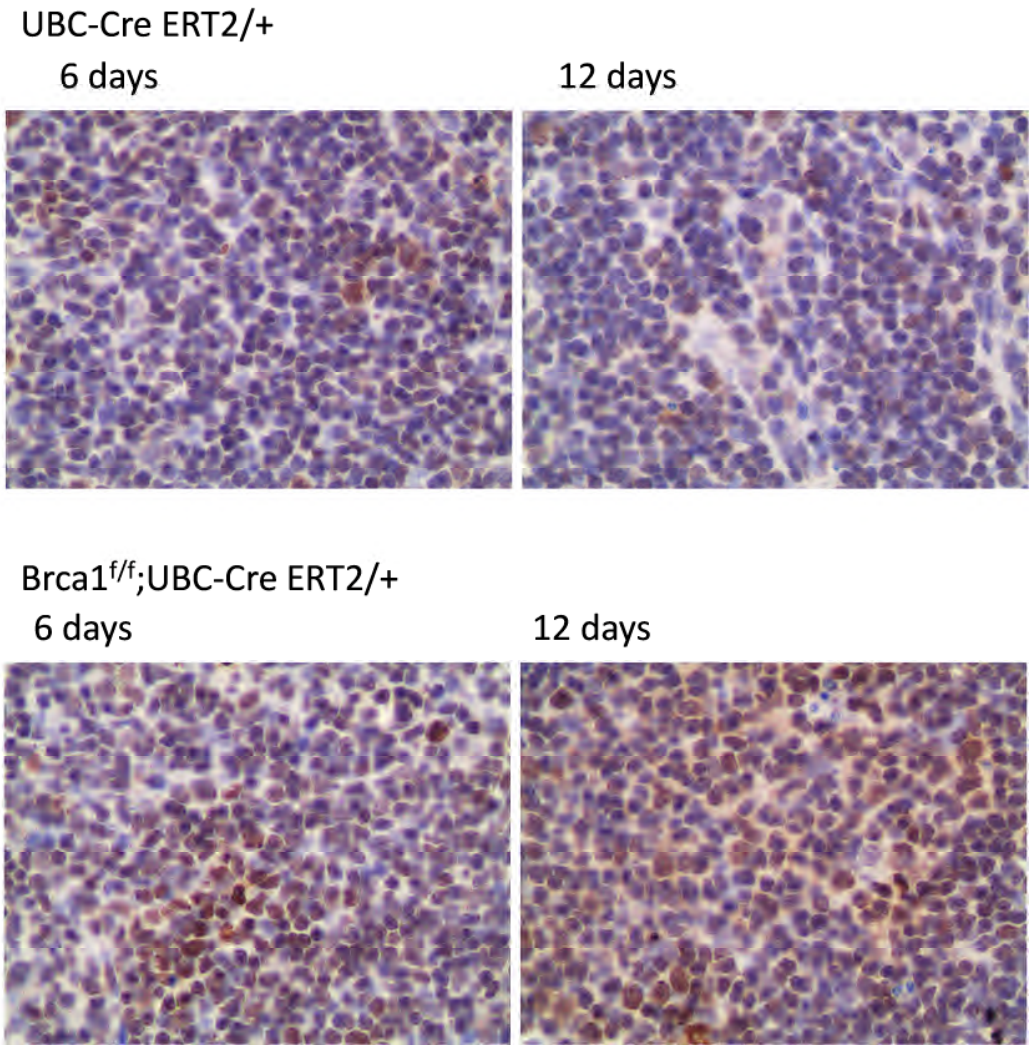

Figure 16. *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+mouse increased γH2AX levels in mammary gland lymph nodes. Representive immunohistochemistry images of mammary gland lymph nodes of UBC-CreERT2/+ (top panel) and Brca1$^{f/f}$;UBC-CreERT2/+ (bottom panel) stained with anti-γH2AX. γH2AX levels were increased after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mouse.

2.4 Deletion of Brca1 Induces p21 in Brca1$^{f/f}$;UBC-CreERT2/+ Mouse

In order to determine the possible functional interplay between p53 and BRCA1 in stress response, we sought to test the effect of *Brca1* deletion on p53 and p21 mRNA and protein levels in TAM-treated *Brca1$^{f/f}$; UBC-CreERT2/+* mice. As shown in Figure 17, p21 mRNA was effectively upregulated after *Brca1* deletion. mRNA of p53 also appeared to increase after *Brca1* deletion, although more replicates need to be performed in order to draw such a conclusion.

To further confirm our observation, we performed IHC to check p21 expression in the liver and mammary gland. After TAM treatment, there was a substantial accumulation of p21 in mice deleted of *Brca1* exons 5-13, whereas control mice showed a clearly lower level of p21 (Fig. 18). In contrast, mammary gland showed no change in p21 level (Fig. 19).

The difference in p21 levels between TAM-treated *Brca1$^{f/f}$;UBC-CreERT2/+* and *UBC-CreERT2/+* mice was more profound, indicating that a loss of BRCA1 induces p53 accumulation. These results are consistent with our previous finding that knockdown of BRCA1 in MCF7 cells led to induction of p53 (Fig. 8A). Together, these data demonstrated that induction of p53 and p21 following BRCA1 loss is important at least to enhance antioxidant capacity given the role of p21 in enhancing NRF2 expression and stability in response to oxidative stress (Chen, Sun et al. 2009). Further experiments need to be done to understand the role of *Brca1* deletion in activation p21 and p53.

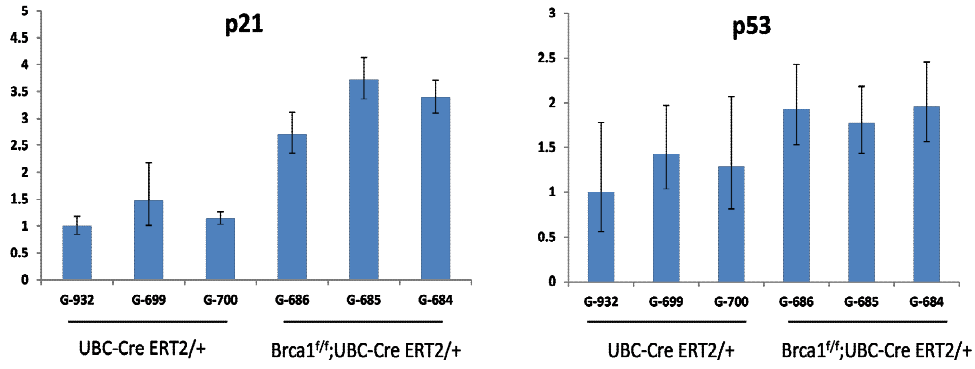

Figure 17. *Brca1* deletion increased p21 and p53 mRNA levels in the liver. Representive qRT-PCR results for the livers of TAM-treated UBC-CreERT2/+ (G-932, G-699, and G-700) and Brca1$^{f/f}$;UBC-CreERT2/+ (G-686, G-685, and G-684) mice. p21 and p53 mRNA expression was tested. Both p21 and p53 mRNA expression were increased after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mice.

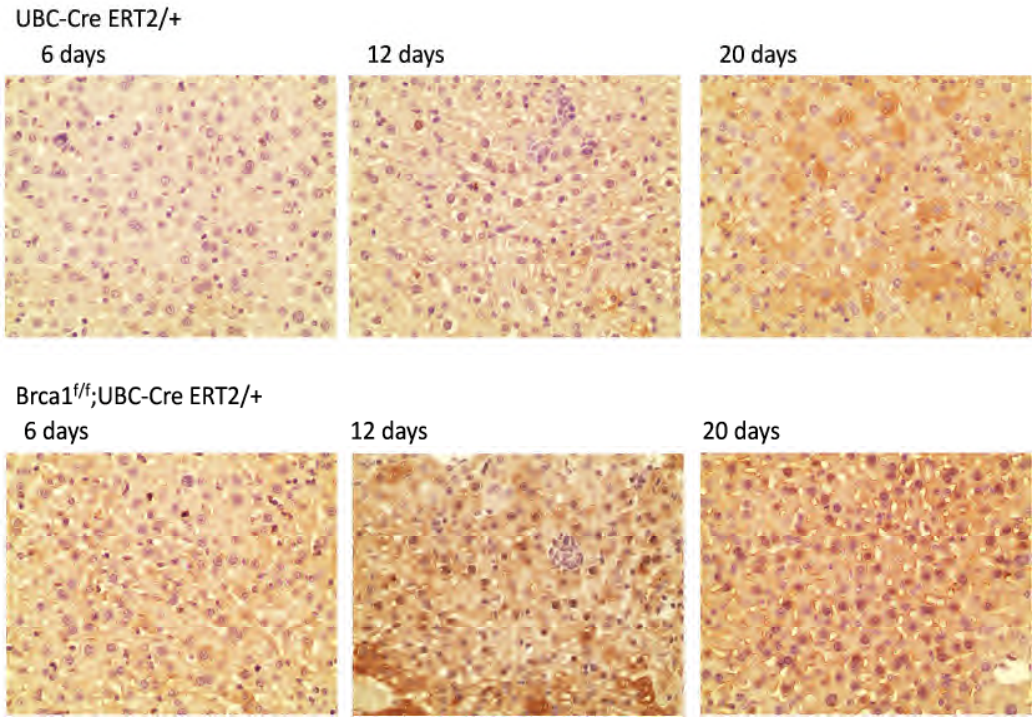

Figure 18. *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+mouse increased p21 protein levels. Representive a IHC for liver of UBC-CreERT2/+ (top panel) and Brca1$^{f/f}$;UBC-CreERT2/+ (bottom panel).p21 protein stained with Anti-p21. p21 protein levels were increased after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mouse compared with control mice on top panel (UBC-CreERT2/+).

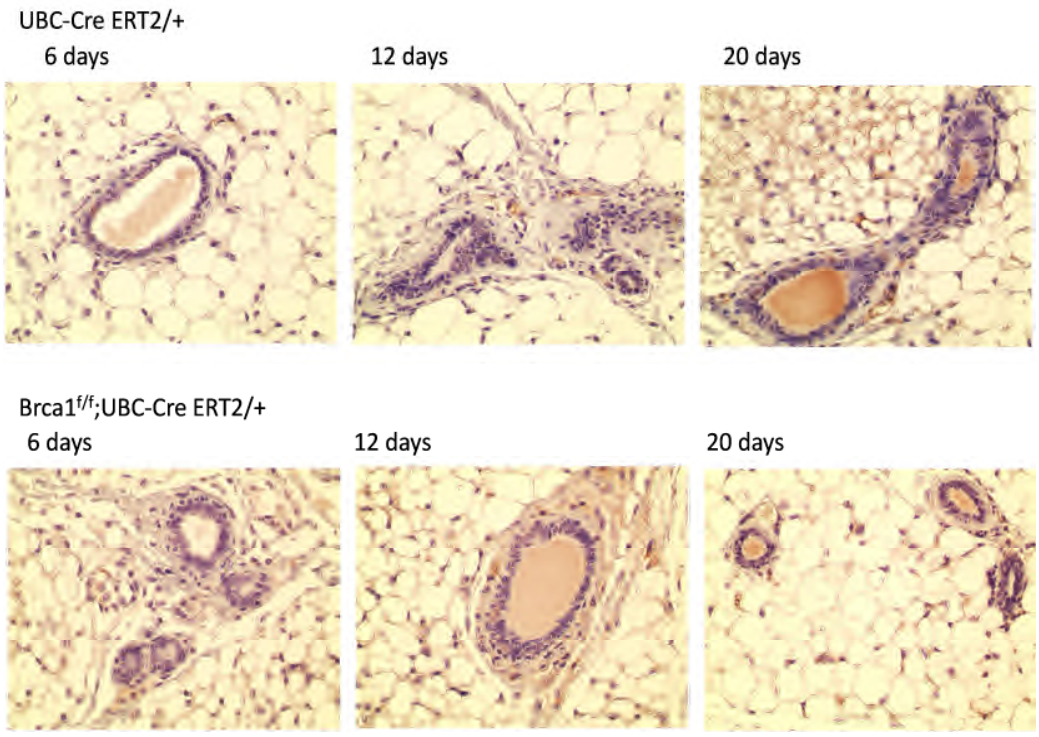

Figure 19. *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+mouse did not change p21 protein levels in mammary gland. Representive a IHC for mammary gland of UBC-CreERT2/+ (top panel) and Brca1$^{f/f}$;UBC-CreERT2/+ (bottom panel).p21 protein stained with Anti-p21. p21 protein levels were not changed after *Brca1* deletion in Brca1$^{f/f}$;UBC-CreERT2/+ mouse compared with control mice on top panel (UBC-CreERT2/+).

Section III: Analysis of SOD2's Role in Sensitizing Breast Cancer Cells to Chemotherapies

3.1 Low Expression of SOD2 is Associated with Better Prognosis in All Tumors

Prediction of tumor metastatic progression is very difficult because of the lack of sufficiently reliable predictive biological markers. In order to identify a new candidate as predictive biomarker of breast cancer metastatic progression, we analyzed gene expression of some antioxidant genes including *SOD1* and *SOD2* in human breast cancers. We found *SOD2* mRNA expression is substantially lower in tumors than in the normal adjacent tissues (Fig. 20A). Further analysis of the METABRIC and TCGA datasets in all breast cancer subtypes showed the basal subtype has the highest *SOD2* mRNA amount among all subtypes (Fig. 20B). Interestingly, most of the basal subtypes are negative for the ER, PR and HER2. Of note, these breast cancer carcinomas referred to as triple negative breast cancers tend to have poor prognosis (Brouckaert, Wildiers et al. 2012). Additionally, we analyzed the correlation between *SOD2* mRNA level and patient survival. We found that high *SOD2* mRNA expression is associated with poor prognosis when all patients are considered, whereas low *SOD2* mRNA expression is associated with better outcome (Fig. 20C). These results suggested a possible functional role of SOD2 in patients' response to breast cancer chemotherapies. Accordingly, we continue our study on the SOD2 expression in breast cancer patients and cell lines.

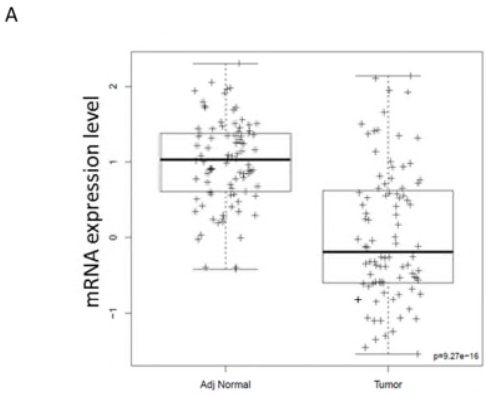

Figure 20. Analyses of *SOD2* gene exexpression in human breast cancer. (**A**) SOD2 mRNA amount in tumors and adjacent normal tissues. (**B**) SOD2 mRNA expression in different subtypes of human breast cancer. Gene expression data in the METABRIC and TCGA datasets were analyzed. (**C**) Kaplan-Meier survival curves of disease-specific survival of all patients with high and low SOD2 expression in their tumors regardless of tumor subtypes.

SOD2 Expression Correlates Negatively with Estrogen Receptor Status in Breast Cancer

To analyze SOD2 expression at the protein level in different breast cancer subtypes, we performed SOD2 IHC on a tissue microarray consisting of tumor samples from breast cancer patients (110 cases). We found SOD2 protein level is negatively correlated with estrogen receptor (ER) status (Fig. 21B). In other words, ER-negative tumors have higher SOD2 protein amount comparing to ER-positive tumors. Estrogen receptor (ER)-negative breast cancers are a group of tumors with poor prognosis and fewer prevention and treatment options compared with ER-positive tumors (Putti, El-Rehim et al. 2005). Our results suggest a possible functional role for SOD2 in response to breast cancer chemotherapies. To verify these results in vitro, we tested SOD2 expression in 9 different breast cancer cells of different subtypes (Fig. 22). Cells were grown to 90% confluency. Cell lysates were collected. A western blot was carried out to profile SOD2 expression in the 9 cell lines. As shown in Figure 22, cells that lack expression for ER, PR, and HER2 have highest expression of SOD2 among other cell lines. In addition, we found HER2-positive cell lines have moderate level of SOD2 among the tested cells, while ER+ cell lines have the lowest expression level of SOD2 (Fig. 22). These results further confirm our microarray analysis and demonstrated that SOD2 expression may play a key role in breast cancer prognosis. Accordingly, we continue focusing our research on the effect of SOD2 expression in responding to chemotherapeutic drugs in breast cancer patients and cell lines.

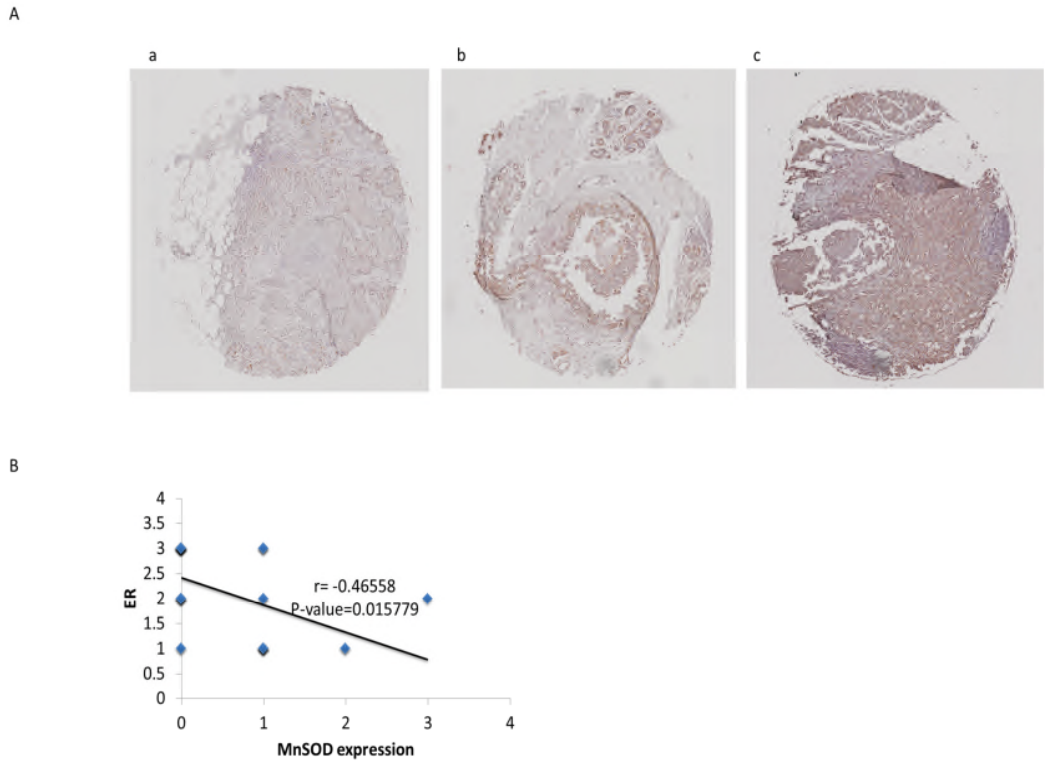

Figure 21. SOD2 protein expression analyses in human breast cancer. (**A**) Representative staining patterns of SOD2 in breast cancer tumors. (a) SOD2 negative tumor (b) SOD2 weakly positive tumor (c) SOD2 strongly positive tumor. (**B**) correlation between ER status and SOD2 expression level. Scoring of SOD2 was performed semi-quantitatively. Staining was considered to be negative (0) if no staining was seen within the tumor, weak positive staining (1) if focal staining was seen within the tumor, strong positive staining (2) if there was a staining in more than 50% of tumor cells, and very strong positive staining (3) if there was a staining in more than 80% of tumor cells. Scores were entered into a standardized Excel worksheet and p value was calculated.

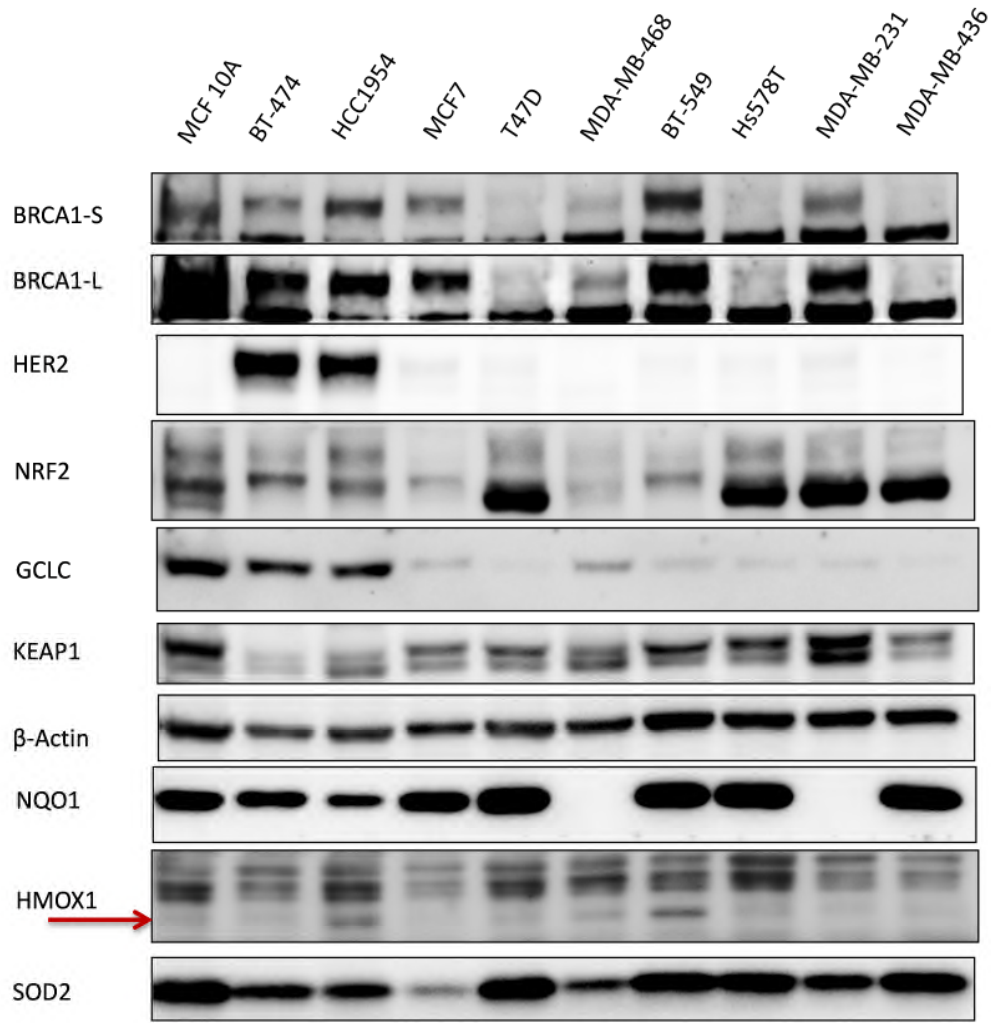

Figure 22. SOD2 expression in ER negative, ER positive and HER2 positive breast cancer cell lines. Representative western blot of breast cancer cell lines: MCF7 and T47D cells are ER positive cells, BT474 and HCC1954 cells are HER2 positive, and MDA-MB-468, MDA-MB-436, MDA-MB-231, Hs578t, and BT-549 cells are triple negative cells. Cells were grown under normal condition (37C, 5% CO2) for 72hrs. Cell lysates were collected. Western blot was performed to check NRF2, GCLC, NQO1, HMOX1, and SOD2 protein expression.

3.3 Low SOD2 Expression Correlates with Poor Prognosis among Patients with ER- or HER+ Breast Cancer

In order to understand the possible effect of SOD2 expression level on the prognosis of patients with different subtypes of breast cancer, we used the Kaplan-Meier plotter to compare relapse-free survival based on SOD2 expression level (high and low expression split by the median). The analysis included all different subtypes of breast cancer stratified by hormone receptor status, HER2 status or luminal/basal classifications. Consistent with the previous finding, low SOD2 expression correlates with better survival when all patients are considered as a whole. Interestingly, among patients with ER-, PR-, or HER2-positive tumors, however, low SOD2 mRNA expression strongly correlates poor prognosis (Fig. 23), suggesting that SOD2 may play a key role in tumor cell response to breast cancer treatment in these patients.

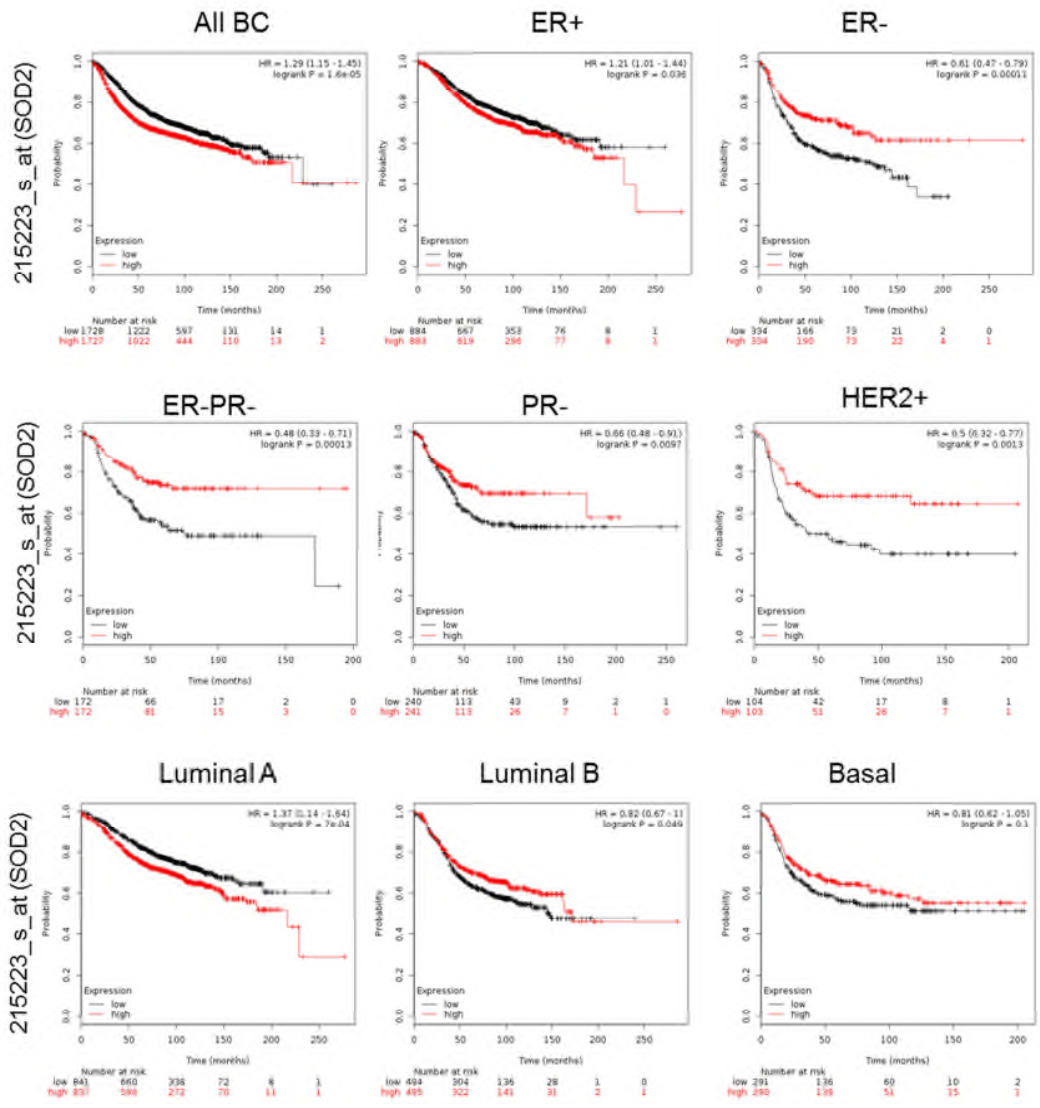

Figure 23. Low SOD2 expression strongly correlates with poor prognosis among patients with ER-negative or HER2-positive breast cancers. KM plotter showing high SOD2 being associated with a poor outcome in all or ER+ and with a good outcome in ER- , ER-PR-, HER, Lumina A, Luminal B and Basal tumors. Survival curves were generated with Kaplan-Meier plotter (kmplot.com).

3.4 Downregulation of SOD2 Causes Cellular Resistance into Breast Cancer Chemotherapies

In order to test potential novel role of SOD2 in driving better response of ER- and HER2+ tumors to treatment, we knocked down SOD2 in the 9 different breast cancer cell lines described in the previous subsection (Section 2.3). We used three different SOD2 siRNAs to deplete SOD2 and two non-targeting siRNAs as controls. We treated the cells with four different breast cancer chemotherapeutic drugs including cisplatin, cyclophosphamide, doxorubicin, and camptothecin. We checked cell viability after 96 hrs of drug treatment using the CellTiter Glo assay that detects the amount of ATP in cells. We first tested the sensitivity of the triple negative MDA-MB-436 and MDA-MB-231 cells depleted of SOD2. Indeed, depletion of SOD2 in these two cells led to clear resistance to all of the four breast cancer chemotherapies (Fig. 24). We confirmed knockdown efficiency of SOD2 by western blot and found knockdown efficiency was approximately 90% complete (Fig. 29). Then, we tested the resistance to breast cancer chemotherapies in the other two triple negative cell lines (MDA-MB-468 and Hs578T) after knockdown of SOD2. The two cell lines showed mildly increased or no difference in sensitivity to breast cancer chemotherapies after knockdown of SOD2 (Fig. 25). We also confirmed the knockdown efficiency by western blot (Fig. 29). These results indicate a potential role of SOD2 in sensitizing cells to chemotherapies.

Next, we further tested the HER2-positive breast cancer cell lines including SKBR3, HCC1954 and ZR-75-30 cells. We knocked down SOD2 for 48 hrs in SKBR3 cells and then treated the cells with the four drugs. After 96 hrs of treatments, cell viability was tested. Indeed, knockdown SOD2 in SKBR3 cells appeared to induce mild resistance to the breast cancer chemotherapies (Fig. 26).

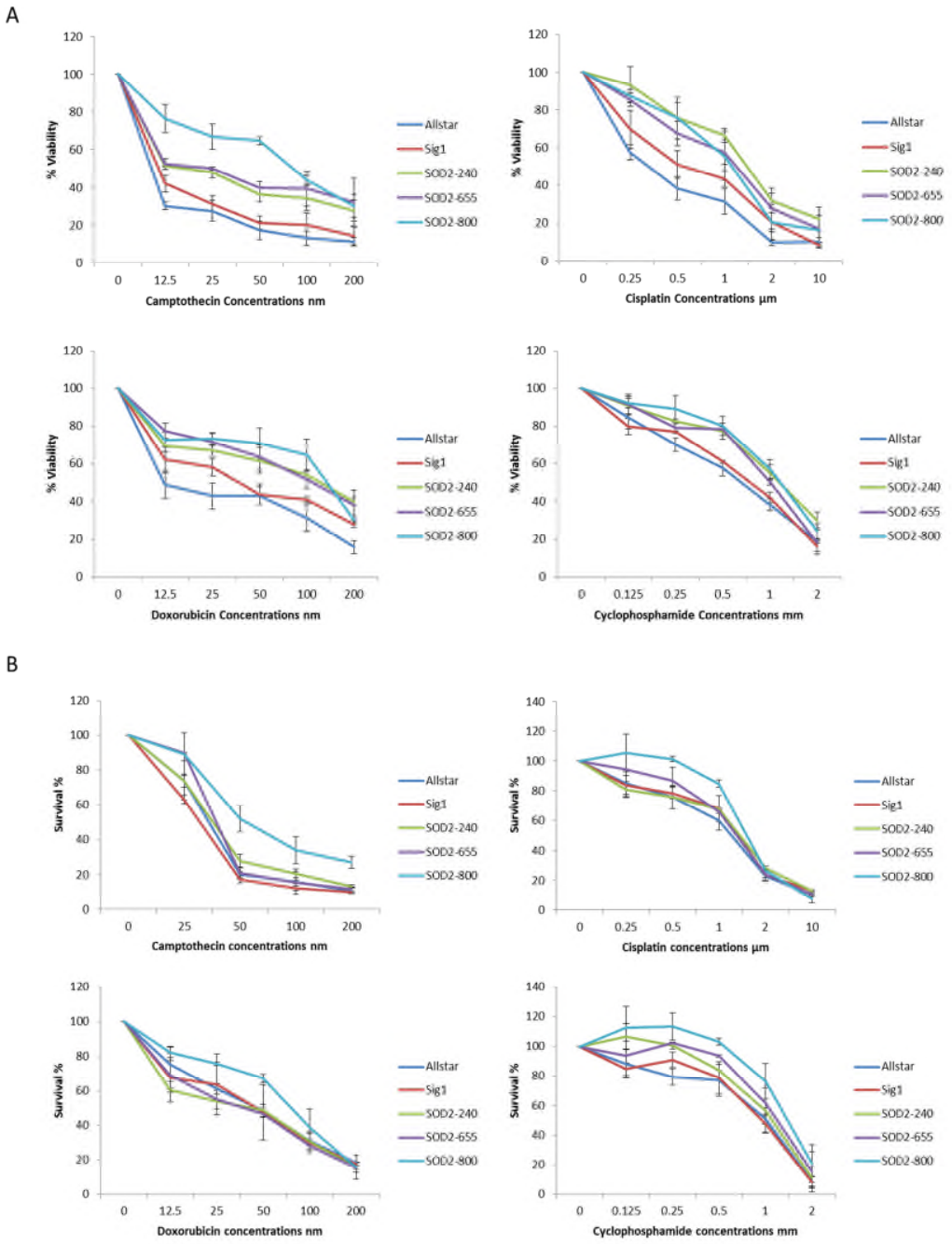

Figure 24. Effect of SOD2 downregulation on cellular resistance to breast cancer chemotherapies. MDA-MB-436 (A) and MDA-MB-436 (B) cells were depleted of SOD2 and then treated with cisplatin, doxorubicin, cyclophosphamide, or camptothecin. Cell viability was measure by CellTiter Glo 96 hr after drug addition. Both cell lines show resistance to breast cancer chemotherapies after SOD2 depletion.

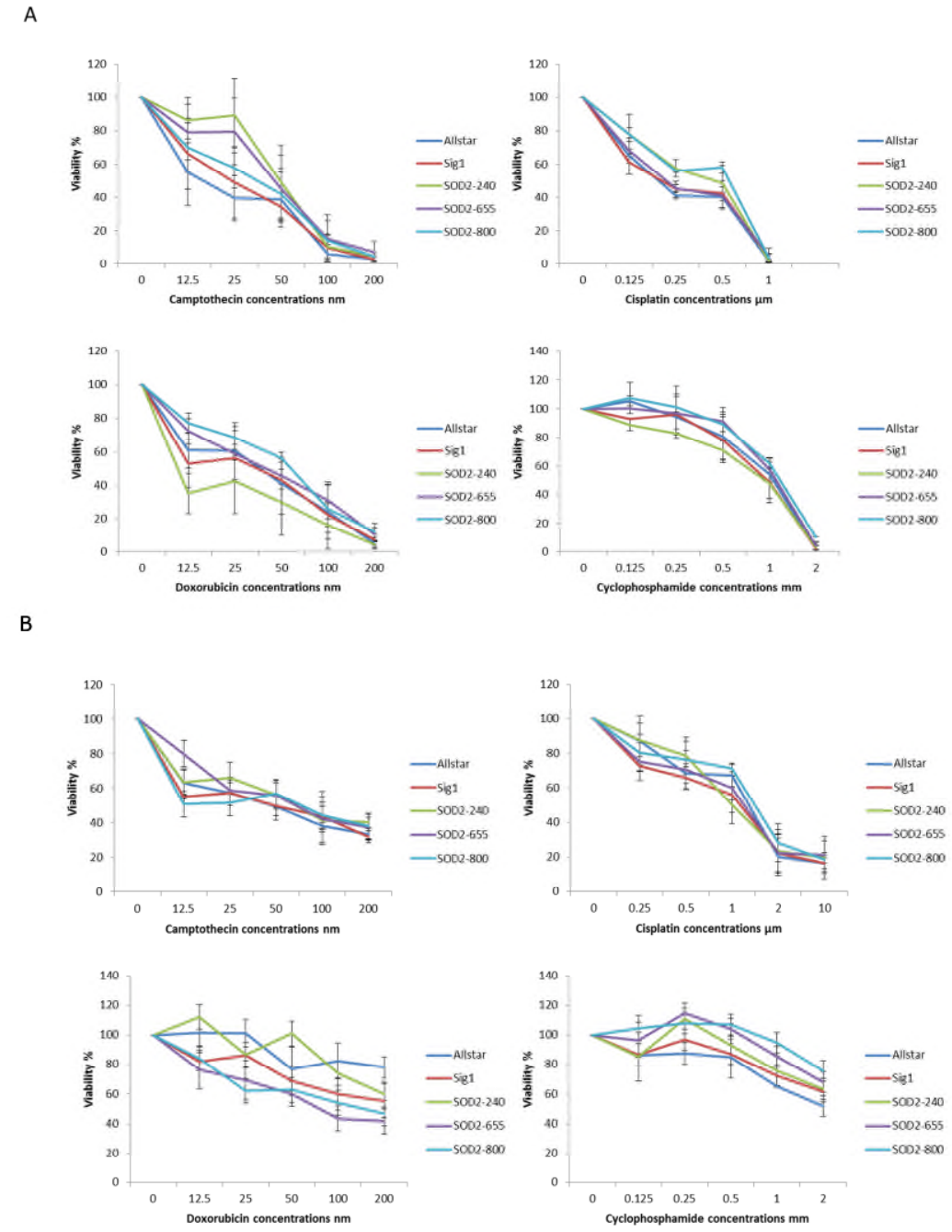

Figure 25. Effect of SOD2 downregulation on cellular resistance to breast cancer chemotherapies. MDA-MB-468 (A) and Hs578T (B) cells were depleted of SOD2 and then treated with cisplatin, doxorubicin, cyclophosphamide,

ZR-75-30 cells showed weak resistance to camptothicin and clear sensitivity to doxorubicin after SOD2 knockdown. Interestingly, ZR-75-30 cells showed no change to cisplatin and cyclophosphamide after SOD2 depletion (Fig. 27). Similarly, HCC1954 did not show any sensitivity or resistance to drugs after SOD2 depletion (Fig. 27).

Moreover, we tested ER positive cells (MCF7 and T47D cells) for cellular sensitivity to the drugs after SOD2 knockdown (Fig. 28 A and B). SOD2 knockdown in MCF7 cells caused mild sensitivity to chemotherapies. Interestingly, knockdown of SOD2 in T47D cells did not appear to cause any

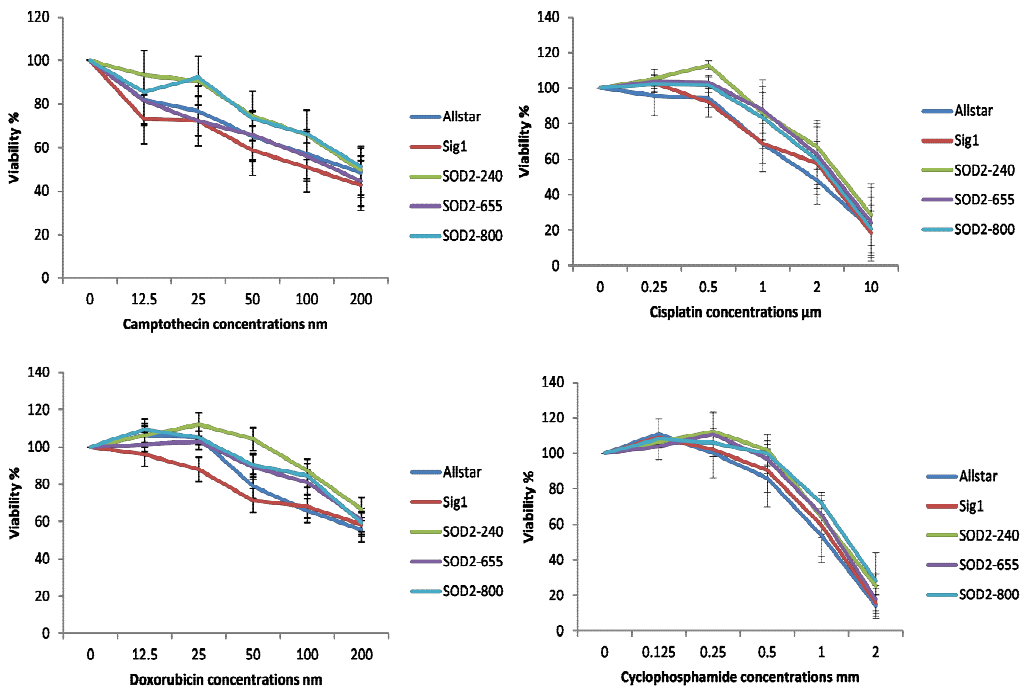

Figure 26. . Effect of SOD2 downregulation on cellular resistance to breast cancer chemotherapies. SKBR3 cells were depleted of SOD2 and then treated with cisplatin, doxorubicin, cyclophosphamide, or camptothecin. Cell viability was measure by CellTiter Glo 96 hr after drug addition. SKBR3 cell lines show mild resistance to breast cancer chemotherapies after SOD2 depletion.

clear difference in cellular response to cyclophosphamide and cisplatin treatments but appeared to induce mild resistance to doxorubicin and camptothecine treatments. It seems that knockdown of SOD2 in ER+ cells with high basal level of SOD2 induce resistance to breast cancer chemotherapies. These results indicated in addition to SOD2, ER may play a role in cellular sensitivity.

In summary, these results overall suggest a significant role of SOD2 in regulating breast cancer cell response to chemotherapies, although the exact effect and the magnitude of its impact are cell type dependent. It should also be pointed out that cell lines may not faithfully recapitulate the drug sensitivity of tumors.

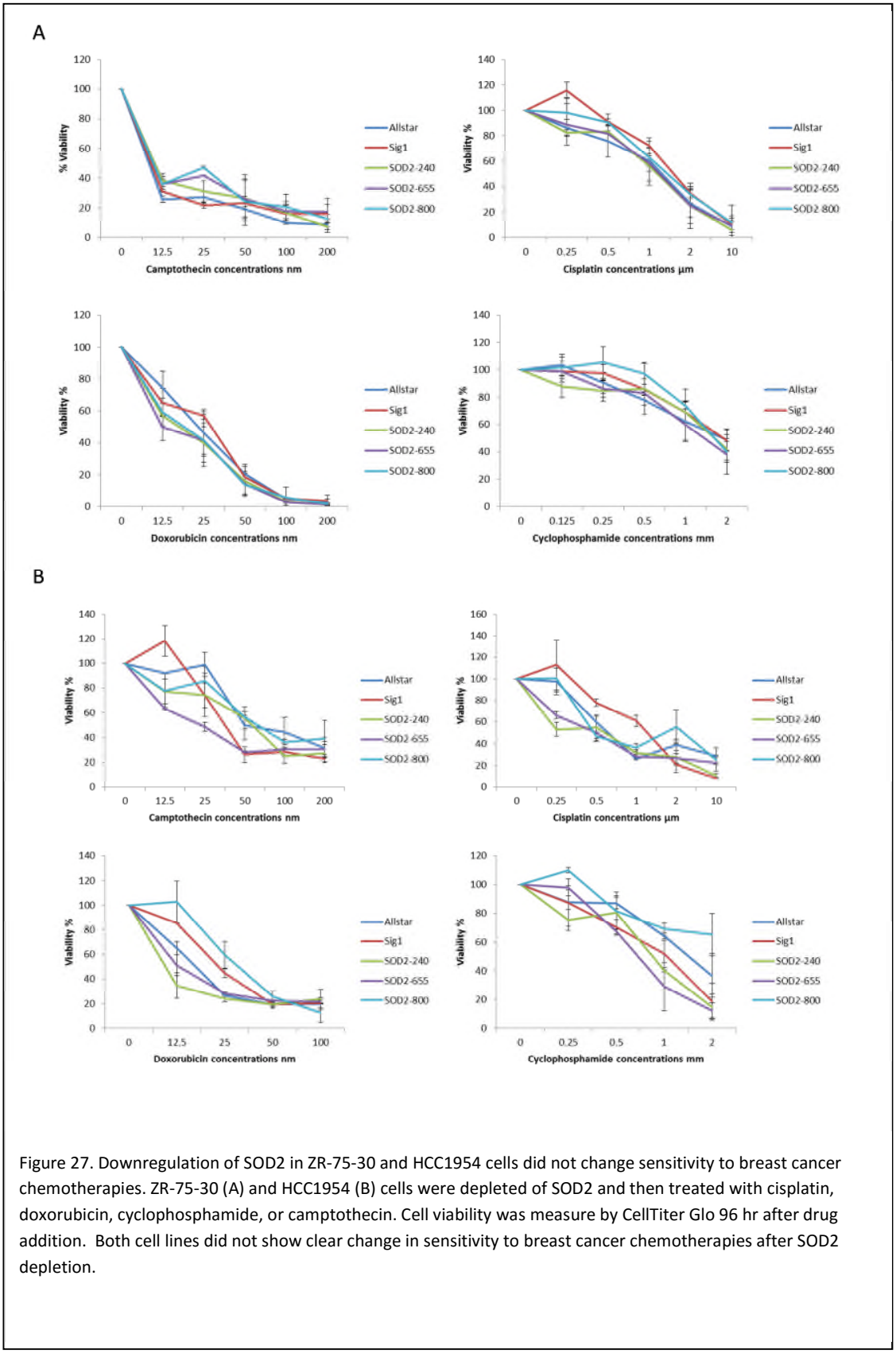

Figure 27. Downregulation of SOD2 in ZR-75-30 and HCC1954 cells did not change sensitivity to breast cancer chemotherapies. ZR-75-30 (A) and HCC1954 (B) cells were depleted of SOD2 and then treated with cisplatin, doxorubicin, cyclophosphamide, or camptothecin. Cell viability was measure by CellTiter Glo 96 hr after drug addition. Both cell lines did not show clear change in sensitivity to breast cancer chemotherapies after SOD2 depletion.

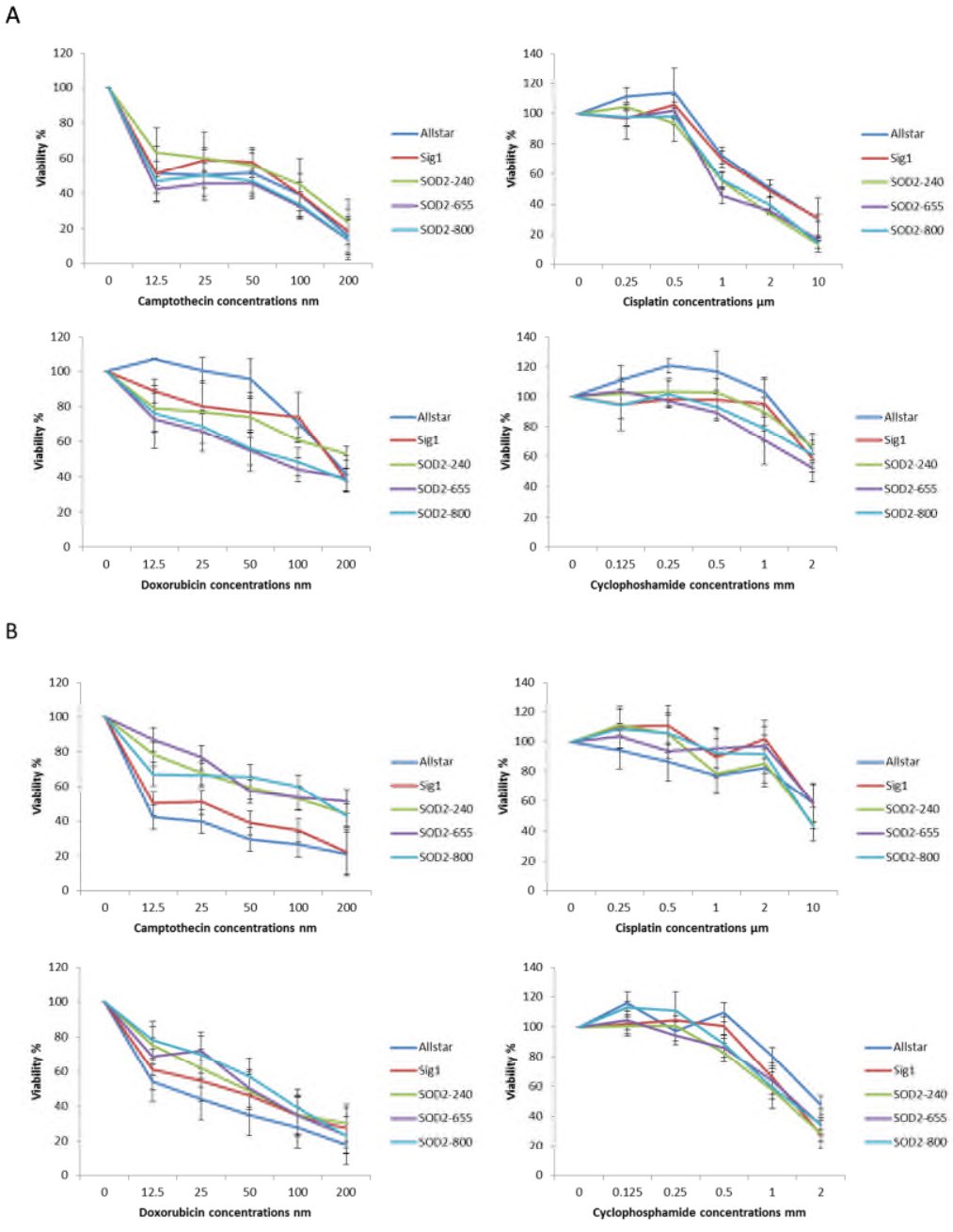

Figure 28. Effect of SOD2 downregulation on cellular resistance to breast cancer chemotherapies. MCF7 (A) and T47D (B) cells were depleted of SOD2 and then treated with cisplatin, doxorubicin, cyclophosphamide, or camptothecin. Cell viability was measure by CellTiter Glo 96 hr after drug addition. T47D show resistance to camtpthecin after SOD2 depletion.

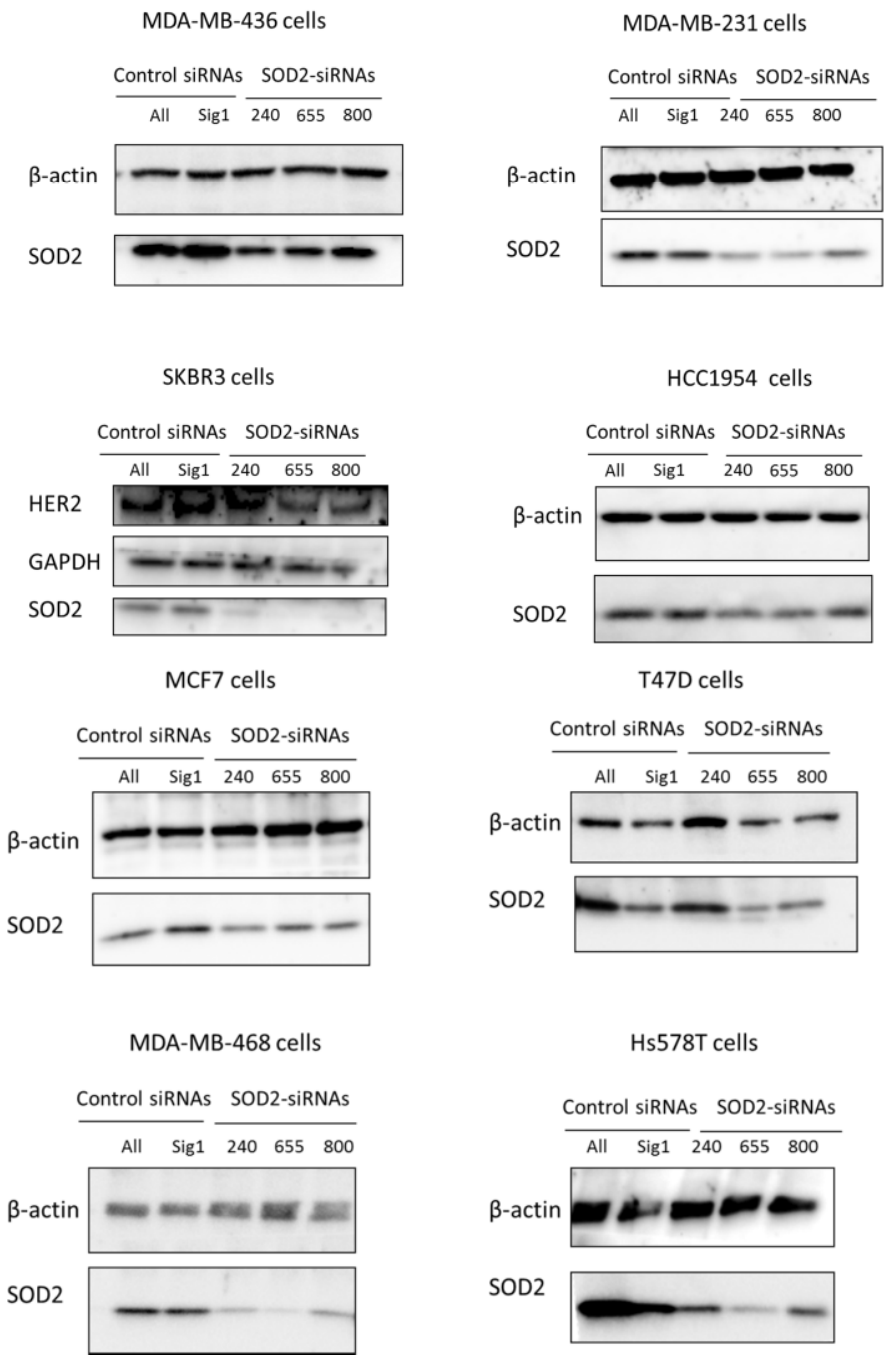

Figure 29. SOD2 depletion in breast cancer cells. Representative is a western blots showing depletion of SOD2 using siRNAs in the triple negative breast cancer cells (MDA-MB-436, MDA-MB-231, MDA-MB-468, Hs578T), HER2 positive cells (SKBR3 and HCC1954), and ER positive cells (MCF7 and T47D).

3.5 SOD2 Depletion Leads to Reduced γH2AX Foci Formation

Our finding that SOD2 depletion promotes cellular resistance to drug treatment prompted us to hypothesize that SOD2 depletion may confer protection against DNA damage. In order to test this hypothesis, we used immunofluorescence assay to detect γH2AX and 53BP1, both markers of DNA double strand breaks, before and after drug treatment. MDA-MB-436 cells were treated with either a pool of SOD2 siRNAs or a control non-targeting siRNA. Forty eight hours after siRNA transfection, cells were treated with 5 μm camptothecin (CPT) for 18 hrs stained with anti-γH2AX and 53BP1 antibodies and analyzed by immunofluorescence. As shown in Figure 30, before CPT treatment, SOD2-depleted cells showed significantly reduced γH2AX foci positive cells compared with cells treated with the control siRNA (Fig. 30), whereas the number of 53BP1 foci positive cells was not changed by SOD2 depletion. After CPT exposure, γH2AX foci were induced in both SOD2-depleted and control siRNA treated cells; however, the induction in SOD-depleted cells was milder. These results suggest that SOD2 depletion may either caused reduced DNA damage or increased DNA repair. It is also possible that depletion of SOD2 causes altered cell cycle distribution, as camptothecin is a topoisomerase I inhibitor that mostly targets S phase cells. These possibilities remain to be investigated. We also tested the effect of drug treatment on apoptosis after SOD2 depletion in MDA-MB-436 cells (data not shown). Unfortunately, we could not detect a significant difference between control siRNAs treated cells and SOD2 depleted cells. This may suggest that SOD2 depletion is not involved in conferring a protection for cells against apoptosis.

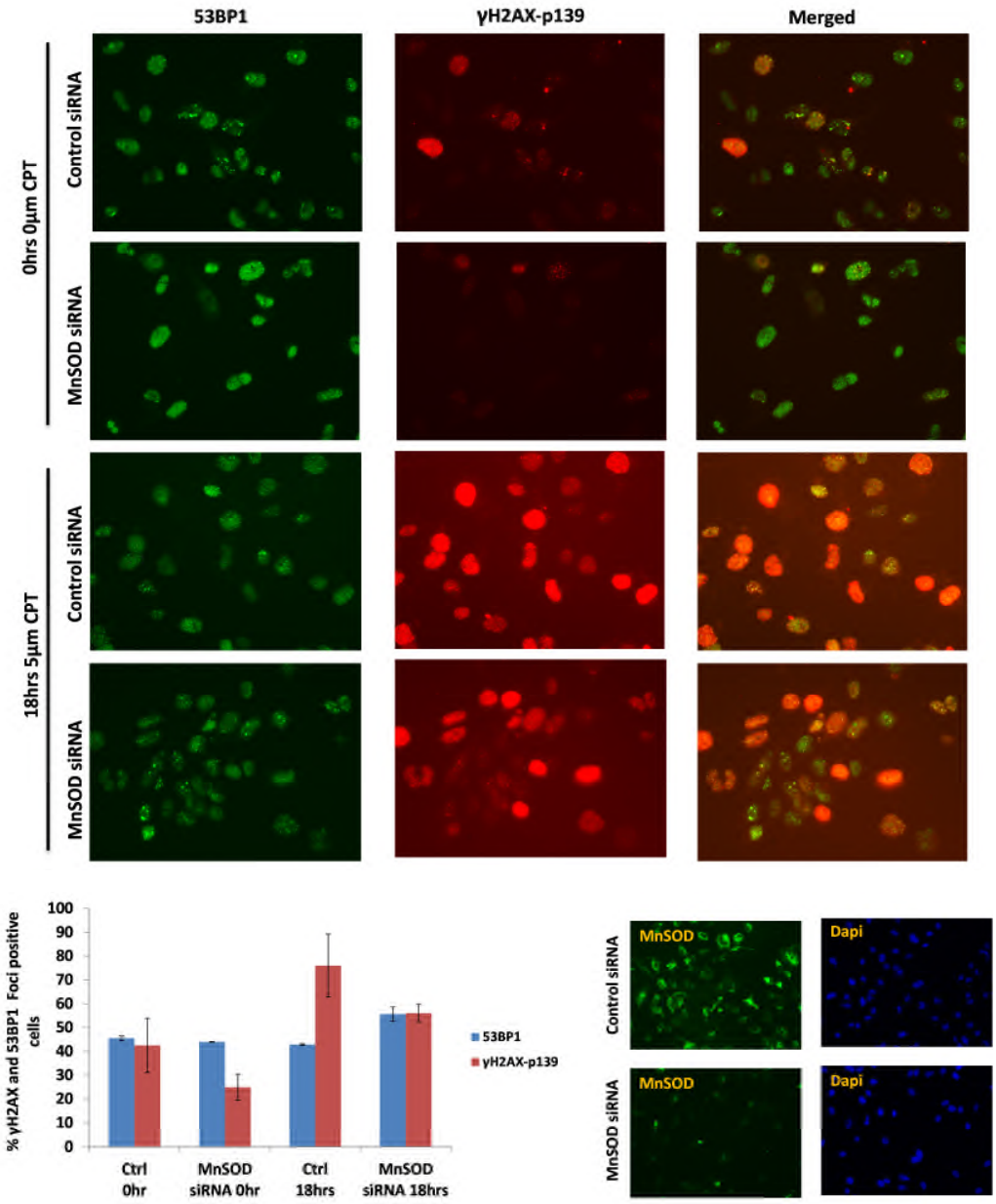

Figure 30. SOD2 depletion leads to reduced γH2AX foci formation in response to camptothecin treatment. MDA-MB-436 cells without and with SOD2 depletion were analyzed by IF before and after CPT treatment. Cells were stained with antibodies against γH2AX and 53BP1.

5. DISCUSSION

Section I

1.1 Downregulation of BRCA1 Increases ROS Levels in Breast Cancer Cells

BRCA1 is important for protecting cells during oxidative stress. Under stress conditions, BRCA1 upregulates the expression of p21 (a cyclin-dependent kinase inhibitor) which interacts directly with NRF2 (Chen, Sun et al. 2009). NRF2 is a master transcription factor that regulates expression of many proteins involved in detoxifying ROS. Furthermore, these antioxidant genes regulated by NRF2 either produce antioxidants such as glutathione (GSH) and NADPH or antioxidant enzymes like glutathione peroxidase (GPx), thiroredoxin (TXN) and superoxide dismutase (SOD2 and SOD1) that help scavenging ROS (Copple, Goldring et al. 2008, Hayes, McMahon et al. 2010).

Our initial experiment of BRCA1 knockdown in U2OS and MCF 10A cells confirmed BRCA1 is important for maintaining ROS at low levels (Fig. 1A and B). Knockdown of BRCA1 in MCF7 and MDA-MB-231 cell lines showed approximately 15-20% increase in ROS levels (Fig 2A and B). This may have been simply because basal ROS levels in cancer cells are higher than normal cells. Therefore, knockdown BRCA1 in the cancer cells may not change ROS levels that much while knockdown BRCA1 in normal cells causes a huge increase in ROS because normal cells are not adapted to these conditions or cells have not developed an alternative mechanism beside NRF2 pathway. However, taken together, these data indicate BRCA1 is required for maintaining redox homeostasis in normal and breast cancer cells.

1.2 BRCA1 Depletion Causes Increased Mitochondrial Superoxide

Our finding of the necessity of BRCA1 to mitigate ROS accumulation led us to think BRCA1 may be necessary for maintaining mitochondrial superoxide level too. Although superoxide anions are regulated through different mechanisms, it is interesting to learn effect of depletion of BRCA1 on superoxide levels. Using independent control and BRCA1 siRNAs we found that MCF-10A cells depleted of BRCA1 fail to maintain superoxide at low levels while control siRNA transfected MCF 10A cells could (Fig. 3).

Moreover, previous reports demonstrate NRF2, which reportedly binds BRCA1, is required for superoxide dismutase regulation (Gorrini, Baniasadi et al. 2013). Therefore, we tested the effect of NRF2 depletion on superoxide levels in MCF-10A cells. Interestingly, knockdown NRF2 showed similar effect on superoxide as BRCA1 knockdown. Our findings support the necessity for BRCA1 and NRF2 to mitigate mitochondrial superoxide does not necessarily mean BRCA1 functions through NRF2 to regulate mitochondrial superoxide. To check this possibility, we analzyed the correlation between NRF2 and SOD2 mRNA expression in human breast cancer. We found SOD2 expression is strongly correlated with NRF2 (Fig. 4A and B). The fact that independent BRCA1 and NRF2 depletions have similar outcome (promoting superoxide accumulation) reduces the likelihood of BRCA1 regulating superoxide through other transcriptional mechanisms.

1.3 BRCA1 Depletion Down-regulates Some Antioxidant Gene Expression

We were able to show the requirement of BRCA1 for ROS homeostasis. The finding led us to further analyze the requirement of BRCA1 for regulation the expression of antioxidant genes.

Using independent control and BRCA1 transfection siRNAs, we found cells depleted of BRCA1 could not maintain normal expression of the tested antioxidant genes (Fig. 5). These results suggested BRCA1 is required for the transcription for the antioxidant genes tested. Importantly, unlike what was reported recently (Gardini, Baillat et al. 2014), downregulation of BRCA1 did not cause any dramatic decrease in *SOD2* mRNA amount. The slight decrease in *SOD2* mRNA led us to think BRCA1 may have different role in SOD2 regulation. Accordingly, we tested the effect of BRCA1 downregulation on SOD2 protein expression using independent control and BRCA1 siRNAs as describe before. Interestingly, we found cells depleted of BRCA1 showed increased SOD2 protein levels. This was true for all of the tested breast cancer cells (Fig. 6). Our findings suggested may negatively regulate the amount of the SOD2 protein. The accumulation of SOD2 protein amount could be due to at least 2 reasons. First, SOD2 may respond to increased ROS levels after depletion of BRCA1 (so that it upregulates indirectly SOD2). Second, BRCA1 may directly promote SOD2 degradation so that its depletion stabilizes SOD2.

To test the first possibility, we treated BRCA1-depleted cells with NAC and checked SOD2 protein by western blotting. As expected, NAC treatment reversed the increase of SOD2 protein amount in BRCA1-depleted cells (Fig. 8). This suggests that the SOD2 upregulation was due to it responding to the increased levels of ROS after BRCA1 depletion. Further experiment need to be done to test second possibility.

1.4 Re-expression of BRCA1 Reverse SOD2 Accumulation in BRCA1-mutant Breast Cancer Cells

Since our cell lines show the increase in SOD2 caused by BRCA1 depletion, we wanted to further confirm this finding. Our lab constructed two vectors expressing wt BRCA1. Consistently with our observation made with stables cells, both versions of BRCA1 vectors were able to partially

restore low or basal levels of SOD2 by 48 hrs after transfection (Fig. 9). This further demonstrated a function of BRCA1 in regulating SOD2 and antioxidant gene regulation. More importantly, the fact that ectopic BRCA1 expression was able to reverse the high expression of SOD2 in BRCA1 mutant cells gave more credibility to our comparisons between our stable and parental cells. Our results indicate BRCA1 is capable of reducing SOD2 in cells and BRCA1 is clearly important in response to oxidative stress as exhibited by the remarkable difference DCF signal between control siRNA and siRNA selectively targeting endogenous BRCA1 in the tested cancer cells (Fig. 1A and B and Fig. 2A and B). Perhaps even more importantly, cells transfected with plasmid 2 constantly displayed a decreased in SOD2 as compared to parental (Fig. 9).

Our data suggest a more general function for SOD2 in ROS scavenging in the absence of BRCA1. This regulation may be relevant in vivo where cells in which BRCA1 is lost develop a compensatory antioxidant capability in the face of compromised NRF2 function. Through two different assays (BRCA1 plasmid transfection and BRCA1 stable cell lines) we have shown there is consistent evidence that BRCA1 can regulate ROS through SOD2.

1.5 p53 and BRCA1 Depletion Share Similar Effects in Increasing ROS

Multiple studies have demonstrated that under low levels of oxidative stress p53 induces a subset antioxidant gene expression, including SOD2, to maintain ROS levels below threshold that induce apoptosis (Liu and Xu 2011, Robbins and Zhao 2012). So the question to be answered is whether p53 will respond to BRCA1 depletion and upregulate SOD2. If this is true, then co-depletion of BRCA1 and p53 will increase ROS even more than silencing of BRCA1 and it will decrease SOD2 expression. High ROS levels induce the expression of prooxidant genes that weakened cellular capacity or suppressed it to promote apoptosis or senescence (Liu and Xu).

We took particular care to ensure our assay conditions did not affect cell viability. BRCA1 and p53 knockdown was for 48 hrs post siRNA transfection in MCF7 cells, and we observed an increase in ROS levels after co-knockdown BRCA1 and p53, as expected (Fig. 8). Remarkably, the increase in ROS levels following co-depletion of BRCA1 and p53 was significantly higher than that under control conditions or after BRCA1 and p53 single depletion. The increase in ROS level in BRCA1-depleted cells under the condition used was not significant as compared to control siRNA transfection. Our co-depletion results indicate p53 was likely responding to ROS increase. The role of p53 in ROS is complex and therefore further analysis needs to be done to explain and confirm our observations.

Section II

2.1 Deletion of Brca1 in brca1$^{f/f}$; UBC-Cre Mouse Liver Causes SOD2 Increased

The overarching question that needs to be answered is whether or not BRCA1 regulates SOD2 negatively in vivo. There is growing evidence that BRCA1 regulates SOD2 through another transcription factors other than NRF2 (Gardini, Baillat et al. 2014). That will require verification by multiple independent investigations to confirm the functional relevance of these factors in SOD2 regulation.

Our data suggest Brca1 deletion is capable by itself to upregulate SOD2 in liver (Fig. 13). Nevertheless, no difference was seen in the mammary glands of Brca1-deleted and control mice (Fig. 12). Additionally, we observed an increase in p21 expression in the liver after deletion of Brca1 (Fig. 18). Our results so far indicate p21 may be also involved in SOD2 upregulation in response to Brca1 deletion. The role of p21 in oxidative stress is well known and it strongly supports our hypothesis (Chen, Sun et al. 2009). However, further analyses are required to confirm the role of p21 in regulating SOD2 in response to BRCA1 deletion.

Section III: ***The Role of SOD2 in Sensitizing Breast Cancer Cells to Chemotherapies***

3.1 Low Expression of SOD2 is Associated with Better Prognosis in All Tumors

Analysis of SOD2 expression in breast cancer patients in the METABRIC dataset revealed that low *SOD2* mRNA level is associated with better prognosis when all patients were considered but with worse prognosis in patients with ER-negative tumors (Fig. 20C). Also, we found the same correlations using the KM plotter (Fig. 23), which uses a different dataset from a different cohort of patients. These findings strongly suggest an important functional relevance of SOD2 in both ER-positive and ER-negative breast cancers. Through analysis of a tissue microarray derived from a cohort of 110 breast cancer patients for SOD2 expression, we identified a significant, inverse correlation between ER status and SOD2 protein levels (Fig. 21), suggesting that ER may directly or indirectly regulate SOD2 expression. Cumulatively, our findings imply that SOD2 levels may impact the response of breast cancer cells to chemotherapies.

3.2 Downregulation of SOD2 Causes Cellular Resistance into Breast Cancer Chemotherapies

In order to properly investigate the role of SOD2 in cancer cell response chemotherapies, one can either overexpress or downregulate SOD2 to assess its effect. As low SOD2 expression is associated with poor prognosis in ER-negative breast cancer (Fig. 23), we knocked down SOD2 in MDA-MB-436 cells (ER-negative with high SOD2 level) and assessed cellular sensitivity to chemotherapeutic agents. Indeed, MDA-MB-436 cells treated with SOD2 siRNAs exhibited increased resistance than control siRNA treated cells (Fig. 24). Interestingly, the opposite was observed in the ER-positive MCF7 cells (Fig. 28). These data are consistent with the results of our analysis of patient survival data (Fig. 23). Remarkably, ER-positive cells with low SOD2 expression became more sensitive to breast cancer chemotherapies after SOD2 knockdown (Fig. 25). This difference between the responses of ER-positive and ER-negative cells to the drug

treatments was only apparent when SOD2 was depleted. Since our siRNAs transfection conditions are not toxic, it is unlikely that the non-targeting siRNAs induced damage to the cells so it is more likely the effects observed were due to SOD2 depletion. Ultimately, SOD2 may independently sensitize at least a subset of ER-negative breast cancer cells to chemotherapies.

3.3 Cells Depleted of SOD2 Exhibit decreased γH2AX Foci Formation

Our drug sensitivity assays used topoisomerase inhibitors drugs such as doxorubicin and camptothecin, both of which kill cancer cells primarily by causing DNA damage. To begin to understand the mechanism behind the chemo resistance caused by SOD2 depletion, we depleted SOD2 in MDA-MB-436 cells and examined γH2AX foci formation, a marker of DNA double strand breaks, following camptothecin treatment. Interestingly, cells depleted of SOD2 showed lower levels of γH2AX foci both before and after camptothecin treatment compared with control siRNA treated cells (Fig. 30), consistent with the increased cellular resistance to the agent following SOD depletion. This finding suggests that loss of SOD2 may either reduce the amount of DNA damage caused by the drug or promote the repair of the DNA damage, which remains to be determined.

6. SUMMARY AND CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Summary and Conclusions

The focus of this research is to identify novel mechanisms that may drive tumorigenesis in *BRCA1*-related hereditary cancers. Understanding the functional consequences of BRCA1 loss can help to guide more effective prevention strategies and therapeutic options. Although BRCA1 protein has well characterized roles in genomic stability maintenance, we continue to discover additional regulatory mechanisms and downstream targets that may be exploited for better personalized medicine.

We found a novel correlation between BRCA1 status and the level of an essential mitochondrial protein, SOD2. We found depletion of BRCA1 in MCF7 cells caused a substantial increase in SOD2 protein level while *SOD2* mRNA was only slightly reduced. In order to test the functional relevance, we compared various BRCA1 proficient and null cells as well as mutant cells reconstituted with wild-type BRCA1. Through multiple assays, we learned that loss of BRCA1 promotes SOD2 protein accumulation in breast cancer cells.

By using KM plotter and TCGA data set of breast cancer patients, we identified low SOD2 mRNA is associated with good prognosis in all breast cancer patients but poor prognosis in patients with ER-negative or HER2-positve tumors, suggesting that SOD2 status may alter response to breast cancer chemotherapies. Indeed, we found that SOD2 influences cellular sensitivity to breast cancer chemotherapies in estrogen receptor (ER) negative breast cancer cell lines with high SOD2 levels. Specifically, depletion of SOD2 in two different ER-negative cell lines caused increased resistance to four different chemotherapeutic agents, whereas depletion of SOD2 in ER positive cell lines (MCF7 cells) appeared to have opposite effect. All of our results strongly suggest that the abundance of SOD2 in tumors can significantly impact therapy response in

patients. The results also suggest that increased SOD2 in BRCA1 null/mutant cells may be a contributing factor for positive therapy response.

Cumulatively, we report novel mechanisms of BRCA1 function in regulating redox homeostasis as well as a new function of SOD2 in ER-negative breast cancer in response to chemotherapies. Our findings may facilitate better understanding of the cellular signaling pathways that can be exploited for more effective and safer treatments.

6.2 Future Directions

Future work will be aimed at extending the ideas explored here to address several other relevant antioxidant protein levels and their response to *BRCA1* loss as well as addressing the mechanisms underlying SOD2 protein level accumulation after depletion of BRCA1. So, it will be interesting to know whether *BRCA1* loss has similar impact on protein expression level of other antioxidant proteins such as *NRF2* downstream targets including HMOX-1, GCLC and NQO1.

It will be important to explore whether BRCA1 regulates SOD2 protein level posttranslationally, or there are other factors that mediates its regulation in response to BRCA1 loss. Treatment with MG132, a proteasome inhibitor, will help addressing whether BRCA1 regulation of SOD2 protein is posttranslational or not. It would also be interesting to extend the current study to address the impact of other proteins such as p53 and its downstream target p21 on SOD2 protein levels following *BRCA1* loss, given the importance role of p53 and p21 in antioxidant response regulation that can enhance or reduce ROS levels. For example, we may test whether BRCA1 and p53 double knockdown can decrease or block SOD2 protein level. It may therefore be possible to provide an explanation why p53 and SOD2 protein levels are both upregulated in response to BRCA1 loss.

One of the more important future extensions of the work will involve the exploring why estrogen receptor negative (ER-) and HER2 amplified cell lines show more resistance to breast cancer chemotherapies following SOD2 depletion. So, it will be important to explore whether SOD2 depletion results in up regulating survival factors such as NFkB that prevent cells from undergoing apoptosis. Also, it will be important to explore whether SOD2 depletion enhances autophagy. Giving that autophagy is a crucial player for mitochondria quality control and in response to therapeutic stresses that also can enhance cellular resistance to chemotherapies. For example, testing the level of LC3-II, an autophagy marker, after SOD2 depletion following drug treatment could provide an explanation why ER- and HER2 positive cells become more resistance after SOD2 depletion. In addition, it will also be important to explore whether SOD2 depletion facilitates the repair of chemotherapy-induced DNA strand breaks by increasing DNA repair protein levels in the cells. Also, it will be important to investigate whether SOD2 depletion results in an induction of aerobic glycolysis, which is associated with therapeutic resistance.

BIBLIOGRAPHY

Acharya, A., I. Das, D. Chandhok and T. Saha (2010). "Redox regulation in cancer: a double-edged sword with therapeutic potential." Oxid Med Cell Longev **3**(1): 23-34.

Aprelikova, O. N., B. S. Fang, E. G. Meissner, S. Cotter, M. Campbell, A. Kuthiala, M. Bessho, R. A. Jensen and E. T. Liu (1999). "BRCA1-associated growth arrest is RB-dependent." Proc Natl Acad Sci U S A **96**(21): 11866-11871.

Bae, G. Y., S. J. Choi, J. S. Lee, J. Jo, J. Lee, J. Kim and H. J. Cha (2013). "Loss of E-cadherin activates EGFR-MEK/ERK signaling, which promotes invasion via the ZEB1/MMP2 axis in non-small cell lung cancer." Oncotarget **4**(12): 2512-2522.

Bae, I., S. Fan, Q. Meng, J. K. Rih, H. J. Kim, H. J. Kang, J. Xu, I. D. Goldberg, A. K. Jaiswal and E. M. Rosen (2004). "BRCA1 induces antioxidant gene expression and resistance to oxidative stress." Cancer Res **64**(21): 7893-7909.

Becuwe, P., M. Ennen, R. Klotz, C. Barbieux and S. Grandemange (2014). "Manganese superoxide dismutase in breast cancer: from molecular mechanisms of gene regulation to biological and clinical significance." Free Radic Biol Med **77**: 139-151.

Brodie, S. G., X. Xu, W. Qiao, W. M. Li, L. Cao and C. X. Deng (2001). "Multiple genetic changes are associated with mammary tumorigenesis in Brca1 conditional knockout mice." Oncogene **20**(51): 7514-7523.

Brouckaert, O., H. Wildiers, G. Floris and P. Neven (2012). "Update on triple-negative breast cancer: prognosis and management strategies." Int J Womens Health **4**: 511-520.

Buisson, R., A. M. Dion-Cote, Y. Coulombe, H. Launay, H. Cai, A. Z. Stasiak, A. Stasiak, B. Xia and J. Y. Masson (2010). "Cooperation of breast cancer proteins PALB2 and piccolo BRCA2 in stimulating homologous recombination." Nat Struct Mol Biol **17**(10): 1247-1254.

Buisson, R. and J. Y. Masson (2013). "[Functions of PALB2 and BRCA2 tumor suppressors in DNA double-strand break repair]." Med Sci (Paris) **29**(3): 301-307.

Bunting, S. F., E. Callen, N. Wong, H. T. Chen, F. Polato, A. Gunn, A. Bothmer, N. Feldhahn, O. Fernandez-Capetillo, L. Cao, X. Xu, C. X. Deng, T. Finkel, M. Nussenzweig, J. M. Stark and A. Nussenzweig (2010). "53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks." Cell **141**(2): 243-254.

Carlioz, A. and D. Touati (1986). "Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life?" EMBO J **5**(3): 623-630.

Chandel, N. S., D. S. McClintock, C. E. Feliciano, T. M. Wood, J. A. Melendez, A. M. Rodriguez and P. T. Schumacker (2000). "Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing." J Biol Chem **275**(33): 25130-25138.

Chapman, M. S. and I. M. Verma (1996). "Transcriptional activation by BRCA1." Nature **382**(6593): 678-679.

Chavez, K. J., S. V. Garimella and S. Lipkowitz (2010). "Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer." Breast Dis **32**(1-2): 35-48.

Cheang, M. C., D. Voduc, C. Bajdik, S. Leung, S. McKinney, S. K. Chia, C. M. Perou and T. O. Nielsen (2008). "Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype." Clin Cancer Res **14**(5): 1368-1376.

Chelikani, P., I. Fita and P. C. Loewen (2004). "Diversity of structures and properties among catalases." Cell Mol Life Sci **61**(2): 192-208.

Chen, C. F., S. Li, Y. Chen, P. L. Chen, Z. D. Sharp and W. H. Lee (1996). "The nuclear localization sequences of the BRCA1 protein interact with the importin-alpha subunit of the nuclear transport signal receptor." J Biol Chem **271**(51): 32863-32868.

Chen, W., Z. Sun, X.-J. Wang, T. Jiang, Z. Huang, D. Fang and D. D. Zhang (2009). "Direct Interaction between Nrf2 and p21Cip1/WAF1 Upregulates the Nrf2-Mediated Antioxidant Response." Molecular Cell **34**(6): 663-673.

Chen, W., Z. Sun, X. J. Wang, T. Jiang, Z. Huang, D. Fang and D. D. Zhang (2009). "Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response." Mol Cell **34**(6): 663-673.

Choudhury, A. D., H. Xu and R. Baer (2004). "Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression." J Biol Chem **279**(32): 33909-33918.

Cobbs, C. S., D. S. Levi, K. Aldape and M. A. Israel (1996). "Manganese superoxide dismutase expression in human central nervous system tumors." Cancer Res **56**(14): 3192-3195.

Conte, P., V. Guarneri and C. Bengala (2007). "Evolving nonendocrine therapeutic options for metastatic breast cancer: how adjuvant chemotherapy influences treatment." Clin Breast Cancer **7**(11): 841-849.

Copple, I. M., C. E. Goldring, N. R. Kitteringham and B. K. Park (2008). "The Nrf2-Keap1 defence pathway: role in protection against drug-induced toxicity." Toxicology **246**(1): 24-33.

Copple, I. M., C. E. Goldring, N. R. Kitteringham and B. K. Park (2008). "The Nrf2–Keap1 defence pathway: Role in protection against drug-induced toxicity." Toxicology **246**(1): 24-33.

Couch, F. J., S. N. Hart, P. Sharma, A. E. Toland, X. Wang, P. Miron, J. E. Olson, A. K. Godwin, V. S. Pankratz, C. Olswold, S. Slettedahl, E. Hallberg, L. Guidugli, J. I. Davila, M. W. Beckmann, W. Janni, B. Rack, A. B. Ekici, D. J. Slamon, I. Konstantopoulou, F. Fostira, A. Vratimos, G. Fountzilas, L. M. Pelttari, W. J. Tapper, L. Durcan, S. S. Cross, R. Pilarski, C. L. Shapiro, J. Klemp, S. Yao, J. Garber, A. Cox, H. Brauch, C. Ambrosone, H. Nevanlinna, D. Yannoukakos, S. L. Slager, C. M. Vachon, D. M. Eccles and P. A. Fasching (2015). "Inherited mutations in 17 breast cancer

susceptibility genes among a large triple-negative breast cancer cohort unselected for family history of breast cancer." J Clin Oncol **33**(4): 304-311.

Cruz-Garcia, A., A. Lopez-Saavedra and P. Huertas (2014). "BRCA1 accelerates CtIP-mediated DNA-end resection." Cell Rep **9**(2): 451-459.

Dang, C. V. (2012). "Cancer cell metabolism: there is no ROS for the weary." Cancer Discov **2**(4): 304-307.

De Lorenzo, S. B., A. G. Patel, R. M. Hurley and S. H. Kaufmann (2013). "The Elephant and the Blind Men: Making Sense of PARP Inhibitors in Homologous Recombination Deficient Tumor Cells." Front Oncol **3**: 228.

Di Paolo, A., C. Racca, P. Calsou and F. Larminat (2014). "Loss of BRCA1 impairs centromeric cohesion and triggers chromosomal instability." FASEB J **28**(12): 5250-5261.

Dine, J. and C. X. Deng (2013). "Mouse models of BRCA1 and their application to breast cancer research." Cancer Metastasis Rev **32**(1-2): 25-37.

Dziaman, T., T. Huzarski, D. Gackowski, R. Rozalski, A. Siomek, A. Szpila, J. Guz, J. Lubinski and R. Olinski (2009). "Elevated level of 8-oxo-7,8-dihydro-2'-deoxyguanosine in leukocytes of BRCA1 mutation carriers compared to healthy controls." Int J Cancer **125**(9): 2209-2213.

Evers, B. and J. Jonkers (2006). "Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects." Oncogene **25**(43): 5885-5897.

Feil, R., J. Wagner, D. Metzger and P. Chambon (1997). "Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains." Biochem Biophys Res Commun **237**(3): 752-757.

Ferguson, L. R., H. Chen, A. R. Collins, M. Connell, G. Damia, S. Dasgupta, M. Malhotra, A. K. Meeker, A. Amedei, A. Amin, S. S. Ashraf, K. Aquilano, A. S. Azmi, D. Bhakta, A. Bilsland, C. S. Boosani, S. Chen, M. R. Ciriolo, H. Fujii, G. Guha, D. Halicka, W. G. Helferich, W. N. Keith, S. I. Mohammed, E. Niccolai, X. Yang, K. Honoki, V. R. Parslow, S. Prakash, S. Rezazadeh, R. E. Shackelford, D. Sidransky, P. T. Tran, E. S. Yang and C. A. Maxwell (2015). "Genomic instability in human cancer: Molecular insights and opportunities for therapeutic attack and prevention through diet and nutrition." Semin Cancer Biol **35 Suppl**: S5-24.

Finn, R. S., M. Martin, H. S. Rugo, S. Jones, S. A. Im, K. Gelmon, N. Harbeck, O. N. Lipatov, J. M. Walshe, S. Moulder, E. Gauthier, D. R. Lu, S. Randolph, V. Dieras and D. J. Slamon (2016). "Palbociclib and Letrozole in Advanced Breast Cancer." N Engl J Med **375**(20): 1925-1936.

Ford, D., D. F. Easton, D. T. Bishop, S. A. Narod and D. E. Goldgar (1994). "Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium." Lancet **343**(8899): 692-695.

Forristal, C. E., K. L. Wright, N. A. Hanley, R. O. Oreffo and F. D. Houghton (2010). "Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions." Reproduction **139**(1): 85-97.

Futreal, P. A., Q. Liu, D. Shattuck-Eidens, C. Cochran, K. Harshman, S. Tavtigian, L. M. Bennett, A. Haugen-Strano, J. Swensen, Y. Miki and et al. (1994). "BRCA1 mutations in primary breast and ovarian carcinomas." Science **266**(5182): 120-122.

Gambino, V., G. De Michele, O. Venezia, P. Migliaccio, V. Dall'Olio, L. Bernard, S. P. Minardi, M. A. Della Fazia, D. Bartoli, G. Servillo, M. Alcalay, L. Luzi, M. Giorgio, H. Scrable, P. G. Pelicci and E. Migliaccio (2013). "Oxidative stress activates a specific p53 transcriptional response that regulates cellular senescence and aging." Aging Cell **12**(3): 435-445.

Gardini, A., D. Baillat, M. Cesaroni and R. Shiekhattar (2014). "Genome-wide analysis reveals a role for BRCA1 and PALB2 in transcriptional co-activation." EMBO J **33**(8): 890-905.

Gorrini, C., P. S. Baniasadi, I. S. Harris, J. Silvester, S. Inoue, B. Snow, P. A. Joshi, A. Wakeham, S. D. Molyneux, B. Martin, P. Bouwman, D. W. Cescon, A. J. Elia, Z. Winterton-Perks, J. Cruickshank, D. Brenner, A. Tseng, M. Musgrave, H. K. Berman, R. Khokha, J. Jonkers, T. W. Mak and M. L. Gauthier (2013). "BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival." J Exp Med **210**(8): 1529-1544.

Hall, J. M., M. K. Lee, B. Newman, J. E. Morrow, L. A. Anderson, B. Huey and M. C. King (1990). "Linkage of early-onset familial breast cancer to chromosome 17q21." Science **250**(4988): 1684-1689.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hart, P. C., M. Mao, A. L. de Abreu, K. Ansenberger-Fricano, D. N. Ekoue, D. Ganini, A. Kajdacsy-Balla, A. M. Diamond, R. D. Minshall, M. E. Consolaro, J. H. Santos and M. G. Bonini (2015). "MnSOD upregulation sustains the Warburg effect via mitochondrial ROS and AMPK-dependent signalling in cancer." Nat Commun **6**: 6053.

Harte, M. T., J. J. Gorski, K. I. Savage, J. W. Purcell, E. M. Barros, P. M. Burn, C. McFarlane, P. B. Mullan, R. D. Kennedy, N. D. Perkins and D. P. Harkin (2014). "NF-kappaB is a critical mediator of BRCA1-induced chemoresistance." Oncogene **33**(6): 713-723.

Hayes, J. D., M. McMahon, S. Chowdhry and A. T. Dinkova-Kostova (2010). "Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway." Antioxid Redox Signal **13**(11): 1713-1748.

Hudis, C. A. and L. Gianni (2011). "Triple-negative breast cancer: an unmet medical need." Oncologist **16 Suppl 1**: 1-11.

Jalal, S., J. N. Earley and J. J. Turchi (2011). "DNA repair: from genome maintenance to biomarker and therapeutic target." Clin Cancer Res **17**(22): 6973-6984.

Kaewpila, S., S. Venkataraman, G. R. Buettner and L. W. Oberley (2008). "Manganese superoxide dismutase modulates hypoxia-inducible factor-1 alpha induction via superoxide." Cancer Res **68**(8): 2781-2788.

Kahlos, K., S. Anttila, T. Asikainen, K. Kinnula, K. O. Raivio, K. Mattson, K. Linnainmaa and V. L. Kinnula (1998). "Manganese superoxide dismutase in healthy human pleural mesothelium and in malignant pleural mesothelioma." Am J Respir Cell Mol Biol **18**(4): 570-580.

Kamarajugadda, S., Q. Cai, H. Chen, S. Nayak, J. Zhu, M. He, Y. Jin, Y. Zhang, L. Ai, S. S. Martin, M. Tan and J. Lu (2013). "Manganese superoxide dismutase promotes anoikis resistance and tumor metastasis." Cell Death Dis **4**: e504.

Kang, H. J., Y. B. Hong, H. J. Kim, O. C. Rodriguez, R. G. Nath, E. M. Tilli, C. Albanese, F. L. Chung, S. H. Kwon and I. Bae (2011). "Detoxification: a novel function of BRCA1 in tumor suppression?" Toxicol Sci **122**(1): 26-37.

Kang, H. J., Y. B. Hong, H. J. Kim, A. Wang and I. Bae (2012). "Bioactive food components prevent carcinogenic stress via Nrf2 activation in BRCA1 deficient breast epithelial cells." Toxicol Lett **209**(2): 154-160.

Kaspar, J. W., S. K. Niture and A. K. Jaiswal (2009). "Nrf2:INrf2 (Keap1) signaling in oxidative stress." Free Radic Biol Med **47**(9): 1304-1309.

Kattan, Z., V. Minig, P. Leroy, M. Dauca and P. Becuwe (2008). "Role of manganese superoxide dismutase on growth and invasive properties of human estrogen-independent breast cancer cells." Breast Cancer Res Treat **108**(2): 203-215.

Kennedy, R. D. and A. D. D'Andrea (2006). "DNA repair pathways in clinical practice: lessons from pediatric cancer susceptibility syndromes." J Clin Oncol **24**(23): 3799-3808.

Kobayashi, E., T. Suzuki and M. Yamamoto (2013). "Roles nrf2 plays in myeloid cells and related disorders." Oxid Med Cell Longev **2013**: 529219.

Li, J. J., L. W. Oberley, D. K. St Clair, L. A. Ridnour and T. D. Oberley (1995). "Phenotypic changes induced in human breast cancer cells by overexpression of manganese-containing superoxide dismutase." Oncogene **10**(10): 1989-2000.

Liu, D. and Y. Xu (2011). "p53, oxidative stress, and aging." Antioxid Redox Signal **15**(6): 1669-1678.

Liu, X., H. Holstege, H. van der Gulden, M. Treur-Mulder, J. Zevenhoven, A. Velds, R. M. Kerkhoven, M. H. van Vliet, L. F. Wessels, J. L. Peterse, A. Berns and J. Jonkers (2007). "Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer." Proc Natl Acad Sci U S A **104**(29): 12111-12116.

Lois, C., E. J. Hong, S. Pease, E. J. Brown and D. Baltimore (2002). "Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors." Science **295**(5556): 868-872.

Ma, Q. (2013). "Role of nrf2 in oxidative stress and toxicity." Annu Rev Pharmacol Toxicol **53**: 401-426.

Martinez-Outschoorn, U. E., R. Balliet, Z. Lin, D. Whitaker-Menezes, R. C. Birbe, A. Bombonati, S. Pavlides, R. Lamb, S. Sneddon, A. Howell, F. Sotgia and M. P. Lisanti (2012). "BRCA1 mutations drive oxidative stress and glycolysis in the tumor microenvironment: implications for breast cancer prevention with antioxidant therapies." Cell Cycle **11**(23): 4402-4413.

Miao, L. and D. K. St Clair (2009). "Regulation of superoxide dismutase genes: implications in disease." Free Radic Biol Med **47**(4): 344-356.

Miar, A., D. Hevia, H. Munoz-Cimadevilla, A. Astudillo, J. Velasco, R. M. Sainz and J. C. Mayo (2015). "Manganese superoxide dismutase (SOD2/MnSOD)/catalase and SOD2/GPx1 ratios as biomarkers for tumor progression and metastasis in prostate, colon, and lung cancer." Free Radic Biol Med **85**: 45-55.

Micalizzi, D. S., S. M. Farabaugh and H. L. Ford (2010). "Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression." J Mammary Gland Biol Neoplasia **15**(2): 117-134.

Miki, Y., J. Swensen, D. Shattuck-Eidens, P. A. Futreal, K. Harshman, S. Tavtigian, Q. Liu, C. Cochran, L. M. Bennett, W. Ding and et al. (1994). "A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1." Science **266**(5182): 66-71.

Miller, C. J., S. S. Gounder, S. Kannan, K. Goutam, V. R. Muthusamy, M. A. Firpo, J. D. Symons, R. Paine, 3rd, J. R. Hoidal and N. S. Rajasekaran (2012). "Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle." Biochim Biophys Acta **1822**(6): 1038-1050.

Mills, G. C. (1957). "Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown." J Biol Chem **229**(1): 189-197.

Monteiro, A. N., A. August and H. Hanafusa (1996). "Evidence for a transcriptional activation function of BRCA1 C-terminal region." Proc Natl Acad Sci U S A **93**(24): 13595-13599.

Naithani, R. (2008). "Organoselenium compounds in cancer chemoprevention." Mini Rev Med Chem **8**(7): 657-668.

Papa, L., M. Hahn, E. L. Marsh, B. S. Evans and D. Germain (2014). "SOD2 to SOD1 switch in breast cancer." J Biol Chem **289**(9): 5412-5416.

Pogoda, K., A. Niwinska, M. Murawska and T. Pienkowski (2013). "Analysis of pattern, time and risk factors influencing recurrence in triple-negative breast cancer patients." Med Oncol **30**(1): 388.

Privat, M., N. Radosevic-Robin, C. Aubel, A. Cayre, F. Penault-Llorca, G. Marceau, V. Sapin, Y. J. Bignon and D. Morvan (2014). "BRCA1 induces major energetic metabolism reprogramming in breast cancer cells." PLoS One **9**(7): e102438.

Putti, T. C., D. M. El-Rehim, E. A. Rakha, C. E. Paish, A. H. Lee, S. E. Pinder and I. O. Ellis (2005). "Estrogen receptor-negative breast carcinomas: a review of morphology and immunophenotypical analysis." Mod Pathol **18**(1): 26-35.

Quiros, I., R. M. Sainz, D. Hevia, O. Garcia-Suarez, A. Astudillo, M. Rivas and J. C. Mayo (2009). "Upregulation of manganese superoxide dismutase (SOD2) is a common pathway for neuroendocrine differentiation in prostate cancer cells." Int J Cancer **125**(7): 1497-1504.

Reczek, C. R., M. Szabolcs, J. M. Stark, T. Ludwig and R. Baer (2013). "The interaction between CtIP and BRCA1 is not essential for resection-mediated DNA repair or tumor suppression." J Cell Biol **201**(5): 693-707.

Rezaei, M., K. Friedrich, B. Wielockx, A. Kuzmanov, A. Kettelhake, M. Labelle, H. Schnittler, G. Baretton and G. Breier (2012). "Interplay between neural-cadherin and vascular endothelial-cadherin in breast cancer progression." Breast Cancer Res **14**(6): R154.

Robbins, D. and Y. Zhao (2012). "Oxidative Stress Induced by MnSOD-p53 Interaction: Pro- or Anti-Tumorigenic?" J Signal Transduct **2012**: 101465.

Rodriguez, J. A. and B. R. Henderson (2000). "Identification of a functional nuclear export sequence in BRCA1." J Biol Chem **275**(49): 38589-38596.

Ruzankina, Y., C. Pinzon-Guzman, A. Asare, T. Ong, L. Pontano, G. Cotsarelis, V. P. Zediak, M. Velez, A. Bhandoola and E. J. Brown (2007). "Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss." Cell Stem Cell **1**(1): 113-126.

Saha, T., J. K. Rih and E. M. Rosen (2009). "BRCA1 down-regulates cellular levels of reactive oxygen species." FEBS Lett **583**(9): 1535-1543.

Salin, M. L., E. D. Day, Jr. and J. D. Crapo (1978). "Isolation and characterization of a manganese-containing superoxide dismutase from rat liver." Arch Biochem Biophys **187**(1): 223-228.

Savage, K. I. and D. P. Harkin (2015). "BRCA1, a 'complex' protein involved in the maintenance of genomic stability." FEBS J **282**(4): 630-646.

Sevilla, F., J. Lopez-Gorge, M. Gomez and L. A. Del Rio (1980). "Manganese superoxide dismutase from a higher plant : Purification of a new Mn-containing enzyme." Planta **150**(2): 153-157.

Sgambato, A., A. Camerini, P. Collecchi, C. Graziani, G. Bevilacqua, A. Capodanno, M. Migaldi, V. Masciullo, G. Scambia, G. Rossi, A. Cittadini and D. Amoroso (2009). "Cyclin E correlates with manganese superoxide dismutase expression and predicts survival in early breast cancer patients receiving adjuvant epirubicin-based chemotherapy." Cancer Sci **100**(6): 1026-1033.

Shakya, R., M. Szabolcs, E. McCarthy, E. Ospina, K. Basso, S. Nandula, V. Murty, R. Baer and T. Ludwig (2008). "The basal-like mammary carcinomas induced by Brca1 or Bard1 inactivation implicate the BRCA1/BARD1 heterodimer in tumor suppression." Proc Natl Acad Sci U S A **105**(19): 7040-7045.

Shinohara, A., H. Ogawa and T. Ogawa (1992). "Rad51 protein involved in repair and recombination in S. cerevisiae is a RecA-like protein." Cell **69**(3): 457-470.

Shuen, A. Y. and W. D. Foulkes (2011). "Inherited mutations in breast cancer genes--risk and response." J Mammary Gland Biol Neoplasia **16**(1): 3-15.

Taguchi, K., H. Motohashi and M. Yamamoto (2011). "Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution." Genes Cells **16**(2): 123-140.

Thakur, S., H. B. Zhang, Y. Peng, H. Le, B. Carroll, T. Ward, J. Yao, L. M. Farid, F. J. Couch, R. B. Wilson and B. L. Weber (1997). "Localization of BRCA1 and a splice variant identifies the nuclear localization signal." Mol Cell Biol **17**(1): 444-452.

Trovesi, C., N. Manfrini, M. Falcettoni and M. P. Longhese (2013). "Regulation of the DNA damage response by cyclin-dependent kinases." J Mol Biol **425**(23): 4756-4766.

Vizoso, F. J., L. O. Gonzalez, M. D. Corte, J. C. Rodriguez, J. Vazquez, M. L. Lamelas, S. Junquera, A. M. Merino and J. L. Garcia-Muniz (2007). "Study of matrix metalloproteinases and their inhibitors in breast cancer." Br J Cancer **96**(6): 903-911.

Vurusaner, B., G. Poli and H. Basaga (2012). "Tumor suppressor genes and ROS: complex networks of interactions." Free Radic Biol Med **52**(1): 7-18.

Wan, X. S., M. N. Devalaraja and D. K. St Clair (1994). "Molecular structure and organization of the human manganese superoxide dismutase gene." DNA Cell Biol **13**(11): 1127-1136.

Wang, T., G. Niu, M. Kortylewski, L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, W. Dalton, R. Jove, D. Pardoll and H. Yu (2004). "Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells." Nat Med **10**(1): 48-54.

Weisiger, R. A. and I. Fridovich (1973). "Superoxide dismutase. Organelle specificity." J Biol Chem **248**(10): 3582-3592.

Wheeler, M. D., M. Nakagami, B. U. Bradford, T. Uesugi, R. P. Mason, H. D. Connor, A. Dikalova, M. Kadiiska and R. G. Thurman (2001). "Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat." J Biol Chem **276**(39): 36664-36672.

Xia, B., Q. Sheng, K. Nakanishi, A. Ohashi, J. Wu, N. Christ, X. Liu, M. Jasin, F. J. Couch and D. M. Livingston (2006). "Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2." Mol Cell **22**(6): 719-729.

Yi, Y. W., H. J. Kang and I. Bae (2014). "BRCA1 and Oxidative Stress." Cancers (Basel) **6**(2): 771-795.

Zelko, I. N., T. J. Mariani and R. J. Folz (2002). "Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression." Free Radic Biol Med **33**(3): 337-349.

Zhang, F., J. Ma, J. Wu, L. Ye, H. Cai, B. Xia and X. Yu (2009). "PALB2 links BRCA1 and BRCA2 in the DNA-damage response." Curr Biol **19**(6): 524-529.

Zhang, H., K. Somasundaram, Y. Peng, H. Tian, H. Zhang, D. Bi, B. L. Weber and W. S. El-Deiry (1998). "BRCA1 physically associates with p53 and stimulates its transcriptional activity." Oncogene **16**(13): 1713-1721.

Zhang, J. (2013). "The role of BRCA1 in homologous recombination repair in response to replication stress: significance in tumorigenesis and cancer therapy." Cell Biosci **3**(1): 11.

Appendix A – Primer Sequences

Primers used for qRT-PCR

Primer Name	Sequence (5' - 3')	Strand
mSOD2-F1 Fwd	GGCCTACGTGAACAATCTCAAC	Sense
mSOD2-R1 Rev	CTGAAGAGCGACCTGAGTTGTAA	Antisense
mSOD2-F2 Fwd	ATCACACCATTTTCTGGACAAAC	Sense
mSOD2-R2 Rev	ACTTCTCAAAAGACCCAAAGTCA	Antisense
mSOD2-F3 Fwd	GCTCTAATCAGGACCCATTGC	Sense
mSOD2-R3 Rev	GTAGTAAGCGTGCTCCCACAC	Antisense
Trp53-F2 Fwd	TAGCTTCAGTTCATTGGGACCATC	Sense
Trp53-R2 Rev	TGCAACAGGCTTTGCAGAATGGA	Antisense
P21 Fwd	CAGGAGCAAAGTGTGCCG	Sense
P21 Rev	CAGAAGACCAATCTGCGC	Antisense
SOD2-1 Fwd	CTACGTGAACAACCTGAACGTC	Sense
SOD2-2 Rev	AACTTCAGTGCAGGCTGAAGAG	Antisense
SOD2-3 Fwd	AACGTCACCGAGGAGAAGTACC	Sense
SOD2-4 Rev	AACTTCAGTGCAGGCTGAAGAG	Antisense
SOD2-5 Fwd	ACAAATTGCTGCTTGTCCAAAT	Sense
SOD2-6 Rev	TAGTAAGCGTGCTCCCACACAT	Antisense

BRCA1 Fwd	GGGTAGTTAGCTATTTCTGGGTGA	Sense
BRCA1 Rev	GTTTCTTCCATTGACCACATCTC	Antisense
GCLC-1 Fwd	CTGGATGATGCTAATGAGTCTGA	Sense
GCLC-2 Rev	CTACTCTCCATCCAATGTCTGA	Antisense
GSTM-1 Fwd	CTGCTACAATCCAGAATTTGAG	Sense
GSTM-1 Rev	CTGAGTAGAGCTTTAGCTTTTCAG	Antisense
NQO1 Fwd	GCAGACCTTGTGATATTCCAGT	Sense
NQO1 Rev	GTCATACATGGCAGCGTAAGTG	Antisense
RPLP0 Fwd	ATCAACGGGTACAAACGAGTCCT	Sense
RPLP0 Rev	AGGCAGATGGATCAGCCAAGAAG	Antisense
GAPDH Fwd	GTCTCCTCTGACTTCAACAGCGAC	Sense
GAPDH Rev	CCAAATTCGTTGTCATACCAGGAA	Antisense
rplp0 Fwd	ACACTCCATCATCAATGGGTACA	Sense
rplp0 Rev	GAAGGCCTTGACCTTTTCAGTAA	Antisense
gapdh Fwd	ACTTCAACAGCAACTCCCACTCT	Sense
gapdh Rev	TTGCTGTAGCCGTATTCATTGTC	Antisense

Duplex siRNA used for gene silencing

Primer Name	**Sequence (5' - 3')**
Non-specific control (NSC) (si-Control)	UUCGAACGUGUCACGUCAAdTdT
Qiagen AllStars Negative Control siRNA	Catalog # SI03650318
Sigma MISSION Universal Negative Control #2	Catalog # SIC002
SOD2 (240)	CCGACCUGCCCUACGACUA
SOD2 (531)	GUUCCUUUGACAAGUUUAA
SOD2 (655)	GGAUCCACUGCAAGGAACA
SOD2 (787)	GAAUGUAACUGAAAGAUAC
SOD2 (800)	AGAUACAUGGCUUGCAAAA
BRCA1 (296)	GGAACCUGUCUCCACAAAGdTdT
BRCA1 (452)	CAAGAAAGUACGAGAUUUAdTdT
BRCA1 (716)	GUGAGAACUCUGAGGACAA
BRCA1 (4459)	GGAAAUGGCUGAACUAGAA
BRCA1 (4862)	CCACACGAUUUGACGGAAA
BRCA1 (5361)	GAAAAUGGGUAGUUAGCUA
BRCA1 (6252)	GGAUCGAUUAUGUGACUUAdTdT
BRCA1 (5900)	CAACAGAAAGGGUCAACAA
NRF2 (786)	UGAAGAGACAGGUGAAUUUdTdT
NRF2 (1095)	UGACAGAAGUUGACAAUUAdTdT

NRF2 (1834)	GAGAAAGAAUUGCCUGUAAdTdT
KEAP1 (1495)	ACAACAGUGUGGAGAGGUAdTdT
KEAP1 (1803)	GAAACAGAGACGUGGACUUdTdT

Primers used for genotyping mouse genomic DNA

Primer Name	**Sequence (5' - 3')**	**Strand**
Brca1 Fwd 5' loxp	AGGTACCAGTTATGAGTTAGTCGTGTGTGCCTGAGTCA	Sense
Brca1 Rev 5' loxp	GCTGAGATTAAAGTGCAGGCCACCACACTCAGTGAT	Antisense
Brca1 Fwd 3' loxp	GCTGTGACATATTCTTACTTCGTGGCACATCTCTCA	Sense
Brca1 Rev 3' loxp	GGCTACCTATAACTACTCTCTAACAACGAAGTGCAA	Antisense
Brca1 Del 5-13 Fwd	AGGTACCAGTTATGAGTTAGTCGTGTGTGCCTGAGTCA	Sense
Brca1 Del 5-13 Rev	GGCTACCTATAACTACTCTCTAACAACGAAGTGCAA	Antisense