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The Tumor Suppressor BRCA1 in IP_3R Calcium Signaling and Apoptosis

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The Tumor Suppressor BRCA1 in IP_3R Calcium Signaling and Apoptosis

by

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Dedication

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The Tumor Suppressor BRCA1 in IP_3R Calcium Signaling and Apoptosis

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The inositol 1,4,5-trisphosphate receptor (IP_3R) is a ubiquitously expressed endoplasmic-reticulum (ER)-resident calcium channel. Calcium release mediated by IP_3Rs influence many signaling pathways including those regulating apoptosis. IP_3R activity is regulated by protein-protein interactions, including binding to proto-oncogenes and tumor suppressors to regulate cell death. Here we show that the IP_3R binds to the tumor suppressor BRCA1. BRCA1 binds directly to the IP_3R and causes destabilization of the IP_3R closed state resulting in an increased open probability and increased calcium release. BRCA1 is recruited to the ER during apoptosis in an IP_3R-dependent manner, and in addition, a pool of BRCA1 protein is constitutively associated with the ER under non-apoptotic conditions. This is likely mediated by a novel lipid binding activity of the first BRCT (BRCA1 C-Terminus) domain of BRCA1. Lastly, phosphatidic acid, which is was identified in a lipid binding screen, is produced at the ER during apoptosis suggesting a mechanism for apoptotic recruitment of BRCA1 to the ER. These findings provide a mechanistic explanation by which BRCA1 can act as a pro-apoptotic protein.

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List of Abbreviations

53BP1	p53 Binding Protein-1
ACCA	Acetyl-CoA Carboxylase alpha
ATM/ATR	Ataxia Telangiectasia Mutated/Ataxia Telangiectasia and Rad3 related
ATP	Adenosine trisphosphate
BARD1	BRCA1 Associated Ring Domain-1
BRCA1	Breast and Ovarian Cancer Susceptibility Gene-1
BRCT	BRCA1 C-Terminal Domain
CFP	Cyan Fluorescent Protein
CICR	Calcium Induced Calcium Release
CytCOx	Cytochrome-c Oxidase
DAG	Diacylglycerol
DGK	Diacylglycerol Kinase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
DT40 TKO	DT40 IP_3R Triple Knock Out
DT40 WT	DT40 IP_3R Triple Wild Type
ER	Endoplasmic Reticulum
FABP	Fatty Acid Binding Protein
FRET	Förster Resonance Energy Transfer
GFP	Green Fluorescent Protein
GST	Glutathione S-Transferase
HBOC	Human Breast and Ovarian Cancer Syndrome
HR	Homologous Recombination
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate Receptor
JNK	c-Jun N-Terminal Kinase
LDH	Lactate Dehydrogenase
MB	Myc Binding Protein
MAM	Mitochondria Associated Membrane
MCU	Mitochondrial Calcium Uniporter
NHEJ	Non-homologous End Joining
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NMR	Nuclear Magnetic Resonance
PA	Phosphatidic Acid
PASS	Phosphatidic Acid biosensor with Superior Sensitivity
pBRCA1	Phospho-BRCA1
PDB	Protein Databank
PI3 kinase	Phosphatidylinositol-4,5-bisphosphate kinase

PIP2	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase C
PLD	Phospholipase D
PML	Promyelocytic Leukemia Protein
PP2A	Protein Phosphatase 2A
PSSM	Position Specific Scoring Matrix
PTEN	Phosphatase and Tensin Homolog
PTX	Paclitaxel
Rb	Retinoblastoma protein
RING	Really Interesting New Gene
SCD	Serine Cluster Domain
siRNA	Small Interfering Ribonucleic Acid
Ub	Ubiquitin
VDAC	Voltage-Dependent Anion Channels
VMD	Visual Molecular Dynamics
YFP	Yellow Fluorescent Protein

Chapter 1: Introduction

IP_3R and BRCA1 Structure and Function in Cancer

Modified in part from:

Structure-Function of the Tumor Suppressor BRCA1

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INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR (IP_3R)

The Inositol-1,4,5-trisphosphate receptor (IP_3R) channel is responsible for calcium signaling from the endoplasmic reticulum (ER) in all cells, and the primary calcium release channel in non-muscle cells. Calcium is released when IP_3R is activated by its ligand IP_3. When activated the channel opens and to becomes permeable to calcium ions. The IP_3R releases calcium from the main cellular calcium store, the endoplasmic reticulum (100uM -700uM Ca^{2+}) down its concentration gradient into the cytosol (50nM-100nM Ca^{2+}) (1). IP_3R calcium release is tightly regulated by cytosolic concentrations of its ligands IP_3, calcium, and ATP as well as post-translational modifications and protein-protein interactions. IP_3R regulation is critical for regulation of the magnitude, frequency, and the temporal and spatial location of calcium release events as these calcium signals affect a variety of cellular outcomes. IP_3R-mediated calcium release affects activation of multiple signaling pathways including but not limited to gene transcription, metabolism, cell proliferation, and apoptosis (2).

The IP_3R channel is a tetramer comprised of four IP_3R subunits. Each subunit is approximately 310 kD, making the assembled channel 1.2 MD (3,4). The channel can be a homo or hetero-tetramer comprised of any combination of three IP_3R subunit isoforms (IP_3R1-3). Each isoform is encoded by an individual gene (ITPR1-3). The protein structure and regulatory mechanisms controlling IP_3R activity have been extensively investigated by multiple research groups and these studies have proven to be critically important for understanding of the IP_3R intracellular function in health and disease.

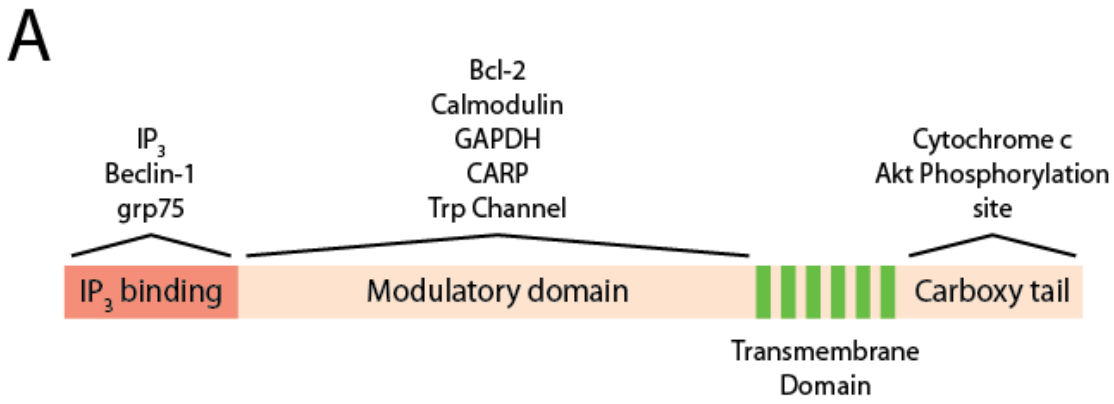
IP_3R Localization

IP_3Rs are primarily localized to the ER, with some presence in the Golgi apparatus, the nuclear envelope and possibly the plasma membrane. IP_3R is also present at mitochondria-associated membranes (MAMs). MAMs are portions of the ER that are in close proximity to mitochondria (10-15nm) (5). IP_3R enrichment at MAMs allows for direct transfer of calcium ions between ER and mitochondria, as well as for the formation of calcium signaling microdomains, or "hotspots", between these two organelles. This is significant in the context of apoptosis, as a large and direct transfer of calcium from ER to mitochondria can cause mitochondrial calcium overload, permeabilization of the outer mitochondrial membrane and release of apoptotic factors (see below). Due to rapid diffusion of ions away from the mouth of the IP_3R into the surrounding cytosol and calcium buffering proteins, calcium concentrations at the mouth of an IP_3R channel can be up to 100X higher than a few microns away. This allows for formation of calcium signaling microdomains and therefore, location-specific calcium signals (1).

IP_3R Structure

The three-dimensional structure of the IP_3R has been studied by several methods (6–11). Due to technical difficulties and potential conformational changes depending on buffer components (i.e. calcium or IP_3), not all structures are in agreement (9,12). However, a general consensus is that the IP_3R channel consists of a large cytoplasmic domain (75-85% of the total protein), a transmembrane pore and a small luminal region comprised of the loops connecting the transmembrane helices (1,7,11). The channel pore consists of transmembrane helices contributed by each IP_3R monomer, creating a four-fold axis of symmetry. Each IP_3 monomer consists of an IP_3 binding domain, a large

coupling/modulatory domain, the pore forming domain and a carboxy-terminal tail domain (Ill 1-1).



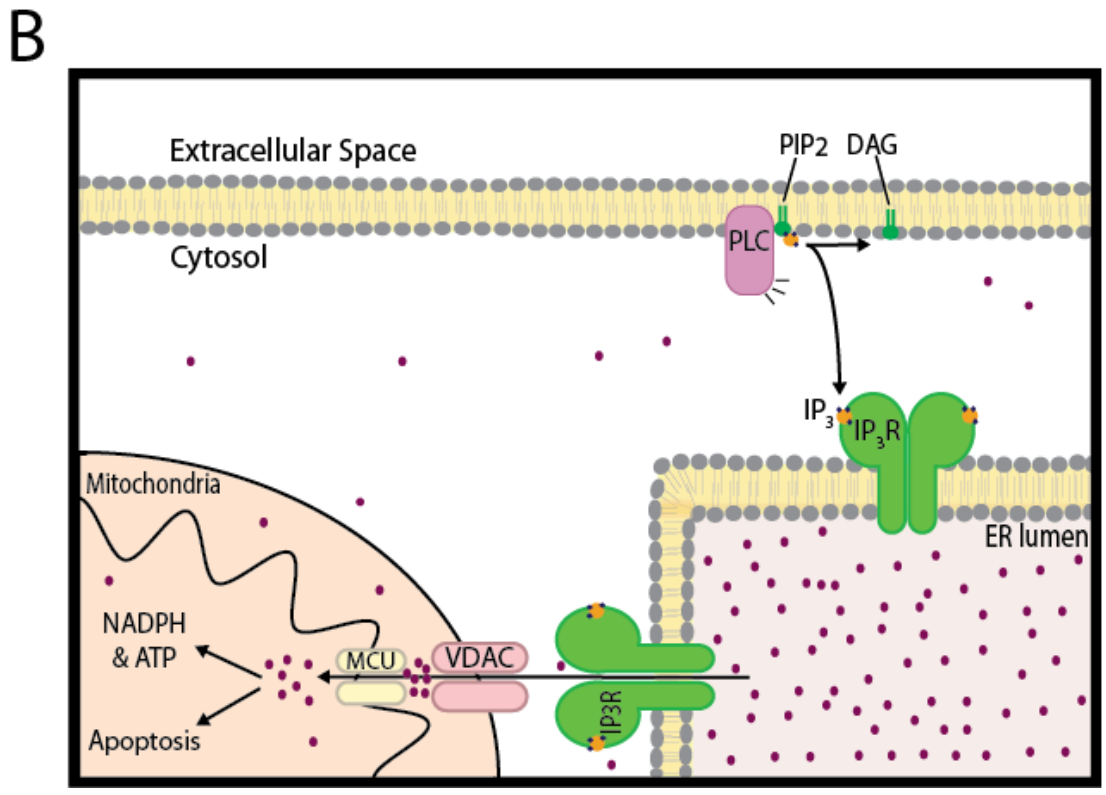


Illustration 1-1. IP_3R structure and function.

A) Each IP_3R subunit consists of an IP_3 binding domain, a modulatory domain, a pore-forming domain, and a carboxy-tail domain. Each domain carries out a different function and contains binding sites for multiple proteins (listed above each domain). B) The IP_3R is activated by its ligand IP_3 to release calcium from the ER into the cytosol. IP_3Rs that are localized to MAMs are in close proximity to mitochondria and are linked physically to VDAC through tethering by grp75. Calcium can be transferred directly from the ER to the mitochondria at these MAMs IP_3R, VDAC, and MCU.

IP_3 Binding Domain

The N-terminal IP_3 binding domain spans amino acids 1-578. This domain can be split into the suppressor subdomain (aa 1-225) and the core IP_3 binding subdomain (aa 276-578) (13). Each core IP_3 binding domain binds one IP_3 molecule with a kD ~10-80nM. A basic pocket including Arg265, Lys508, and Arg 511 is required for IP_3 binding (13). The suppressor domain decreases the IP_3 binding affinity of the core IP_3 binding domain. However, deletion of the suppressor domain causes complete inhibition of IP_3R activity (13). To resolve this controversy, it has been suggested that the suppressor domain may couple the IP_3 binding activity into gating activity. Therefore, the suppressor domain inhibits IP_3 binding but is necessary for channel activity. It has been suggested that IP_3 binding and gating functions may be physically linked by the suppressor domain. This may occur through interaction between the suppressor domain and the pore domain, specifically the linker between transmembrane helices S4 and S5 (14).

IP_3R Modulatory Domain

The modulatory domain is the largest IP_3R domain consisting of the region between the IP_3 binding domain and the pore forming domain and includes 1700 amino acids (aa 586-2276). This domain is the site of many regulatory interactions including Bcl-2, calmodulin, GAPDH, CARP, and TRP channels (reviewed in (1) and (2)). IP_3R regulatory

proteins modulate IP_3R activity by tuning the amplitude and/or frequency of calcium releases to the needs of the cell. Due to the modulation of the IP_3R by many proteins, the IP_3R acts as an integrator of multiple signaling pathways. The signals of these pathways converge at the IP_3R and one integrated calcium signal is produced. Likewise, many mutations and deletions in this domain have sizable effects on the function of the channel (15–17).

IP_3R Carboxy-Terminal Domain

The carboxy-terminal tail domain is comprised of the region between the last transmembrane helix and the carboxy-terminus of the IP_3R (150aa). This domain contains sites for several protein-protein interactions including Bcl-XL, cytochrome c, and Huntingtin, Huntingtin Associated Protein-1 (HAP1), and contains a cleavage site for caspase-3 (reviewed in (1), (18–21)). Many of these interactions increase the sensitivity of the IP_3R. This suggests an important role for the carboxy-terminal tail domain in the regulation of channel activity.

IP_3R Function

IP_3R gating is regulated for the most part by two ligands, calcium and IP_3 (1). The channel is regulated biphasically by calcium. Increasing doses of IP_3 increase channel activity while calcium regulates the activity of the IP_3R biphasically. If saturating doses of IP_3 are present (10μM), very low and very high concentrations of calcium inhibit the channel, while concentrations from about 500nM-10uM activate the channel with an open probability of 0.8 (22). If subsaturating levels of IP_3 are present (10nM), the IP_3R is more sensitive to calcium inhibition, resulting in a more narrow range of activating calcium

concentrations, about 100nM-200nM. These concentrations activate the channel with a maximum open probability near 0.2 (23). Thus, increased IP_3 concentrations make the channel less sensitive to calcium inhibition, resulting in higher open probability and, therefore, more robust calcium release. This may be due to a conformational change caused by IP_3 binding that disrupts or masks a calcium binding site (24). ATP also modulates IP_3R activity by allosterically sensitizing the IP_3R to calcium, thus lowering the calcium concentration required to activate the channel. This effect is isoform specific. (25).

Biphasic regulation of IP_3R by calcium allows for several types of calcium signals. Elementary calcium signals are caused by opening of individual IP_3Rs (blip) or a cluster of IP_3Rs (puff) (26). Individual channels or clusters of IP_3Rs can influence adjacent IP_3Rs through calcium-induced calcium release (CICR), when the calcium released by a single IP_3R or a cluster of IP3Rs activates adjacent IP_3Rs, resulting in calcium waves. High calcium concentrations at an initially open IP_3R causes channel closure as the CICR calcium wave propagates across the surface of the ER (27).

While very similar, each isoform has a slightly different affinity for ligands and each isoform may be regulated differently by regulatory proteins such as kinases, phosphatases, and binding proteins (1,28). The cell type-specific expression profile and the subcellular localization may be different between IP_3R isoforms. Furthermore, multiple splice variants have been identified for each isoform allowing for great diversity of isoform/spliceoform variability in each IP_3R tetramer (29,30). Potential diversity of IP_3Rs based on tetramer makeup as well as expression levels, splicing, localization, and the regulatory effects of various IP_3R binding proteins and calcium binding proteins produce a wide variety of potential calcium signaling outcomes. The resulting calcium signals are

unique in their frequency, amplitude, and location (temporal and spatial). The overall "fingerprint" of each calcium release is interpreted by the cell machinery into a message for a variety of cellular outcomes including apoptosis.

IP_3R and Apoptosis

IP_3R has an undoubtable central role in apoptosis. IP_3R-null cells are resistant to several types of apoptotic stimuli (31). Numerous pro-apoptotic and pro-survival signals converge at the IP_3R and are interpreted and translated into one calcium signal (2). IP_3R-mediated pro-apoptotic calcium signals can affect multiple pathways however, a major effect is direct calcium transfer from ER to mitochondria at MAMs. IP_3Rs are linked physically to the mitochondrial channel VDAC through tethering by the protein grp75 (32). Calcium uptake across the mitochondrial inner membrane is mediated by mitochondrial calcium uniporter (MCU) (reviewed in (33)). While small calcium transfers are pro-survival and are required by mitochondrial enzymes for NADPH production and therefore ATP production, large calcium transfers cause mitochondrial calcium overload (33,34). Calcium overload causes loss of mitochondrial membrane potential, mitochondrial outer membrane permeabilization, and release of pro-apoptotic factors including cytochrome c (35). Mitochondrial outer membrane permeabilization may determine the fate of a cell by committing it to the apoptotic pathway (36,37). Many pro-apoptotic stimuli require an intact calcium signaling network in order to induce apoptosis (reviewed in (38,39)). Many proteins involved in apoptotic signaling regulate IP_3R activity. Important proteins involved in apoptotic signaling that regulate IP_3R activity include Bcl-2, Bcl-XL, cytochrome c, and p53. These proteins regulate IP_3R activity by regulating the size and or frequency of IP_3R calcium releases. In other words, these proteins IP_3R activity to the correct amplitude or

frequency required for either promotion of or protection from apoptosis. The involvement of IP_3R regulation in the signaling networks of these important pro- and anti-apoptotic proteins highlights the importance of the IP_3R and IP_3R calcium release in apoptotic signaling. Altered calcium signaling caused by deregulated IP_3R-mediated calcium signaling in cancer cells may allow cells to overcome stress and allow for survival and growth.

IP_3R and Cancer

Due to the integration of IP_3R in apoptotic processes and the high influence of IP_3R calcium signaling on apoptotic progress, it seems inevitable that tumor suppressors and oncogenes target and regulate IP_3R function. Under normal conditions, tumor suppressors tend to support apoptotic progression and inhibit proliferation and tumorigenesis. However, when tumor suppressors are deregulated, by multiple possible mechanisms, anti-apoptotic signals and proteins are left unchecked. Tumor suppressors including PML, Beclin-1, PTEN and p53 regulate the IP_3R either directly (Beclin-1, PTEN) or indirectly (PML, p53). While their interaction with IP_3R is typically not the only function of these tumor suppressors, it is clear that the IP_3R has a strong role in multiple tumor suppressor pathways. Interestingly, some reports also show that IP_3R expression and localization may be related to cancer growth and regulation (40–43).

Promyelocytic Leukemia Protein (PML)

The Promyelocytic Leukemia Protein (PML) is a tumor suppressor that indirectly regulates IP_3R function to increase IP_3R activity. In the nucleus, PML is present in structures called nuclear bodies where PML acts as a scaffold for several proteins including

PTEN, p53, PP2A, and Akt. PML acts as a switch to initiate activation of pro-apoptotic proteins and suppress anti-apoptotic proteins through its scaffolding function (44). When shuttled into the cytosol, PML also interacts with IP_3R indirectly through a multi-protein complex (45). In the absence of PML, Akt phosphorylates the IP_3R which reduces calcium release activity and allows only enough ER-mitochondria calcium transfer to promote NADPH and therefore, ATP production and survival (reviewed in (46)). PML recruits PP2A to the site of the IP_3R/Akt interaction. PP2A dephosphorylates the phosphorylated IP_3R to increase IP_3R activity and allows increased calcium transfer from ER to mitochondria. PML can be inactivated, mainly by chromosomal translocations which cause deregulations of several pathways including apoptosis. PML deficiency may cause hyperphosphorylation of IP_3Rs due to the lack of PP2A to counteract the phosphorylation activity of Akt. Hyperphosphorylated IP_3Rs are less active and transfer less calcium to the mitochondria which would make PML-deficient cells resistant to apoptotic stimuli (46,47). In this case, PML acts as a pro-apoptotic scaffold to recruit an IP_3R regulatory protein, PP2A, into proximity of the IP_3R.

BECLIN-1

The tumor suppressor Beclin-1 is required for both induction and execution of autophagy (48). Beclin-1 heterozygous mice show decreased levels of cellular autophagy, increased cellular proliferation and increased tumor number (49). Beclin-1 gene deletion or Beclin-1 protein down-regulation has been seen in several types of human cancers and cancer cell lines (50). Beclin-1 has two unique interactions, one direct and one indirect. Beclin-1 interacts directly with the IP_3R IP_3 binding domain. Beclin-1 also indirectly interacts with the IP_3R by competing for Bcl-2 binding (51,52). Pro-survival Bcl-2

decreases IP_3R-dependent apoptosis, therefore removal of Bcl-2 from IP_3Rs would remove this pro-survival signal (reviewed in (46)). During starving conditions, the Beclin1/IP3R interaction seems to increase, causing increased calcium signaling and increased autophagic flux (52). Due to the double-edged relationship between autophagy and apoptosis, the effect of the Beclin-1/IP_3R interaction on apoptosis is slightly unclear at this time. However, it is clear that the tumor suppressor Beclin-1 has an effect on IP_3R calcium signaling and therefore most likely has an effect on apoptosis.

P53

The p53 tumor suppressor is mutated in 50% of all tumors. Additionally, the majority of the remaining tumors have other genetic alterations that cause down regulation of p53 function or expression (53). This is not surprising due to the array of pro-apoptotic and anti-proliferative functions of p53. p53 is considered the caretaker of the genome and acts as a major sensor of cellular stress through many pathways, both internal (e.g. DNA damage) and external (e.g. chemotherapeutic drugs) (reviewed in (53,54)). Under non-apoptotic conditions, p53 is present at very low levels in the cell due to inhibition by the protein Mdm2. Mdm2 promotes proteasomal degradation of p53 through ubiquitination (55). Under stress conditions, inhibition of p53 is released, allowing p53 to accumulate and promote cell death or cell cycle arrest primarily through transcriptional activation.

p53 affects IP_3R indirectly through its interaction with Akt. Under non-apoptotic conditions Akt is phosphorylated and activated by PI3 kinase (56). Phosphorylated Akt phosphorylates other pro-survival substrates, including IP_3R and Mdm2. Akt phosphorylation of IP_3R causes inhibition of the channel, allowing only small releases of calcium that fuel mitochondrial metabolism. Akt-mediated phosphorylation of the IP_3R

prevents pro-apoptotic calcium overload (46). Akt-mediated phosphorylation of Mdm2 increases its inhibitory effect on p53. However, when a cell is stressed and p53 stabilizes and accumulates, p53 inhibits the pro-survival activity of the PI3 kinase/Akt pathway and therefore IP_3R activity.

Stabilization and accumulation of p53 has three separate but related effects on IP_3R activity (Ill 1-2). First, p53 activates transcription of PTEN, which inhibits PI3 kinase (57). Inhibition of PI3 kinase and consequently, inhibition of Akt blocks phosphorylation of IP_3R. Decreased Akt-mediated phosphorylation of IP_3R would allow for increased ER-mitochondria calcium transfer and potential apoptotic mitochondrial overload. Second, p53 causes activation of executioner caspase-3. Caspase-3 has many cleavage substrates including Akt and IP_3R. While the implication of degradation of Akt results in loss of the kinase function and therefore decreased Akt-mediated IP_3R phosphorylation, the physiological importance of caspase-3 cleavage of IP_3R is debatable as only a small fragment is cleaved (21,58). Third, p53 activates transcription of cyclin G, which recruits the phosphatase PP2A to Mdm2 to reverse the Akt-mediated phosphorylation of Mdm2. This results in inhibition of the p53inhibitor, Mdm2 (59). While this inhibition does not have any direct effect on IP_3R activity, stabilization and accumulation of p53 results in increased PTEN expression and caspase-3 activation. It has been suggested that p53-induced down regulation of Akt signaling may irreversibly commit a cell apoptotic cell death (60). Lack of p53 expression due to mutation, deletion, or down regulation would leave the PI3K/Akt pathway unchecked, causing Akt-mediated IP_3R hyperphosphorylation and thus preventing large ER-mitochondria calcium transfers and mitochondrial calcium overload.

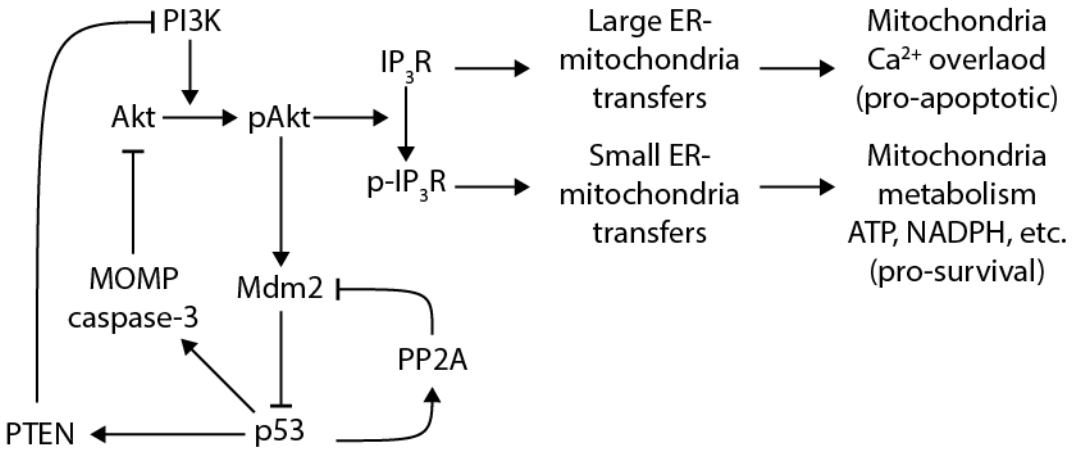


Illustration 1-2. The effect of p53 on the PI3 kinase/Akt pathway.
P53 stabilization and accumulation negatively regulates the PI3 kinase/Akt pathway. Depending on the Akt-mediated phosphorylation status of the IP_3R, two outcomes are possible. Loss of Akt-mediated phosphorylation allows for large ER-mitochondria calcium transfers that lead to mitochondrial calcium overload and apoptosis. Akt-mediated hyperphosphorylation allows for small ER-mitochondria transfers that lead to mitochondrial metabolism, ATP production, and cell survival.

PHOSPHATASE AND TENSIN HOMOLOG (PTEN)

The phosphatase and tensin homolog protein, or PTEN, is a lipid and protein phosphatase and a tumor suppressor. PTEN mutations associate with many cancers and cancer predisposition syndromes making it one of the most common cancer mutations after p53 (61). The PTEN tumor suppressor function is traditionally considered to be carried out through its inhibition of the PI3 kinase/Akt pathway (described above). However, new evidence has emerged suggesting another pro-apoptotic function for PTEN through direct modulation of IP_3R3 (62). PTEN dephosphorylates Akt-mediated phosphorylation of IP_3R3. This is dependent on the protein phosphatase activity of PTEN and independent of the PTEN lipid phosphatase activity. Removal of Akt-mediated phosphorylation by PTEN causes increased calcium transfer from ER to mitochondria and increased apoptosis as measured by cleaved caspase-3. This direct effect on IP_3R3 combined with its upstream effect on the PI3 kinase /Akt pathway (as described above) suggests that the tumor suppressor PTEN could have a large effect on apoptotic IP_3R calcium signaling. Clearly, deregulation of PTEN expression or activity would lead to increased Akt-mediated phosphorylation, hyperphosphorylation of IP_3R and decreased ER-mitochondrial calcium transfer, allowing for evasion of apoptosis and cell survival.

OTHER TUMOR SUPPRESSORS

It has been known for many years that the IP_3R is central to apoptotic initiation and progression. The relatively recent discoveries of tumor suppressors that interact with IP_3Rs makes apoptotic IP_3R calcium signaling even more relevant to human health. With the number of currently known tumor suppressors that act at least partially through regulation of IP_3R calcium signaling, it is conceivable that other tumor suppressors may share the

same target. In the following chapters we will show that the tumor suppressor BRCA1 also interacts both physically and functionally with the IP_3R to increase the sensitivity of the channel to IP_3 and to increase apoptotic calcium release.

BREAST AND OVARIAN CANCER SUSCEPTIBILITY GENE-1 (BRCA1)

Clinical Significance of BRCA1 in Breast and Ovarian Cancers

Hereditary Breast and Ovarian Cancer (HBOC) is a syndrome resulting in an increased lifetime risk for developing breast and/or ovarian cancer. The genetic basis of HBOC is an inherited germline mutation in one allele of either the BRCA1 or BRCA2 genes and subsequent loss of heterozygosity in somatic tissues (63). Some of the trademarks of this syndrome include multiple family members with breast and/or ovarian cancer, personal history of both breast and ovarian cancer, development of breast or ovarian cancer at an early age, and family or personal history of male breast cancer (63).

Mutations in BRCA1 and BRCA2 are responsible for the majority of HBOC cases while 10% of sporadic ovarian cancer cases and 3-5% of sporadic breast cancer cases are associated with BRCA1 or BRCA2 mutations (63). In the presence of a BRCA1 mutation, women have a 70-80% lifetime risk of developing breast cancer and a 50% risk of developing ovarian cancer. Women carrying a BRCA2 mutation have a 50-60% lifetime risk of developing breast cancer and a 30% risk of developing ovarian cancer (64). BRCA1 and BRCA2 genes belong to the tumor suppressor gene family for their capacity to repair damaged DNA through a process known as DNA double-strand break repair (65). Therefore, an inherited mutation in either of these genes combined with loss of heterozygosity predisposes cells to chromosomal instability and greatly increases the probability of malignant transformation and cancer development. Interestingly, multiple

other potential functions have been proposed for the BRCA1 and BRCA2 proteins that may have an impact on their tumor suppressor function (66).

The management of HBOC syndrome is an evolving area, and clearly much more research is needed to understand the molecular basis of cancer progression in these patients. The linkage of BRCA1 and BRCA2 to early-onset hereditary breast cancer was discovered in 1990 and 1994, respectively (67,68). Since then, BRCA genotyping is now used to determine patient counseling, management decisions, and prognosis of this syndrome (69). However, inconsistent and limited data exist regarding the clinical course of BRCA-mutated patients after cancer develops (70). A published meta-analysis for BRCA1-related tumors reported a worse outcome among the breast cancer patients carrying a mutated BRCA gene (69), while BRCA1 mutated ovarian cancer patients had a more favorable clinical outcome (71). Other studies have reported that both BRCA1-mutated breast and ovarian tumors have a better outcome (70,72). This is likely due to increased sensitivity of BRCA mutated cells to chemotherapeutics targeting DNA, such as anti-metabolites, alkylating agents, and topoisomerase inhibitors (73). However, more research into the molecular basis by which the BRCA proteins functions as a tumor suppressor and the clinical significance is clearly needed.

Over 1700 unique BRCA1 mutations have been reported to the Breast Cancer Information Core Database (74). Of these mutations, 858 have been confirmed as being "clinically significant." Clinically significant mutations cause an increased risk of cancer and result in a protein with reduced function or no protein product. Three domains of the BRCA1 protein are mutated in cancer patients with relatively high frequency. These domains include the RING domain (exons 2-7), a region encoded by exons 11-13, and the

BRCT domain (exons16-24) (Ill. 1-3). The RING domain functions as an E3 ubiquitin ligase. The amino acids encoded by exons 11-13 contain protein binding domains for a number of diverse proteins. The BRCT domain is a phosphoprotein binding domain with specificity for proteins phosphorylated by ATM/ATR kinases.

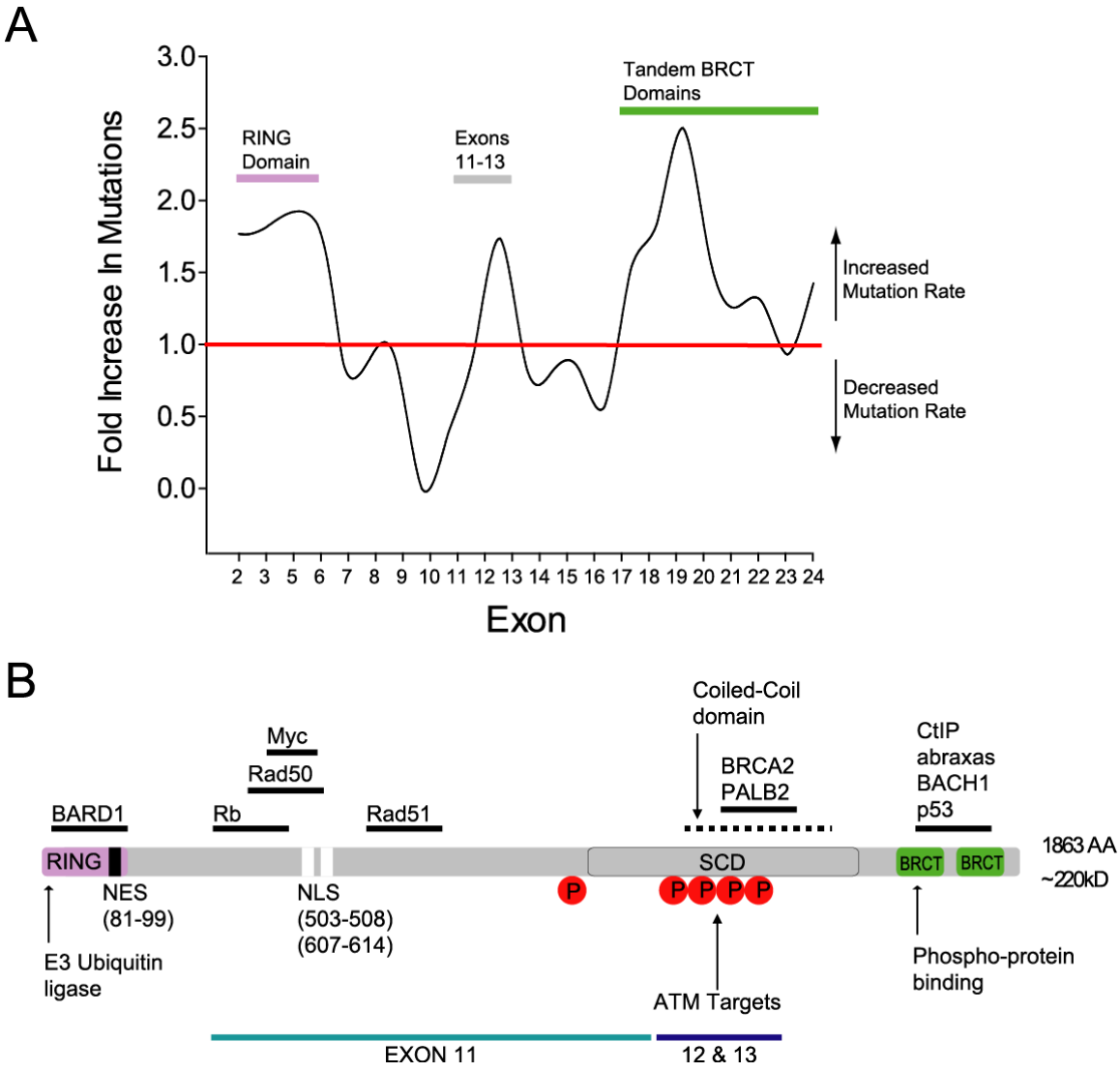


Illustration 1-3. BRCA1 mutations occur at the highest rates in the RING domain, exons 11-13, and the BRCT domain.

A) BRCA1 clinically relevant mutations from the Breast Cancer Information Core (BIC). Fold increase in mutations were calculated as mutations per codon length of each exon/total mutations per total BRCA1 codons. 1.0 on the y-axis indicates the total average mutations per codon for BRCA1. Corresponding domains are indicated above the graph. B) Domain map of BRCA1. RING, serine containing domain (SCD), and BRCT domains are indicated. NES and NLS sequences are also depicted. Horizontal solid black lines indicate protein binding domains for the listed binding partners. Red circles mark phosphorylation sites.

Understanding the structural biology of BRCA1 and BRCA2 is important for elucidating both physiologic and pathophysiologic functions of these proteins. As shown in Table 1, multiple structures have been solved for the BRCA1 RING and BRCT domains and associated proteins, including clinically relevant mutants. In this review we will focus on the structural basis by which the BRCA1 protein functions as a tumor suppressor, and highlight the importance of these studies to understanding the pathophysiology and clinical outcomes of breast and ovarian cancers.

Table 1. Summary of published BRCA1 structure

Domain	Method	Description	PDB	Ref.
RING	NMR	BRCA1/BARD1 RING-domain heterodimer	1JM7	(75)
BRCT	X-Ray	BRCA1 BRCT repeat region	IJNX	(76)
BRCT	X-Ray	BRCA1 BRCT mutation M1775R	1N5O	(77)
BRCT	X-Ray	BRCA1 BRCT mutant M1775K	2ING	(78)
BRCT	NMR	BRCA1 BRCT-c domain	1OQA	(79)
BRCT+ peptide	X-Ray	BRCA1 BRCT with BACH1 phosphopeptide	1T29	(80)
BRCT+ peptide	X-Ray	BRCA1 BRCT with BACH1 phosphopeptide	1T15	(81)
BRCT+ peptide	X-Ray	BRCA1 BRCT with CtIP phosphopeptide	1Y98	(78)
BRCT+ peptide	X-Ray	BRCA BRCT with Acetyl-CoA Carboxylase 1 phosphopeptide	3COJ	(82)
BRCT+ peptide	X-Ray	BRCA1 BRCT with phosphopeptide	1T2V	(77)
BRCT+ peptide	X-Ray	BRCA1 BRCT V1809F with phosphopeptide	1T2U	(77)
BRCT+ peptide	X-Ray	BRCA1 BRCT with a minimal recognition tetrapeptide (amidated C-terminus)	3K0H	(83)
BRCT+ peptide	X-Ray	BRCA1 BRCT with a minimal recognition tetrapeptide (free carboxy C-terminus)	3K0K	(83)
BRCT+ peptide	X-Ray	BRCA1 BRCT D1840T with a minimal recognition tetrapeptide (amidated C-terminus)	3K15	(83)
BRCT+ peptide	X-Ray	BRCA1 BRCT D1840T with a minimal recognition tetrapeptide (free carboxy C-terminus)	3K16	(83)
BRCT+ peptide	X-Ray	BRCA1 BRCT G1655D with phosphopeptide	3PXA	(84)
BRCT+ peptide	X-Ray	BRCA1 BRCT T1700A with phosphopeptide	3PXB	(84)
BRCT+ peptide	X-Ray	BRCA1 BRCT R1699Q with phosphopeptide	3PXC	(84)
BRCT+ peptide	X-Ray	BRCA1 BRCT R1835P with phosphopeptide	3PXD	(84)
BRCT+ peptide	X-Ray	BRCA1 BRCT E1836K with phosphopeptide	3PXE	(84)

BRCA1 RING Domain

The RING (Really Interesting New Gene) domain of BRCA1 consists of a RING finger and two flanking alpha helices encompassing amino acids 1-109 (exons 2-7) (75,85). Through seven conserved cysteine residues and one conserved histidine residue, the RING finger coordinates two Zn^{2+} atoms which stabilize the RING structure (86,87). The RING finger forms a globular structure with a core three-strand β-sheet and a central helix, while the flanking helices align perpendicular to the RING finger (Ill. 1-4). The RING finger, which is a highly conserved domain found in a large number of proteins, is responsible for the E3-ubiquitin ligase activity of BRCA1 (88). The N and C-terminal helices are responsible for the interaction of BRCA1 with BARD1 (BRCA1 Associated RING Domain protein 1), a major BRCA1 binding partner that also contains a RING domain (89). The ubiquitin ligase activity of BRCA1 is dramatically increased by formation of the BRCA1/BARD1 heterodimer (90). As with all E3-ubiquitin ligases, ubiquitination of a substrate can only occur through interaction with an E2 ubiquitin-conjugating enzyme. UbcH5, as well as other E2 enzymes, binds to the surface of BRCA1 opposite the binding interface with BARD1 (91). The large number of cancer-predisposing mutations that affect the interaction of BRCA1/BARD1 or BRCA1/UbcH5, as well as the RING E3 ligase function, suggest that the ubiquitin ligase activity of BRCA1 is essential for its tumor suppressor function (but see (92)).

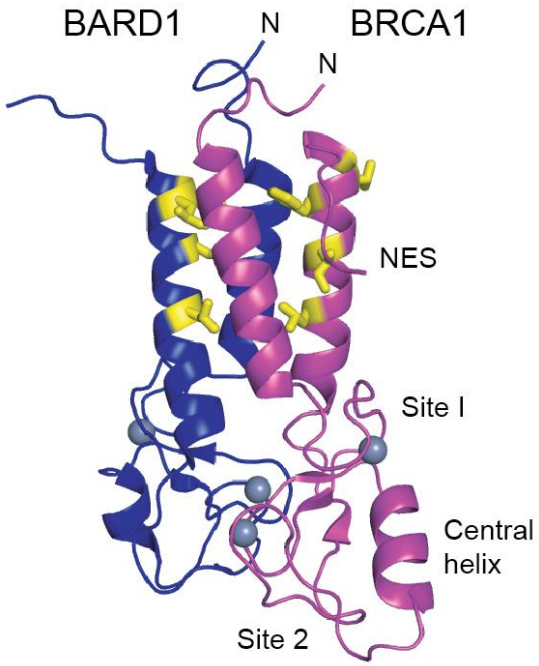


Illustration 1-4. Structure of BRCA1 RING domain in complex with BARD1 RING domain.

The RING domain contains a RING finger and two flanking alpha helices. The RING finger consists of a core of β-strands, a central helix, and two Zn2+ binding sites. BRCA1 (pink) forms a heterodimer with the RING domain of BARD1 (blue). Critical NES residues are highlighted in yellow. N-termini of each strand are labeled. Structural model is derived from PDB accession number 1JM7 and rendered using POLYVIEW-3D (93).

STRUCTURE-FUNCTION OF THE BRCA1 RING DOMAIN

We have gained the most information about the structure-function of the BRCA1 RING domain from the structure of the BRCA1/BARD1 heterodimer (75). BARD1 also contains a RING domain with sequence and structural homology to BRCA1, including two flanking alpha helices. The N-terminal alpha-helix of BRCA1 aligns in an antiparallel fashion with the C-terminal alpha helix of BARD1. Conversely, the C-terminal alpha-helix of BRCA1 is antiparallel to the N-terminal alpha-helix of BARD1 (Ill. 1-4)) (75). The four-helix bundle creates a large buried hydrophobic region and stabilizes the heterodimer, while interactions between the BRCA1 RING finger and the flanking alpha-helices maintain the orientation of the RING finger with respect to the flanking alpha-helices. The interaction between BRCA1 and BARD1 both increases the ubiquitin ligase activity of BRCA1 and causes the nuclear export sequence (NES), located on the C-terminal helix of the RING domain of both BRCA1 and BARD1, to be buried (75,94,95). The buried NES in the four-helix bundle results in nuclear retention of the two proteins. The four-helix bundle contains the majority of the interactions between BRCA1 and BARD1, however a few inter-RING interactions may occur as well (75). As stated above, the RING finger of BRCA1 consists of a small three-strand antiparallel β-sheet and a central helix. Two Zn^{2+} atoms stabilize the structure within the RING finger and are coordinated by Zn^{2+} binding loops named Site I and Site II. Site I is made up of four cysteine residues, while Site II contains three cysteine residues and one histidine residue. The Zn^{2+} binding residues are highly conserved and characteristic of RING fingers found in many other proteins. Additionally, the spacing between the Zn2+ binding residues is conserved among many

RING fingers. Conversely, a central helix is present in some RING fingers, but not all (75).

Ubiquitination of substrates occurs in a three-step process. First, an E1 ubiquitin-activating enzyme activates an ubiquitin (Ub) molecule, which is transferred to an E2 ubiquitin-conjugating enzyme. The E3 brings together the E2 and substrate to complete the ubiquitination process. The human genome encodes ~40 E2 enzymes, which rely on ~1000 E3 ubiquitin ligases for their specificity (96). RING E3 ubiquitin ligases, including BRCA1, act solely as scaffolds by binding to the E2 via the RING finger domain, while the substrate binds to another domain on the E3. This brings the substrate close enough to the E2 to allow for the transfer of Ub from the E2 to the substrate. The presence of the E2 ubiquitin-conjugating enzyme, UbcH5, dramatically increases BRCA1/BARD1 ubiquitination activity in vitro [28]. NMR structures of BRCA1/BARD1/UbcH5c show that loops of UbcH5c bind to a groove formed by the two Zn2+ binding sites and the central helix of the RING finger of BRCA1, and that UbcH5c has no interaction with BARD1 (91). Several other E2 proteins have been shown to interact with the BRCA1/BARD1 heterodimer in a yeast-two hybrid study (97). Targets of BRCA1 E3 ligase activity in vivo include estrogen receptor-alpha, progesterone receptor, CtIP, and histone protein H2A with resulting alterations in gene activation, DNA repair, and DNA condensation (98–102).

BRCA1 is also subject to autoubiquitination in in vitro experiments. Depending on the specific E2 interaction, either mono or poly-autoubiquitination can occur. Additionally, Lys63, Lys48, and Lys6 polyubiquitin chains can be conjugated to BRCA1. Two modes of BRCA1/BARD1 autoubiquitination have been established. “Substrate-specific” monoubiquitination by the E2s UbcH6, Ube2e2, UbcM2, Ube2w and UbcH5

result in the conjugation of a single Ub residue to BRCA1 (97). "Ubiquitin-specific" E2s, Ubc13, Ube2k and UbcH5 recognize monoubiquitinated BRCA1 and stimulate the conjugation of Lys6, Lys48, and Lys63 polyubiquitin chains to BRCA1 (97). Thus, different E2 enzymes mediate the mono and polyubiquitination of BRCA1 in vitro.

CANCER RELATED MUTATIONS

Mutation of the cysteine residues that coordinate the Zn^{2+} atoms have been reported as clinically important, indicating that they result in altered function and an increased risk of cancer. Mutation of residues in Site I result in altered folding of the RING domain (75). A more complete study of Site II residue mutations found altered structure by mass spectrometry and reduced Zn^{2+} binding at Site II (103). This study reported that BRCA1/BARD1 heterodimerization was not affected by Site II mutations, however a later study by the same group reported that several Site I and Site II mutations caused not only a decrease in ubiquitin ligase activity, but also a decrease in co-immunoprecipitation of BRCA1 and BARD1 (91). These studies suggest that mutation of Site I and Site II residues may affect BRCA1 ubiquitin ligase activity by either decreasing BRCA1/BARD1 heterodimerization or BRCA1/UbcH5 interaction. Another study has shown that the E3-ubiquitin ligase activity of BRCA1 is inhibited by platinum (Pt)-based alkylating chemotherapeutic drugs (104,105). Cisplatin forms adducts through its Pt atom with His117 of BRCA1, causing conformational changes and inhibiting the E3-ubiquitin ligase activity in vitro (105). Other Pt-based drugs had similar functional effects. Transplatin, carboplatin and oxaliplatin all reduced the E3 ligase activity of BRCA1 at therapeutically relevant concentrations (104). The large number of RING domain mutations that result in

increased risk of breast cancer and the effect of chemotherapeutic drugs on RING domain activity suggest an important role for the RING domain in tumor suppression (but see (92)).

BRCA1 Exons 11-13

Exons 11-13 cover over 65% of the sequence of BRCA1 and encode two nuclear localization sequences (NLS) and binding sites for several proteins, including retinoblastoma protein (RB), c-Myc, Rad50, and Rad51 (reviewed in (106)). The amino acids encoded by these exons also contain portions of a coiled-coil domain that mediates interactions with PALB2, as well as a portion of a serine-containing domain (SCD) that is phosphorylated by ATM (Ill. 1-5). No atomic-level structures have been determined for exons 11-13 of BRCA1. Despite the fact that exons 11-13 contain a large percentage of the clinically relevant mutations, very little is known about the structure or function of this region when compared to the RING or BRCT domains (74). Interestingly, BRCA1 exon 11-13 binding partners are involved in a wide range of cellular pathways. Myc is a transcription factor for a large number of genes. Rad50, Rad51, and PALB2 are involved in DNA repair. RB controls cell cycle progression. The large number of mutations occurring in this region, many with loss of large portions of sequence, suggest that this region is important for the tumor suppressor function of BRCA1.

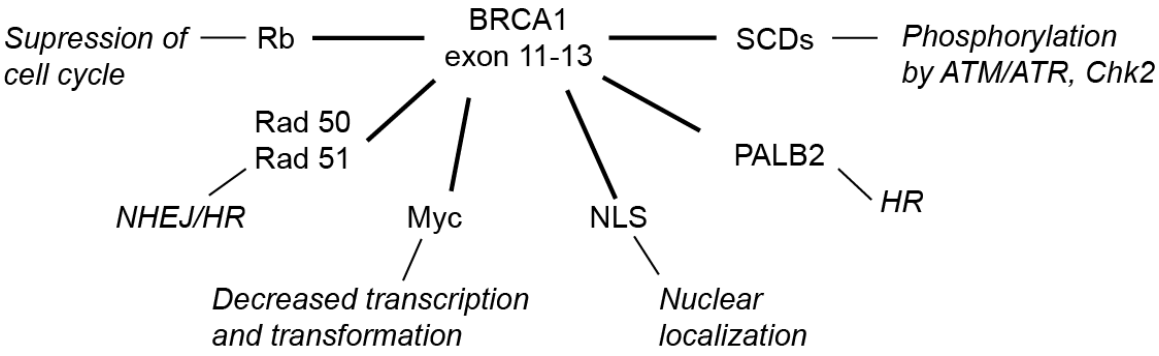


Illustration 1-5. BRCA1 Exons 11-13 have multiple functions.
The amino acids encoded by BRCA1 exons 11-13 have binding domains for several proteins including retinoblastoma (RB), Rad 50, Rad51, c-Myc, and PALB2 (a scaffold for BRCA2). BRCA1 exons 11-13 also contain a nuclear localization signal (NLS) and a serine cluster domain (SCD).

RETINOBLASTOMA PROTEIN (RB)

The phosphoprotein RB is a well-known tumor suppressor that controls growth by regulating progression through the cell cycle (107). BRCA1 interacts with the hypo-phosphorylated form of RB via BRCA1 exon 11. Specifically, amino acids 304-394 were found to be responsible for binding to the ABC domain of RB (108). Over-expression of BRCA1 in cells expressing wild type RB causes suppression of cell cycle progression. Deletion of the region of BRCA1 that mediates BRCA1/RB binding inhibits BRCA1-dependent suppression of cell cycle progression (108). This suggests that the exon 11-mediated interaction between BRCA1 and RB causes cell cycle arrest through actions of RB. This finding also indicates that exon 11 is responsible for BRCA1-dependent cell cycle arrest, and this may also be dependent on the BRCA1/RB interaction.

RAD50 AND RAD51

Rad50 and Rad51 are two proteins involved in DNA repair. Rad50 functions in a complex that includes MreII and Nbs1. This complex is involved in both non-homologous end joining (NHEJ), as well as homologous recombination (HR). An interaction between BRCA1 and Rad50, and therefore with the Rad50/MreII/Nbs1 complex, has been established. This interaction requires BRCA1 exon 11 amino acids 341-748 (109). BRCA1 recruits the Rad50/MreII/Nbs1 complex to sites of DNA double strand breaks to facilitate DNA repair. BRCA1-null mouse embryonic fibroblast cells exhibit decreased levels of NHEJ activity, which suggests that BRCA1 is involved in the NHEJ process through interaction with the Rad50/MreII/Nbs1 complex. Rad51 is a homologue of the yeast protein RecA and binds to ssDNA, facilitating homologous recombination (HR). BRCA1 is associated with Rad51 during both mitotic and meiotic cells via amino acids

758-1064 (110). BRCA1 association with Rad50 and Rad51 suggests a role for exon 11 in both NHEJ and HR processes of DNA repair.

c-Myc

The transcription factor c-Myc also interacts with BRCA1. Reports have indicated that c-Myc promotes transcription of up to 15% of the genome, making it a major hub for transcriptional activation (111). BRCA1 has two c-Myc binding sites (known as MB1 and MB2). MB1 is located only in exon 11 (a.a. 433-511) while MB2 is located in exons 8-11 (a.a. 175-303) (112). In SVD-P5 cells co-transformed with c-Myc/Ras, transfection with BRCA1 significantly decreased the ability of these cells to form transformed foci (112). This suggests that the transformation activity of c-Myc/Ras is inhibited by BRCA1 expression. Additionally, the transcriptional activity of Myc is decreased by BRCA1 (112). Thus, suppression of the oncogenic activities of c-Myc may account for some of the tumor suppressor activity of BRCA1.

Nuclear Localization Sequences

Exon 11 contains two nuclear localization sequences (NLS). Amino acids 501-507 (NLS1) and 607-614 (NLS2) are both recognized by importin-α machinery to mediate BRCA1 transport from the cytosol to the nucleus. While both sequences are recognized by importin-alpha, NLS1 is the most critical sequence because mutation of this sequence inhibits all interactions between BRCA1 and importin-α (113). Mutation of the NLS sequences results in altered subcellular localization of BRCA1, with a shift toward cytosolic localization. Clearly, mutations of BRCA1 NLSs causing cytosolic expression of BRCA1 would decrease the tumor suppressor activity of BRCA1 due to the loss of

BRCA1's DNA repair activity and subsequent increase in unrepaired mutations and chromosomal abnormalities.

PALB2

A putative coiled-coil domain spanning exons 11-13 in BRCA1 (a.a. 1364-1437) contains the binding site for PALB2. At this site, PALB2 acts as a scaffold to bring together BRCA1 and BRCA2 to form a complex of the three proteins, which is involved in HR during DNA repair. Both BRCA1 and PALB2 contain coiled-coil domains that mediate the interaction of the two proteins. Through modeling of the coiled-coil domain of BRCA1 and PALB2, the interaction sites were mapped to the predicted a-face of the PALB2 helix containing Lys14, Leu21, Tyr28, Leu35, and Glu42, and the predicted a and d-faces of BRCA1 (114). Mutations in the coiled-coil region of BRCA1 led to the discovery of the PALB2 binding site on BRCA1, since mutations reported in this region (Met1400Val, Leu1407Pro, and Met1411Thr) inhibit interaction between BRCA1 and PALB2 (114).

SERINE CLUSTER DOMAIN

BRCA1 contains a domain called the serine cluster domain (SCD). A portion of the SCD of BRCA1 is located in exons 11-13, and spans from amino acids 1280-1524. The region has a concentrated amount of putative phosphorylation sites, and is phosphorylated by ATM/ATR kinases in vitro and in vivo. ATM and ATR are kinases activated by DNA damage. Phosphorylation of BRCA1 causes recruitment of BRCA1 to sites of double strand breaks. SCDs are common in ATM/ATR targets and include multiple DNA damage response proteins (115). Serines 1189, 1457, 1524, and 1542 can all be

phosphorylated in vivo, while additional serines can be phosphorylated in vitro (116). Mutation of these serine residues are seen clinically, and may affect localization of BRCA1 to sites of DNA damage and DNA damage response function.

BRCA1 BRCT Domain

The BRCA1 C-terminal (BRCT) domain was originally identified in BRCA1, but it is also a conserved domain in multiple other proteins (most being involved in DNA damage repair). BRCT domains can occur as a single BRCT domain, as a tandem repeat (as found in BRCA1), as multiple repeats, or fusions between two domains (reviewed in (117)). The BRCA1 BRCT domain mediates phosphoprotein interactions between BRCA1 and proteins phosphorylated by ATM and ATR, two kinases activated by DNA damage (reviewed in (118)). BRCT domains are classified into two categories based on their ability to recognize phosphoproteins. Class-I BRCT domains can recognize phosphoserine (pSer) residues, while Class-II BRCT domains can recognize both pSer and phosphothreonine (pThr) residues. The BRCA1 BRCT domain recognizes the sequence pSer-X-X-Phe in its phosphorylated binding partners, and is therefore a Class-I BRCT domain. Binding partners for the BRCA1 BRCT domain include BACH1, CtIP, and CCDC98/abraxas (119–121). While the main function of the BRCA1 BRCT domain is modulating interactions between BRCA1 and phosphoproteins, BRCT domains, including the BRCT domain of BRCA1, can also mediate DNA binding and non-phosphoprotein interactions (122).

STRUCTURE-FUNCTION OF THE BRCA1 BRCT DOMAIN

Amino acids 1650-1863 of BRCA1 consist of two tandem BRCT repeats connected by a 22 amino acid linker (76). Each BRCT repeat consists of three α-helices packed around a four-strand β-sheet (Ill. 1-6A). The two BRCT repeats interact in a head-to-tail fashion through the interaction between α-helix 2 of BRCT1, and α-helices 1 and 3 of BRCT2 through mainly hydrophobic residues. The architecture of the tandem BRCT allows the BRCA1 BRCT to recognize both a pSer and the 3+ aromatic residue in a bipartite manner in two separate recognition pockets in the cleft between BRCT1 and BRCT2 (Ill. 1-6B) (77,81) (also reviewed in (117)). The pSer residue forms hydrogen bonds with Ser1655 and Lys1702 and the backbone amine group of Gly1656, all within the N-terminal BRCT1 (81). The 3+ phenylalanine residue fits into the hydrophobic core created by the two BRCT repeats, while the main chain backbone of the 3+ phenylalanine forms hydrogen bonds with R1699 of α-helix 1 of the N-terminal BRCT domain (Ill. 1-6C) (81). The size and subsequent rigidity of the hydrophobic core of the interface between the two BRCT repeats dictates the strict consensus sequence required for substrate recognition by the BRCA1 BRCT domain. The consensus sequence pSer-X-X-Phe facilitates recognition of targets such as CtIP, BACH1, and abraxas which are all phosphorylated in response to DNA damage (reviewed in (117)).

The BRCA1 BRCT domain has been shown to bind directly to DNA double strand breaks (DSB) by electron microscopy, however the in vivo relevance of this interaction is unknown (122). While structural studies of the interaction between the BRCA1 BRCT domain and double strand breaks have yet to be carried out, models have been developed with predicted interactions between the BRCT of replication factor RFCp140 and DNA

double strand breaks. The BRCT domain of RFCp140 recognizes the terminal 5' phosphate of a 3' overhanging DNA double strand break, as well as the major groove of the DNA adjacent to the double strand break (123). It is unknown whether or not the BRCT domain of BRCA1 binds to DNA in a similar manner. BRCT domains have also been shown to interact with some proteins in a phosphorylation-independent manner, however this has been much less studied and not well characterized in BRCA1 (reviewed in (117)).

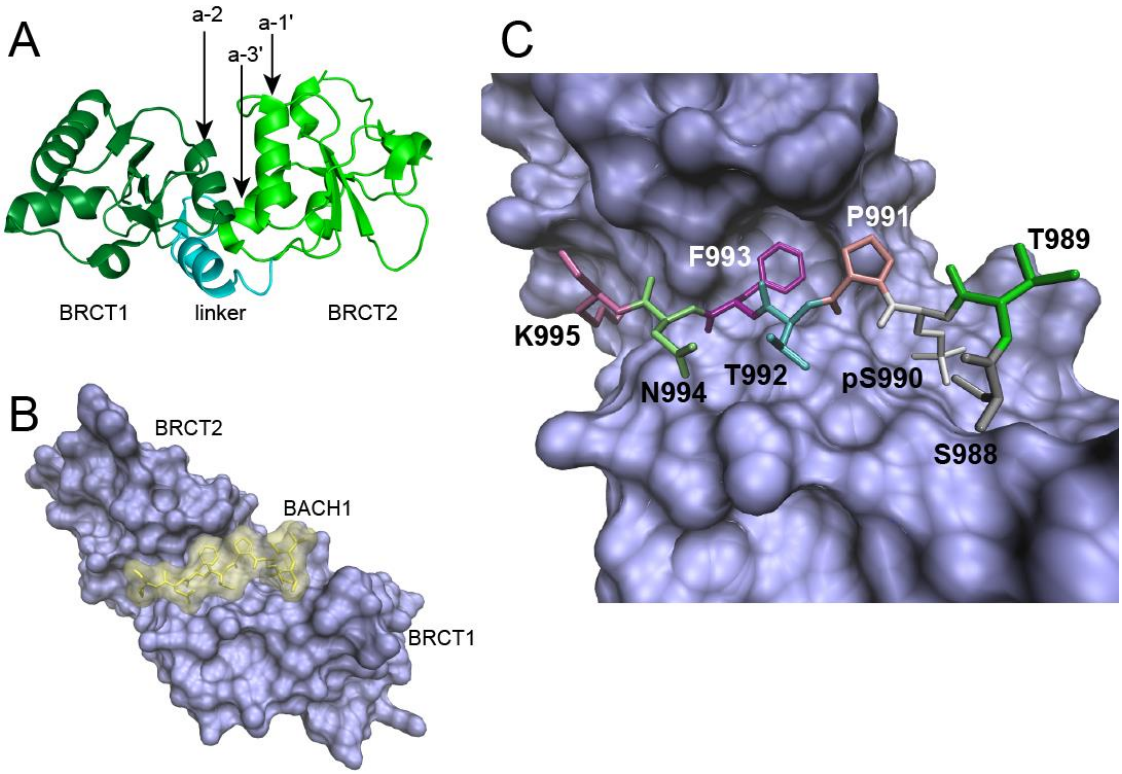


Illustration 1-6. BRCA1 BRCT tandem repeats recognize phosphoproteins.

A) BRCT1 and BRCT2 pack together in a head-to-tail orientation and are connected by a linker helix. Helix 2 from BRCT1 and helices 1 and 3 from BRCT2 form a hydrophobic core and stabilize the structure. Rendering was generated using POLYVIEW-3D (93). Structural model is derived from PDB accession number 1T29. B) The cleft between BRCT1 and BRCT2 forms the binding pocket for proteins phosphorylated by ATM and ATR. The BRCA1 BRCT domains are shown in blue, and a fragment of BACH1 is shown in yellow. C) A magnification of the BRCA1 BRCT/BACH1 binding pocket. The consensus sequence for BRCA1 BRCT recognition of phosphoproteins is 990pSer-X-X-Phe993. The BRCA1-binding region of a phosphopeptide derived from BACH1 is shown. Phospho-Ser990 (pS990) interacts with Ser1655 and Lys1702 of BRCA1, which form a basic pocket. The 3+ Phe993 fits into a hydrophobic pocket created by the two BRCT domains (Phe1704, Met1775, Leu1839). Lysine 995 (K995) forms a salt bridge with Asp1840 and Glu1836. Rendering of B and C was generated using Visual Molecular Dynamics (VMD) (124). Structural model in B and C is derived from PDB accession number 1T15.

BRCA1 BRCT CANCER PREDISPOSING MUTATIONS

Multiple studies have identified mutations in the BRCT domain of BRCA1 in breast and/or ovarian cancers, and these mutations give insight into the function of the domain (125–127). Specifically, mutation of hydrophobic residues within the hydrophobic core of the BRCT domain inhibits the ability of BRCA1 to recognize phospholigands (81). This would suggest that mutation of a residue required for recognition of a substrate would impede the ability of BRCA1 to carry out its role in the DNA damage repair pathway. An interesting example causes BRCA1 to fall into a "similarity trap" (128). Typically, phosphorylated p53 has a much higher binding affinity for 53BP1 (p53 binding protein 1), than BRCA1. Both 53BP1 and BRCA1 interact with p53 through their tandem BRCT domains, however with different affinities. Two cancer-causing mutations in BRCA1, Phe1695Leu and Asp1733Gly cause BRCA1 to bind p53 with similar affinity to 53PB1

(128)[66]. This suggests that these mutations of BRCA1 in the BRCT domain could force BRCA1 into a similarity trap, causing 1) BRCA1 to bind p53 with higher affinity than wild-type BRCA1, and 2) competition for 53BP1 binding to p53. Thus, it is likely that these mutations in the BRCA1 BRCT domain lead to altered p53 function possibly contributing to the cancer phenotype. Another study has shown that cancer-causing mutations in other areas of the BRCA1 BRCT domain can alter the backbone structure of the BACH1 binding pocket [67]. This suggests that mutations that affect the BACH1 binding pocket are not limited to just the residues in direct contact with the phosphopeptide. The number of cancer causing mutations in this region suggests that this domain is critical for tumor suppression.

TUMOR SUPPRESSOR SHUTTLING

As stated previously and discussed in the proceeding chapters, we have found that the tumor suppressor BRCA1 regulates IP_3R function at the ER membrane. However, BRCA1 is classically considered to be a nuclear protein due to its role in DNA damage repair. Relatively recently, several studies have identified BRCA1 localization to non-nuclear organelles, as well as non-nuclear functions of BRCA1 (as reviewed in(129)). This is supported by the identification and characterization of regulated shuttling of BRCA1 into and out of the nucleus (94,113,130,131). Interestingly, other tumor suppressors that regulate IP_3R function also undergo regulated shuttling across the nuclear envelope (reviewed in (132)). p53 and BRCA1 are by far the most thoroughly studied of the proteins reviewed here in regards to nuclear export and import. Therefore they will be the focus here.

Nuclear Import and Export of p53

P53 has multiple functions. The active function of p53 is partially determined by its subcellular location. As described above, nuclear p53 activates transcription of several genes, including p21, PTEN, Bax, and Apaf1. Nuclear p53 is also involved in DNA damage and replications. Non-nuclear p53 is involved in centrosome duplication and mitochondrial outer membrane permeabilization during apoptosis (133). Regulated shuttling pathways allow for nuclear import and export of p53 in order to carry out these functions of p53. P53 localization is regulated by an NES, an NLS, post-translational modifications, DNA damage, and p53 tetramerization.

Two types of NES sequences have been identified in p53. First, a set of C-terminal NES sequences were identified. These NES sequences are masked when p53 forms a tetramer, reducing export of the tetramer (134). An N-terminal NES sequence was also identified that remains exposed after p53 tetramer formation. These NES sequences can also be modified by post-translational modifications. Ubiquitination of the C-terminal portion of a p53 subunit causes exposure of the C-terminal NES sequence (135). Conversely, DNA damage-induced phosphorylation of the N-terminal NES sequence causes inhibition of the NES sequence (136).

Nuclear import of p53 is mediated by three NLS sequences that are recognized by the importin alpha/beta complex (137,138). Nuclear import, as well as export, can also be affected by protein-protein interactions causing sequestration of p53 in a cellular compartment as well as genetic mutations. For example, the glucocorticoid receptor anchors p53 in the cytosol, restricting nuclear import. Lastly, mutation of p53 can affect p53 subcellular localization. Not surprisingly, mutations that affect NES or NLS sequences

or any mutations that affect key post-translational modification sites or protein-protein interaction sites cause altered p53 localization.

Nuclear Import and Export of BRCA1

Similar to p53, BRCA1 has several functions that depend on its subcellular localization (129). Nuclear BRCA1 participates in homologous recombination (HR) and repair of damaged DNA as well as protection of stalled replication forks during DNA replication (see previous sections). Non-nuclear BRCA1 participates in centrosome duplication and BRCA1 localization has also been suggested at the mitochondria and the ER ((139) and reviewed in (129)). Also, non-nuclear expression of BRCA1 increases cell death, however the mechanism is poorly understood (but will be examined experimentally in the proceeding chapters) (140).

BRCA1 localization is regulated by NLS and NES sequences, as well as a "piggy-backing" mechanism through an interaction with BARD1 and a sequestration mechanism through interaction with organelle specific proteins (Ill. 1-7). The BRCA1 NES and NLS sequences are located at the N-terminus. The NLS sequence is recognized by the importin machinery for nuclear import (113). The NES, located in the BRCA1 RING domain, is recognized by CRM-1 for nuclear export (94). The NES sequence can be masked, however, through a protein-protein interaction that buries the sequence. The protein BARD1 binds to BRCA1 through heterodimerization of the RING domain of each protein (see above for more details) (75,89). This interaction causes nuclear retention of BRCA1 (130). BRCA1 can also enter the nucleus through a "piggy-backing" mechanism. The BRCA1/BARD1 heterodimer can be imported using only the BARD1 NLS sequence. Therefore, if the BRCA1 NLS sequence is mutated, nuclear import can still occur.

Other mechanisms have been suggested to affect BRCA1 localization. DNA damage causes recruitment of BRCA1 to DNA damage-induced foci. Multiple DNA damage induced BRCA1 complexes can cause recruitment of BRCA1 to these foci. Not only does DNA damage induce nuclear retention, DNA damage can cause recruitment of non-nuclear BRCA1 into the nucleus (141). Cytoplasmic sequestration of BRCA1 through interaction with either BRAP2 and Bcl-2 as well as multiple genetic mutations have been identified in BRCA1 patient tumors that affect BRCA1 localization (131,139,142,143).

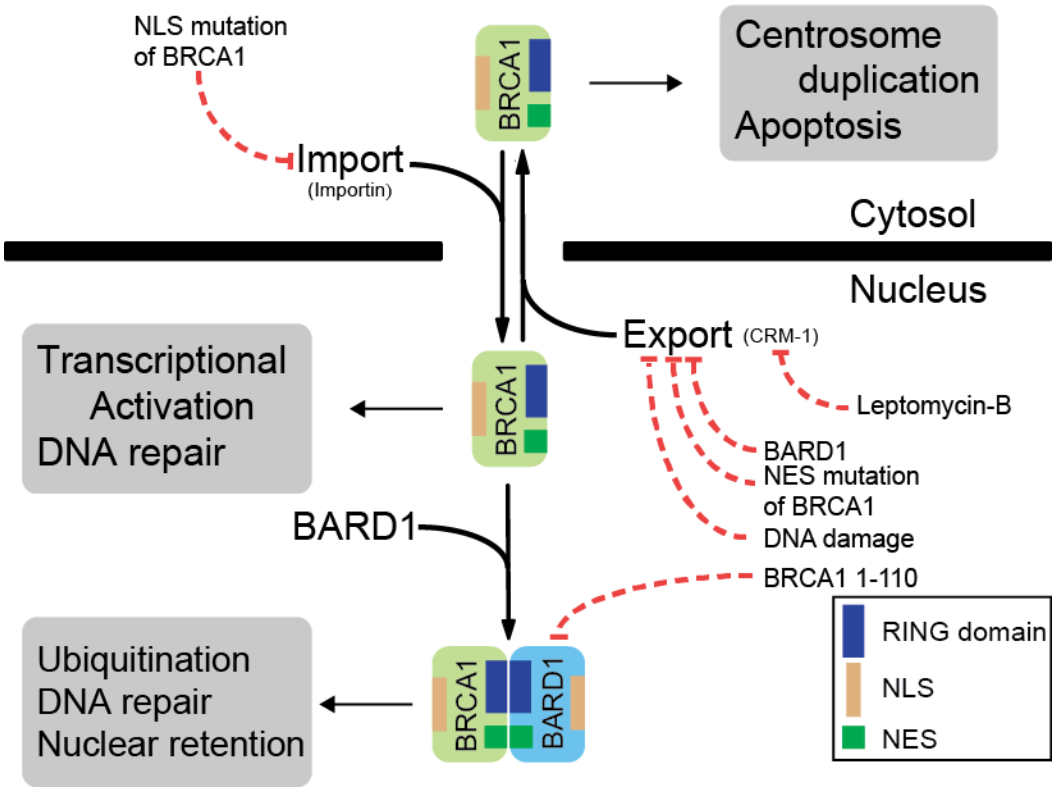


Illustration 1-7. Nuclear import and export of BRCA1.
BRCA1 is exported from the nucleus through recognition of its NES (orange rectangle) by the CRM-1 exporter. BRCA1 is imported from the cytosol through recognition of its NLS (green square) by importin-a. BARD1 and BRCA1 heterodimerize in the nucleus through interaction of their RING domains (purple rectangle). Interaction of BRCA1 and BARD1 also shields the NES sequence of both proteins, therefore sequestering BRCA1 in the nucleus.

Nuclear Import and Export of Beclin-1, PML, and PTEN

Interestingly, each of the remaining IP_3R targeting tumor suppressors have some form of regulated nuclear import and export. However, much is left to be understood about each protein. Beclin-1 and PML each have an identified NES, however not much is known about the nuclear import and export dynamics of either protein (132,144). PTEN has no identified NLS sequences, however nuclear PTEN localization has been identified in numerous reports. Ubiquitination of PTEN regulates its nuclear import. Mono-ubiquitination of PTEN at K289 causes nuclear import, while K289E mutation eliminates nuclear import of PTEN (145).

SIGNIFICANCE

In the following chapters we will show a physical and functional interaction between BRCA1 and IP_3Rs. Similar to the other tumor suppressors listed above, BRCA1 may carry out its tumor suppressor function at least in part by binding directly to the IP_3R carboxy-terminal domain and sensitizing the channel, causing increased release of calcium. Similar to p53, BRCA1 is involved in DNA replication and repair in the nucleus and has

non-nuclear functions. It is possible that BRCA1, acting as a stress sensor similar to p53 and PML, associates with ER membranes where it interacts with the IP_3R. As described above, BRCA1 genetic deletion results in a high risk of breast and or ovarian cancers. Studies have shown that BRCA1 down-regulation is present in numerous patient tumors, suggesting that lack of BRCA1 expression is also contributory to the development of sporadic breast cancers (146,147). By thoroughly understanding pro-apoptotic functions of BRCA1, therapeutic targets may be identified to mimic the effect of wild-type expression. For example, small molecule modulators of proteins targeted by BRCA1 during apoptosis may be able to mimic BRCA1-mediated apoptosis in BRCA1-null or BRCA1 under-expressing cells.

Chapter 2: Experimental Methods

Modified in part from:

The BRCA1 Tumor Suppressor Binds to Inositol 1,4,5-Trisphosphate Receptors to Stimulate Apoptotic Calcium Release

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Materials and Antibodies

Paclitaxel was purchased from Sigma-Aldrich and resuspended in dimethyl sulfoxide (DMSO). The Cell Signaling Technology anti-BRCA1 antibody (cat# 9010) was used on HeLa cell lysates and immunoprecipitations due to questions regarding the specificity of the widely used MS110 monoclonal BRCA1 antibody (148). We tested all other commercially available BRCA1 antibodies and determined the Cell Signaling rabbit anti-BRCA1 antibody to be the most specific. The specificity of this antibody in detecting endogenous human BRCA1 was confirmed in knockdown experiments (Fig. 3-3A). The Bethyl anti-BRCA1 antibody (A301-378A) was used on DT40 lysates as this antibody reacts with the chicken BRCA1 isoform. The IP_3R-1 antibody has been described previously (19). The actin antibody used for co-immunoprecipitation was purchased from AbCam (ab6276). Anti-LRP130 antibody was purchased from ThermoScientific (PA5-22034). Anti-α-fodrin clone AA6 was from EMD Millipore (MAB1622). Cytochrome c oxidase, histone H1, and lactate dehydrogenase-HRP antibodies were purchased from Invitrogen (338500), Cell Signaling (2576), and Chemicon (no longer sold) respectively. Sigma-Aldrich anti-GST antibody (G1160) was used to detect GST-tagged proteins on PIP strips. Fura-2, AM (F1221) and Mag-Fura-2, AM (M1292), and Rhod-2-AM (R1244) were purchased from Molecular Probes. Paclitaxel was purchased from LC laboratories (P-9600). GSK 2606414 PERK inhibitor was purchased from EMD Millipore (cat# 516535).

Cell lines and transfections

HeLa (CCL-2), UWB1.289 (CRL-2945), and UWB1.289-BRCA1 (CRL-2946) cell lines were purchased from ATCC and maintained in the ATCC suggested media. DT40

and DT40 TKO cells were cultured as described (19). HeLa cells were transfected with Lipofectamine 2000 (Invitrogen). DT40 and DT40 TKO cells were transfected with the Invitrogen Neon transfection system using 1 pulse of 1375 volts for 40 milliseconds.

Protein purification and in vitro binding

GST-tagged BRCA1 RING (a.a. 1-112), BRCA1 BRCT (a.a. 1528-1863), GST-IP_3R modulatory domain (a.a. 922-1581), and GST-IP_3R tail domain (a.a. 2589-2749) were purified using glutathione-agarose beads (Pierce). GST was cleaved from GST-BRCA1 RING with thrombin protease (GE Health Sciences). BRCA1 RING (225μg) free of cleaved GST was conjugated to 0.1 g cyanogen bromide activated agarose (Sigma-Aldrich) to make RING-agarose. *In vitro* binding assays were performed using RING-agarose as bait and 200μg of GST-IP_3R modulatory domain and GST-IP_3R tail domain as prey in PBS with 1% Triton-X100.

PIP strip binding

PIP strips were purchased from Echelon Biosciences (P-6001). Binding was performed as suggested by the manufacturer using 5μg/ml of GST-RING and GST-BRCT.

Subcellular Fractionation

Cell homogenization and purification of the 10,000 xg pellet (P2), 100,000 xg pellet (P3), 100,000 xg supernatant (S3),and 100,000 xg pellet (P3) fractions were prepared as described previously (19). The 1000 xg pellet (P1) was resuspended in 1mL of buffer A (1.62M sucrose, 10mM HEPES pH 7.5, 2mM $MgCl_2$) and underlaid with 326μl of buffer B (2.3M sucrose, 10mM HEPES pH 7.5, 2mM $MgCl_2$) and centrifuged for one hour at

40,000 rpm. The supernatant was removed by aspiration. P1 pellets were resuspended in buffer A. Resuspended pellets were sonicated in a bath sonicator in ice water for 30 minutes before being passed through a 23g needle 10 times to shear genomic DNA.

Cell Death Assay

Propidium iodide and caspase-3 measurements were performed as described previously (149).

Cytosolic Calcium Imaging

HeLa cells were transfected with full length YFP-BRCA (94). After 48 hours, cells were loaded with 2.5 μM Fura-2 in imaging buffer composed of 1% BSA, 107mM NaCl, 20mM HEPES, 2.5mM $MgCl_2$, 7.25 mM KCl, 11.5 mM glucose, 1mM $CaCl_2$ for 30 minutes at room temperature. The solution was replaced with imaging solution without Fura-2 for an additional 30 minutes prior to imaging. Coverslips were then imaged on a Nikon TiS inverted microscope with a 40X oil objective as recently described (150). Responses to 100nM, 1μM, and 10μM histamine were recorded in YFP-BRCA1 cells and adjacent YFP-negative control cells from four separate coverslips. A total of 25 individual YFP-BRCA1 positive and 24 YFP-negative cells were quantified and pooled from the four coverslips. Peak release was determined in the MetaFluor acquisition software. Oscillation frequency was determined manually, where an oscillation was defined as a rise and fall of the Fura-2 ratio of at least 0.01 units. This same threshold was used to determine percent responding cells at 100nM histamine. All cells responded at 1 μM and 10 μM histamine, and thus were not quantified. For the siRNA knockdown experiments, a similar approach was used. HeLa cells were transfected with two different siRNAs targeting human BRCA1

(Ambion/Life Technologies siRNAs s458 and s459). The total amount was 12.5 pmoles/well of a six well dish. Transfected cells were identified by co-transfection with YFP. Cells were imaged after 48 hours. We found that both siRNAs efficiently knocked down BRCA1 expression (Figure 3-3A), however siRNA s458 also upregulated IP_3R-1 expression (Figure 3-3A). Thus, only siRNA s459 was used for the calcium imaging experiments. The siRNA data was quantified exactly as above, with the exception that the control cells were transfected with control siRNA (control siRNA, medium GC content, Life Technologies #12935-300) on separate coverslips and identified by YFP expression exactly as described for the BRCA1 targeted siRNA.

Mitochondrial calcium imaging

Mitochondrial calcium was quantified by Rhod-2 imaging exactly as described (150). The response to 10μM histamine was quantified and pooled from five coverslips comprising 18 YFP-BRCA1 and 18 YFP-negative cells.

ER Calcium Imaging

HeLa cells were loaded with 5μM Mag-Fura-2 AM in imaging solution (1% BSA, 107mM NaCl, 20mM HEPES, 2.5mM $MgCl_2$, 7.25 mM KCl, 11.5 mM glucose, 1mM $CaCl_2$) for 20 minutes at room temperature. To obtain cytosolic access and image ER calcium, cells were permeabilized with 120μg/ml saponin in intracellular solution (125mM KCl, 19mM NaCl, 10mM HEPES, 1mM EGTA, 0.4 mM $CaCl_2$) until permeabilization was obvious via imaging (approximately 1 min). Images were taken every 3 seconds during continuous recording of the response to various additions. Measurement of steady

state changes in cytosolic calcium after 24 hours of paclitaxel treatment (Fig. 3D) was performed using Fura-2 as described previously (15).

IP_3R Single Channel Recording

Recombinant rat IP_3R-1 was stably expressed in triple IP_3R knockout DT40 cells. Single channel recording by the nuclear patch clamp technique was performed exactly as described (151). The recording solution contained 140mM KCl, 10mM HEPES, 5mM NaATP, 100μM BAPTA, 200nM free calcium, and 1μM IP_3. Channels were recorded at -100mV, sampled at 20 kHz, and filtered at 5 kHz. A minimum of 15s of recording from patches containing a single channel were used for analysis. The total number of channels analyzed for each condition are indicated over the bar graph in Figure 4B. Where indicated, either 30nM BRCA1 GST-RING or 30nM GST alone were included in the patch pipette.

YFP-BRCA1 CFP-IP_3R FRET Imaging

HeLa cells were transfected with YFP-BRCA1 (94) and CFP-IP_3R (19). After 48 hours, cells were imaged on a Nikon TiS inverted microscope with a 100X SuperFluor objective, and images were aquired every 30 seconds with a Photometrics Evolve EMCCD camera. Exitation and emmission filters for aquiring donor and acceptor channels were from Chroma Technology (set 89002). Cells were maintaned at 37^{o}C during imaging. Data was quantified by taking a region of interest from the cytosol and nucleus, and taking the ratio of the acceptor flourescence (exc. 430nm, emm. 535nm) over the donor fluorescence (exc. 430nm, emm. 470nm) for each region of interest. The data in Fig. 3B were pooled from 20 cells (CFP-IP_3R/YFP-BRCA1) and 22 cells (CFP-IP_3R/YFP) from at least three separate experiments. Data was quantified by taking a region of interest from the cytosol

and nucleus, and taking the ratio of the acceptor flourescence (exc. 480nm, emm. 620nm) over the donor fluorescence (exc. 430nm, emm. 525nm) for each region of interest.

GFP-PASS dsRED-ER FRET Imaging

HeLa cells were transfected with GFP-PASS (152) and pDsRed2-ER (Clontech-632409) using Lipofectamine 3000. After 48 hours imaging was performed as described above using beam splitter (86007bs) excitation (480nm and 565nm) and emission (525nm and 620nm) filters from Chroma Technology.

Fatty Acid Binding Site Prediction

To screen for a putative fatty acid binding region in BRCA1, a Position Specific Scoring Matrix (PSSM)-based method described previously in Ko et. al. was used (153). An initial PSSM library was defined from the experimentally determined Fatty Acid Binding Protein (FABP) regions in 42 well-characterized lipid binding crystal structures collected from the protein databank (154–157). This initial PSSM library was leveraged to search for more FABP regions using psi-blast and thus expanded to 1185 FABP-specific PSSM libraries. Human BRCA1 was aligned with this expanded FABP specific PSSM library. All the positive alignments were recorded. From these positive PSSMs a residue score was calculated that represents the occurrence of identical and similar residues from each query-PSSM alignment above threshold. Briefly, using Smith-Waterman algorithm alignments were re-evaluated in the query sequence with profiles that were positive, as in (158,159). Then, raw scores for each residue were calculated by scoring a value=2 for identities and value=1 for positive substitutions from each alignment. These values were summed for all alignments at each position to obtain a total raw residue score.

Chapter 3: The BRCA1 Tumor Suppressor Binds to Inositol 1,4,5-Trisphosphate Receptors to Stimulate Apoptotic Calcium Release

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INTRODUCTION

The IP_3R is a ligand-gated calcium channel localized primarily to ER membranes. The IP_3R is a tetrameric protein, and each subunit consists of an N-terminal ligand binding domain, a C-terminal transmembrane pore domain and an intervening modulatory domain. Calcium release through the IP_3R regulates multiple cellular processes including but not limited to gene expression, metabolism, and apoptosis (2,38). Numerous IP_3R protein-protein interactions allow for signal integration of diverse signaling pathways (2). Several proto-oncogenes and tumor suppressors interact with and regulate the IP_3R, including Bcl-2, PTEN, and PML (17,45,46). These proteins regulate IP_3R activity and apoptotic calcium release through multiple mechanisms. In general, proto-oncogenes cause reduced IP_3R activity, while tumor suppressors cause increased IP_3R activity to regulate cell death (46).

BRCA1 (Breast and Ovarian Cancer Susceptibility Gene-1) is a tumor suppressor well known for its function during homologous recombination and repair of DNA double strand breaks (64,160,161). The presence of nuclear export and import signals suggests regulated transport of BRCA1 into and out of the nucleus (94,113). Several studies have suggested a pro-apoptotic role for BRCA1 linked to its nuclear export and cytosolic localization, however the mechanisms by which BRCA1 stimulates cell death once outside of the nucleus are unclear (129,130,140).

Here we show that BRCA1 stimulates apoptosis through a physical and functional interaction with the IP_3R. BRCA1 is recruited to the IP_3R during apoptosis and stimulates apoptotic calcium release and cell death. Thus, BRCA1 mediates its pro-apoptotic effects by binding to and modulating apoptotic calcium release through the IP_3R.

RESULTS

BRCA1 binds to the IP3R

In a yeast-two-hybrid experiment using the C-terminal tail domain of IP_3R (a.a. 2589-2749) as bait, the RING domain of BRCA1 was isolated as an interacting clone (a.a. 1-112). To confirm this direct interaction, we used purified recombinant protein in an *in vitro* binding experiment. Purified RING domain was covalently conjugated to cyanogen bromide activated agarose beads and the ability of purified GST-IP_3R tail domain and modulatory domain (a.a. 923-1582) to bind to the conjugated beads was tested. The RING domain of BRCA1 was able to specifically pull down IP_3R tail domain, suggesting a direct protein-protein interaction between BRCA1 and the IP_3R tail (Fig. 3-1A). In order to test for a physical interaction between full length BRCA1 and IP_3R in cells, we performed a co-immunoprecipitation experiment. Using an IP_3R antibody we were able to co-immunoprecipitate endogenous IP_3R-1 with endogenous BRCA1 in HeLa cells (Fig. 3-1B). We could not perform a reciprocal co-immunoprecipitation of IP_3R with BRCA1, as we were unable to identify an antibody suitable for immunoprecipitation of BRCA1. This may be related to epitope masking in Triton-X100 lysates of BRCA1. Regardless, yeast-2-hybrid, co-immunoprecipitation, and direct binding of purified components strongly suggest a direct physical interaction between BRCA1 and IP_3R mediated by the BRCA1 RING domain and the IP_3R tail domain.

The existence of BRCA1 nuclear import and export signals indicate regulated transport of the protein into and out of the nucleus. However, the subcellular localization of BRCA1 has been a subject of debate (162). To determine the subcellular localization of BRCA1, we performed subcellular fractionation using differential centrifugation of HeLa cell homogenates. We used HeLa cells because IP_3R function, subcellular localization,

and protein interactions during apoptosis have been extensively studied and are well characterized in this cell line (19,163). Furthermore, HeLa cells express endogenous wild-type BRCA1 (164). After differential centrifugation, the purity of the individual fractions was confirmed by stripping and re-probing the same blot with IP_3R-1 (endoplasmic reticulum), histone H1 (nucleus), cytochrome c oxidase (mitochondria), and lactate dehydrogenase (cytosol). BRCA1 was expressed in all cell fractions except the 100,000 xg supernatant (representing cytosol), and surprisingly the majority of BRCA1 was localized to the 100,000 xg microsomal ER-enriched fraction (Fig. 3-1C). IP_3Rs in this fraction also specifically bind to cytochrome c to regulate apoptotic calcium release (19). Interestingly, the phosphorylated form of BRCA1 was also found in the 10,000 xg pellet which is enriched in IP_3R and mitochondria, consistent with a previous report (164). Thus, non-nuclear BRCA1 is abundant and localized to the same subcellular compartments as the IP_3R, and could potentially modulate its activity.

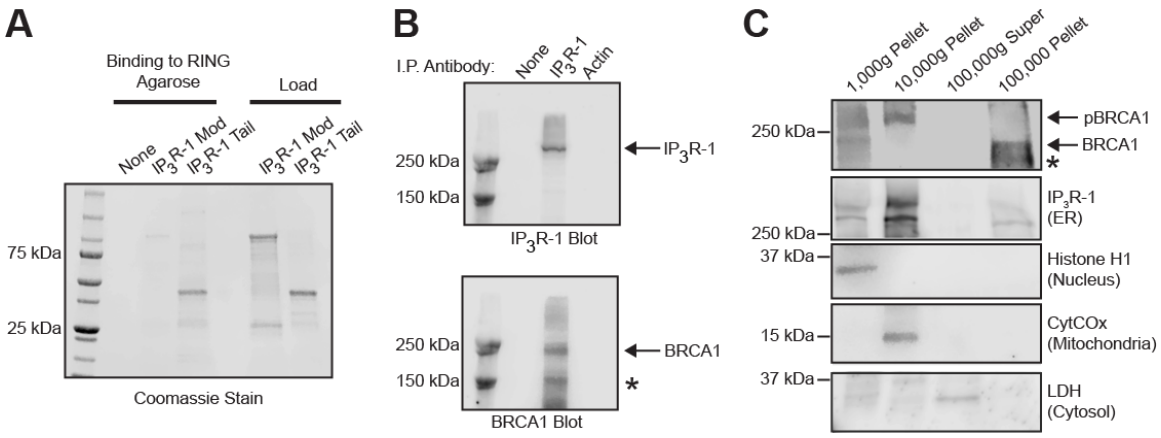

Figure 3-1. BRCA1 binds to the IP$_3$R.
A) In vitro binding of purified GST-tagged fragments of IP3R to RING-agarose beads. First three lanes include RING-agarose beads, either alone (lane 1) or mixed with the indicated IP3R domains. Load lanes include 5 g of the indicated GST-tagged protein. B) Immunoprecipitation of HeLa lysates with no antibody (lane 1), IP3R-1 antibody (lane 2), or actin antibody (lane 3) and Western blotted with either IP3R-1 (top) or BRCA1 (bottom) antibodies. *indicates a possible spliceoform of BRCA1 that is detected by the antibody. In our hands, commercial antibodies to BRCA1 were not suitable for reciprocal co-immunoprecipitation. C) Subcellular fractionation by differential centrifugation of HeLa cells. BRCA1 expression was detected at the predicted molecular weight (labeled BRCA1) and a slower migrating band which is likely hyperphosphorylated (labeled pBRCA1). *indicates a possible spliceoform of BRCA1 that is detected by the antibody. Fraction purity was evaluated with organelle controls lactate dehydrogenase (LDH) (cytosol), IP3R-1 (endoplasmic reticulum), cytochrome c oxidase (CytCOx; mitochondria), and histone H1 (nucleus). Panels B and C in this figure utilized HeLa cell lysates.

BRCA1 modulates IP3R function

We next chose to examine the effect of BRCA1 on IP$_3$R function by expressing full length BRCA1 fused to the C-terminus of yellow fluorescent protein (YFP) in HeLa cells and examining the response to escalating doses of histamine. HeLa cells were used in these

experiments because of their ease of transfection and well-characterized calcium release characteristics after histamine challenge (165–167). The YFP-BRCA1 fusion protein has been previously characterized and is functionally comparable to the wild type protein (130,168). As shown in Fig. 3-2A, YFP-BRCA1 is localized to both nuclear and non-nuclear compartments. We measured calcium release in response to 100nM, 1μM, and 10μM histamine in cells expressing YFP-BRCA1 and adjacent cells not expressing the fusion protein. As shown in Fig. 3-2B-D, BRCA1 expression significantly sensitized HeLa cells to 100nM histamine challenge, increasing both peak release (Fig. 3-2C-D) and the number of responding cells (Fig. 3-2E). Expression of BRCA1 did not appear to affect peak release of calcium in response to 1μM histamine or a saturating dose of 10μM histamine (Fig. 3-2F-G). However, at both of these doses there is a significant increase in the oscillation frequency, which would be expected to have profound implications for downstream signaling events (169,170). Thus, expression of BRCA1 has significant effects on calcium signaling through IP_3R-coupled pathways. It has been shown that BRCA1 the BRCA1 binding protein and homologue BARD1 are targeted to mitochondria (164,171), and our subcellular fractionation results indicate a significant amount of BRCA1 is present in a fraction which also contains mitochondria (Fig 3-1C). We hypothesized that BRCA1 may facilitate calcium transfer into mitochondria. To test this hypothesis, we stimulated HeLa cells with 10μM histamine and measured calcium uptake into mitochondria using Rhod-2. As shown in Fig. 3-2I, BRCA1 expression has no effect on calcium uptake into mitochondria.

The above results indicate that BRCA1 has significant effects on IP_3R-coupled calcium release induced by histamine, but does not evaluate if BRCA1 has a direct effect

on IP_3R function. In order to directly measure IP_3R activity, we used saponin permeabilized cells loaded with MagFura-2 to measure ER calcium release induced by IP_3 addition to the bath. MagFura-2 imaging of ER stores is a well-established method for measuring ER calcium store depletion in response to IP_3R activation (31,32). Cell membrane permeabilization also allows for access to the cytosol and the ability to directly activate IP_3R with addition of IP_3 to the bath (174). We found that cells expressing YFP-BRCA1 released significantly more calcium after addition of both sub-saturating (200nM) and saturating (10μM) doses of IP_3 (Fig. 3-2J-K). This indicates that expression of BRCA1 directly increases IP_3R activity, which is consistent with the functional effects on histamine-induced calcium release.

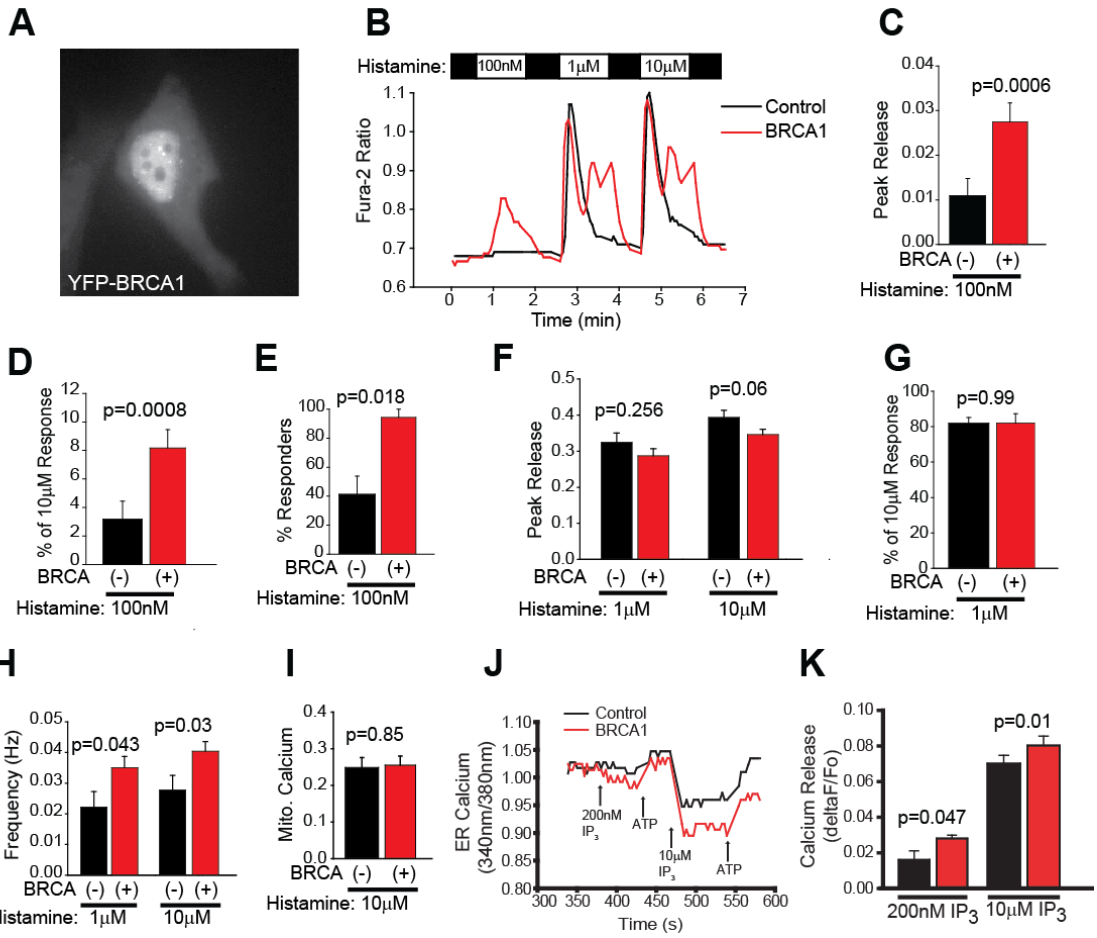
A
YFP-BRCA1
B
Histamine:
100nM
1μM
10μM
Control
BRCA1
Fura-2 Ratio
Time (min)
C
p=0.0006
Peak Release
BRCA (-) (+)
Histamine: 100nM
D
p=0.0008
% of 10μM Response
BRCA (-) (+)
Histamine: 100nM
E
p=0.018
% Responders
BRCA (-) (+)
Histamine: 100nM
F
p=0.256
p=0.06
Peak Release
BRCA (-) (+) (-) (+)
Histamine: 1μM 10μM
G
p=0.99
% of 10μM Response
BRCA (-) (+)
Histamine: 1μM
H
p=0.043
p=0.03
Oscillation Frequency (Hz)
BRCA (-) (+) (-) (+)
Histamine: 1μM 10μM
I
p=0.85
Mito. Calcium
BRCA (-) (+)
Histamine: 10μM
J
Control
BRCA1
ER Calcium (340nm/380nm)
200nM IP3
ATP
10μM IP3
ATP
Time (s)
K
p=0.047
p=0.01
Calcium Release (deltaF/Fo)
200nM IP3
10μM IP3

Figure 3-2. Overexpressed BRCA1 modulates IP3R function.
A) Representative image of a YFP-BRCA1 expressing HeLa cell demonstrating both nuclear and non-nuclear localization. B) Representative single cell traces of calcium release induced by 100nM, 1μM, and 10μM histamine. In this and all subsequent panels, data from control cells are black and BRCA1 expressing cells are red. C) Peak release (change in ratio) induced by 100nM histamine pooled from all cells from four coverslips (n=24 control, n=25 BRCA expressing). D) Release induced by 100nM histamine normalized to the response to a saturating dose of histamine in the same cell. The results are comparable to panel C. E) Percent of cells responding to 100nM histamine. F) Peak release (change in ratio) induced by 1μM and 10μM histamine pooled from all cells as in panel C. G) Release induced by 1μM histamine normalized to the response to a saturating dose of histamine in the same cell. H) Oscillation frequency in response to 1μM and 10μM histamine. No cells oscillated in response to 100nM histamine. I) Change in mitochondrial calcium (deltaF/F0 of Rhod-2 fluorescence) in response to 10μM histamine (n=18 control and 18 BRCA1 expressing cells from five coverslips). J) Representative MagFura-2 traces of ER calcium release in response to IP3 of two cells on one coverslip. One cell is transfected with YFP-BRCA1 (Red). Media was exchanged at the points indicated with the solutions indicated. The traces begin after permeabilization and ER calcium store loading. Data is reported as the ratio of 340nm/380nm, and is indicative of the relative calcium concentration. K) Calcium released (deltaF/F0) from the ER after addition of a sub saturating (200nM) and saturating (10□M) dose of IP3. Control n=11 for 200nM, and n=40 for 10uM; BRCA1 n=10 for 200nM, n=28 for 10uM. Data in panels C-I and K is presented as the mean +/- s.e.m. p values are indicated above each set of bars. All data in this figure was derived from HeLa cells.

The experiments in Fig. 3-2 examined the effects of overexpressed BRCA1 on IP_3R function. We next wished to determine if endogenous BRCA1, which we found to bind endogenous IP_3R-1 (Fig. 3-1B), also modulates calcium release. We obtained two commercial siRNA duplexes targeting human BRCA1 and determined their efficiency in knocking down endogenous BRCA1 expression in HeLa cells. We found that both siRNA

duplexes almost completely abrogated BRCA1 expression in HeLa cells (Fig. 3-3A). We found that one of the siRNA complexes (s458) also upregulated IP_3R-1 expression, which may reflect a compensatory mechanism. Regardless, further experiments were performed with the siRNA duplex which did not affect IP_3R expression (s459). Knockdown of BRCA1 expression had the opposite effect of overexpression, including decreased peak release, decreased oscillation frequency, and a dramatic reduction in the number of percent responders to 100nM histamine compared to cells expressing control siRNA (Fig. 3-3B-E). In addition, BRCA1 knockdown also decreased peak release to 1 and 10 μM histamine (Fig. 3-3F). Thus, endogenous BRCA1 has significant effects on the activity of endogenous IP_3R channels in HeLa cells.

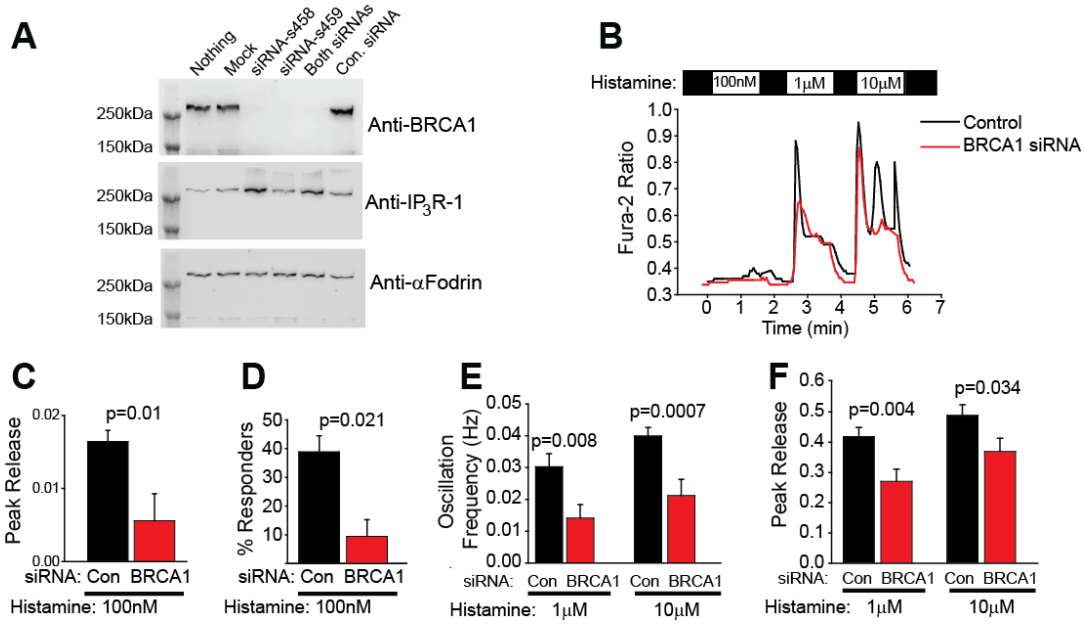

Figure 3-3. Endogenous BRCA1 modulates IP_3R function.

A) Western blot of HeLa cells with siRNA-mediated knockdown of endogenous BRCA1. Two siRNAs (s458, s459) alone and in combination significantly reduced endogenous BRCA1 expression, whereas a control siRNA did not. Probing with an anti-IP_3R-1 antibody revealed that siRNA s458 also increased IP_3R levels. Blotting with anti-α-fodrin was used as a loading control. As full length α-fodrin is very efficiently cleaved during cell death (58), this result also indicates the siRNAs had limited or no toxicity. Finally, this result also validates the specificity of the BRCA1 antibody used in this study. B) Representative traces of histamine release in BRCA1 knockdown cells or cells tranfected with control siRNA. The control siRNA is commercially available and is not homologous to any sequence in the vertebrate transcriptome (Life Technologies catalog number 12935-300). In this and all subsequent panels, control siRNA is in black and BRCA1 siRNA is in red. C) Peak release in control and BRCA1 knockdown cells in response to 100nM histamine. D) Percent responders in control and BRCA1 knockdown cells in response to 100nM histamine. E) Oscillation frequency in response to 1μM and 10μM histamine in control and BRCA1 knockdown cells. F) Peak release in control and BRCA1 siRNA expressing cells in response to 1μM and 10μM histamine. For panels C-F, n=36 for control siRNA and n=34 BRCA1 siRNA expressing cells. All data in this figure was derived from HeLa cells.

BRCA1 increases IP_3R open probability

To determine the direct effects of BRCA1 on IP_3R activity, we recorded single channel currents on isolated nuclei which express recombinant rat IP_3R-1 ((151); see methods). As shown in Fig. 3-4A-B, when the BRCA1 GST-RING domain was included in the patch pipette the open probability of the IP_3R-1 channel in the presence of a subsaturating concentration of IP_3 (1 μM) was dramatically increased (control: 0.21 +/- 0.02, GST-RING: 0.57 +/- 0.06, GST only: 0.21 +/- 0.02). This was due to a destabilization of the closed state of the channel (Fig. 3-4C). Thus, the BRCA1 RING domain directly and potently increases IP_3R activity by modulating channel gating.

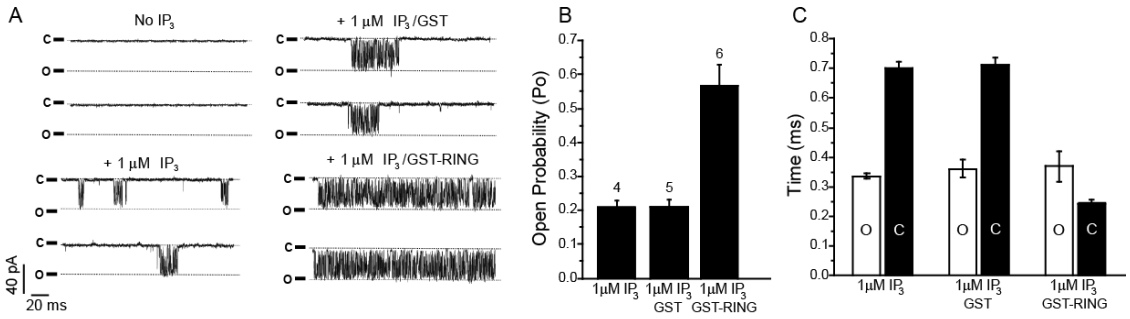

Figure 3-4. BRCA1 increases IP_3R single channel activity.
A) Representative traces of nuclear patches of triple IP_3R knockout DT40 cells expressing recombinant rat IP_3R-1. Traces were recorded with no IP_3 in the pipette, 1µM IP_3, 1µM IP_3 with 30nM GST, or 1µM IP_3 with 30nM GST-BRCA1 RING domain. B) Open probably (Po) of IP_3R-1 channels recorded in the presence of IP_3, IP_3 and GST, or IP_3 and GST-BRCA1 RING domain. The number of channels analyzed are indicated above the bars. C) Mean open (O; white) and closed (C; black) times for each condition.

BRCA1 binds to IP3Rs during cell death to stimulate calcium-dependent apoptosis

We hypothesize that BRCA1 binding to IP_3R increases during cell death, and this is an essential component of its tumor suppressor activity. In order to measure changes in the BRCA1/IP_3R interaction in living cells during apoptosis, we used the FRET pair CFP-IP_3R and YFP-BRCA1. We used the clinically relevant chemotherapeutic paclitaxel to induce apoptosis, and measured dynamic changes in the FRET ratio (and thus binding) in HeLa cells transfected with CFP-IP_3R-1 and YFP-BRCA1 or YFP alone (Fig. 3-5A). We deliberately avoided DNA damaging chemotherapeutics such cisplatin so that the DNA repair activity of BRCA1 would not confound the results. We found a significant increase in cytosolic FRET after treatment with paclitaxel (1μM) in cells expressing both CFP-IP_3R-1 and YFP-BRCA1 (Fig. 3-5B). The kinetics of association are consistent with the time course of activation of cell death proteins such as JNK1 in response to paclitaxel treatment (175). We saw no increase in FRET after treatment with paclitaxel in the nucleus of cells transfected with CFP-IP_3R-1 and YFP-BRCA1 (Fig. 3-5B) or in cells transfected with CFP-IP_3R-1 and YFP only (Fig. 3-5C). These results indicate that BRCA1 is recruited to IP_3R channels on the ER during apoptosis, and support the hypothesis that under resting (i.e., non-apoptotic) conditions only a subpopulation of IP_3Rs are bound to BRCA1.

We next examined the effect of BRCA1 expression on paclitaxel-induced apoptosis. In these experiments we used the patient derived, BRCA1-mutated cell line UWB 1.289 (UWB) isolated from an ovarian carcinoma and this same cell line stably rescued with wild type BRCA1 (UWB-BRCA1). The BRCA1 mutations in the parental UWB line eliminate expression of the BRCA1 protein. Treatment of UWB cells with paclitaxel for 24 hours did not cause an elevation of cytosolic calcium, whereas UWB cells

with rescued BRCA1 expression had significantly elevated calcium consistent with apoptotic calcium release (Fig. 3-5D). This indicates that BRCA1 expression is required for paclitaxel-induced apoptotic calcium release *via* the IP_3R. Measurement of apoptosis by both caspase-3 enzymatic activity and propidium iodide staining both indicated that BRCA1 expression was required for efficient paclitaxel-induced cell death of ovarian carcinoma cells (Fig. 3-5E-F). This suggests that BRCA1 expression restores paclitaxel-sensitivity to the BRCA1-null cells. This is consistent with similar findings by other groups (176).

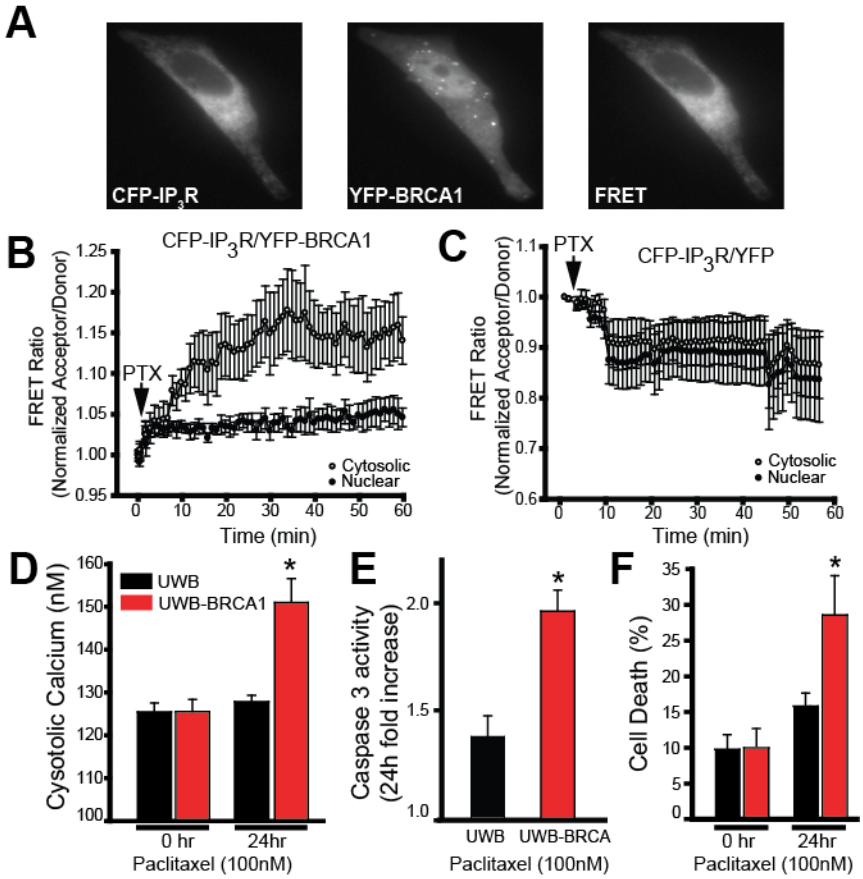

Figure 3-5. BRCA1 interaction with IP_3R increases during apoptosis and mediates cell death.

A) Representative images of CFP-IP3R, YFP-BRCA1, and the FRET signal (CFP excitation, YFP emission; see methods). B) Live cell imaging of FRET dynamics in HeLa cells expressing YFP-BRCA1 and CFP-IP3R. Cells were treated with 1uM paclitaxel (PTX) at the time indicated. Data from FRET ratio of cytosolic and nuclear regions of interest (normalized acceptor/donor) is reported on the x-axis. The data is pooled from 20 cells from 5 separate experiments, and is presented as the mean +/- s.e.m. C) Live cell imaging of FRET dynamics in cells expressing YFP only and CFP-IP3R. Cells were treated identically to panel B. The data is pooled from 22 cells from 5 separate experiments. D) Cytosolic calcium measurements of cells treated with 100nM paclitaxel for 0 and 24 hours. Cytosolic calcium concentration was measured by the ratiometric calcium indicator Fura-2 AM. E) Fold increase in caspase-3 activity in cells treated with 100nM paclitaxel for 24 hours compared to cells treated with DMSO. Caspase-3-like activity was assessed by measuring the linear rate of cleavage of a fluorescent substrate (Ac-DEVD-R110). F) Cell death percentage as measured by propidium iodide staining of cells treated with 100nM paclitaxel for 0 and 24 hours. For D-F the data is pooled from at least three separate experiments. * $p<0.05$. Data in all panels is presented as the mean +/- s.e.m. The data panels A-C was obtained from HeLa cells. The data in panels D-F are from UWB cells or UWB cells stably expressing BRCA1.

DISCUSSION

In this study, we have established for the first time a possible mechanism through which BRCA1 exerts its pro-apoptotic function. Our data suggests a physical and functional interaction between BRCA1 and IP_3R-1 which increases IP_3R activity. We have also shown that BRCA1 is recruited to the ER during apoptosis. While BRCA1 mutations and loss of expression are considered a contributing cause of hereditary breast and ovarian cancer, studies have suggested that BRCA1 down-regulation may be present in a large number of breast tumors without mutations in BRCA1 (146,147,177). Furthermore, it has been shown that cytosolic localization of BRCA1 can be correlated with better disease prognosis (178). We suggest that the pro-apoptotic function of BRCA1 is to increase IP_3R-mediated apoptotic calcium release. This has significant implications for both hereditary and non-hereditary breast and ovarian cancers as BRCA1 expression mediates cellular responses to chemotherapeutics (176). The addition of yet another tumor suppressor,

BRCA1, as a regulator of IP_3Rs adds even more importance to the need to understand the role of calcium homeostasis in tumor progression.

Chapter 4: BRCA1 is Recruited to the ER via a Novel Lipid Binding Domain

Modified in part from:

The BRCA1 Tumor Suppressor Binds to Inositol 1,4,5-Trisphosphate Receptors to Stimulate Apoptotic Calcium Release

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INTRODUCTION

In Chapter 3 we investigated and identified a physical and functional interaction between IP_3R and BRCA1. The IP_3R-BRCA1 interaction occurs at the ER between the RING domain of BRCA1 and the carboxy-tail domain of the IP_3R and causes increased IP_3R open probability. BRCA1 causes increased agonist-induced calcium release as well as apoptotic calcium release. We also found that the IP_3R-BRCA1 interaction increases during paclitaxel-induced apoptosis. This suggests either recruitment of BRCA1 from other organelles to the ER or recruitment of ER-associated BRCA1 to IP_3Rs. As BRCA1 nuclear import and export is regulated by several factors including retention by organelle specific proteins (e.g. BARD1, BRAP), it is possible that the interaction between BRCA1 and IP_3R has an effect on BRCA1 localization (130,142).

Here we investigate the role of IP_3R expression on BRCA1 localization and recruitment to the ER. We show here that BRCA1 ER localization as well as apoptotic recruitment of BRCA1 to the ER is partially dependent on IP_3R expression. We also show that a pool of BRCA1 associates with the ER independent of an IP_3R expression, likely through a protein-lipid interaction between BRCA1 and ER membrane lipids. In support of this we found that BRCA1 has lipid binding activity and binds to several lipids including phosphatidic acid (PA). Paclitaxel is a known microtubule stabilizer, however recent findings have proposed that ER-stress is a more likely mechanism responsible for paclitaxel-induced apoptosis, and that ER-stress markers, including PERK activation, are elevated before any evidence of mitochondrial apoptosis (179). In addition, PERK was recently shown to have a lipid kinase function which produces PA (180). We found that

paclitaxel treatment causes rapid PA production at the ER suggesting a possible mechanism for recruitment of BRCA1.

RESULTS

BRCA1 Recruitment to the ER is Dependent on IP_3R Expression

We examined whether IP_3R expression is required for BRCA1 localization in non-nuclear compartments. We used DT40 cells as well as DT40 IP_3R triple knockout (DT40 TKO) cells. This is the only cell line currently available deficient in all three IP_3R isoforms (31). We transiently transfected DT40 and DT40 TKO with YFP-BRCA1 and nuclear localized DsRed (Fig. 4-1A, left panel), and scored cells with nuclear only or nuclear and cytosolic expression in a blinded manner. The number of cells with BRCA1 non-nuclear localization was significantly higher in the DT40 cells expressing IP_3R compared to DT40 TKO cells (Fig.4-1A, right panel). This indicates that IP_3R expression is a partial determinant of BRCA1 localization outside of the nucleus.

To determine if BRCA1 is recruited to ER membranes during apoptosis, we treated DT40 and DT40 TKO cells with paclitaxel and purified ER-enriched 100,000 xg fractions as in Fig. 3-1C. BRCA1 ER localization increased with paclitaxel treatment, and this increase was dependent on IP_3R expression (Fig. 4-1B). Interestingly, loss of IP_3R expression did not completely prevent constitutive ER localization of BRCA1. This suggests that a sub-population of BRCA1 is associated with the ER independent of both IP_3R expression and apoptosis. We hypothesized that recruitment of BRCA1 to the ER may be mediated by an intrinsic lipid binding activity in the BRCA1 protein.

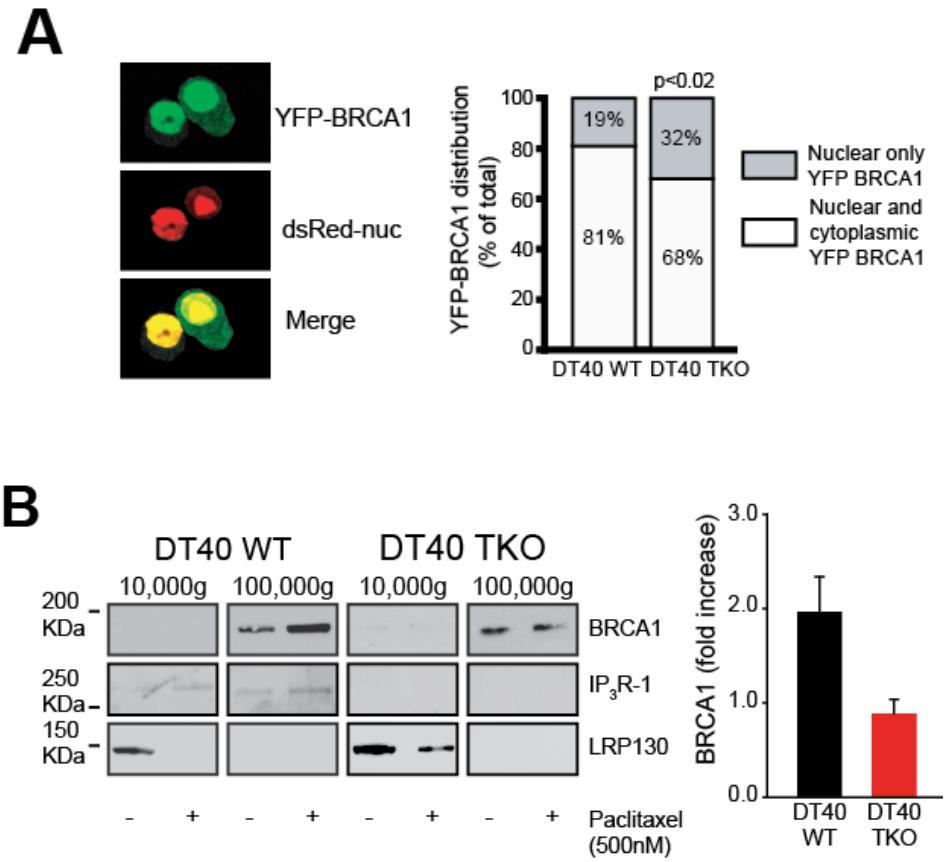
A
YFP-BRCA1
dsRed-nuc
Merge
YFP-BRCA1 distribution (% of total)
p<0.02
19%
81%
32%
68%
DT40 WT
DT40 TKO
Nuclear only YFP BRCA1
Nuclear and cytoplasmic YFP BRCA1
B
DT40 WT
DT40 TKO
10,000g
100,000g
10,000g
100,000g
200 KDa
250 KDa
150 KDa
BRCA1
IP3R-1
LRP130
Paclitaxel (500nM)
BRCA1 (fold increase)
C
DT40 WT
DT40 TKO
Caspase-3 Activity Fold Increase
8h
16h
24h
32h
40h
48h
DMSO
500nM Paclitaxel

Figure 4-1. BRCA1 is recruited to the ER during apoptosis.
A) Distribution of YFP-BRCA1 in nucleus only versus nuclear and cytosol localization in wild type DT40 cells or DT40 cells with triple IP3R knockout (TKO). Nuclear localization was established by co-localization with nuclear-localized DsRed. Representative confocal images are shown in the left panel. Images of cells were scored in a blinded manner as being nuclear only or having both nuclear and cytosolic expression. DT40 n=122, DT40 TKO n= 143. Significance was determined by Fisher's exact test using a 2x2 contingency table ($p=0.0157$). B) Subcellular localization of BRCA1 in DT40 cells treated with or without paclitaxel. DT40 and DT40 TKO cells were treated with 500nM paclitaxel for 48 hours. Cells were harvested and fractionated using differential centrifugation as in Fig. 1C. 20µg of each fraction was used for western blotting with anti-BRCA1 antibody (top row). IP3R antibody was used to show purity of the ER-enriched fraction (middle panel). LRP130, a mitochondrial marker, was used to show the absence of mitochondria proteins in the microsomal ER-enriched fraction (bottom panel). The loss of LRP130 from the mitochondrial fraction after paclitaxel treatment may be related to its putative role in taxol-mediated cell death signaling (181,182). For simplicity, only the mitochondria and ER fractions are shown. The amount of BRCA1 protein present in the ER (100,000xg) fraction was quantified and graphed from three separate experiments, and presented as the fold increase over basal (no paclitaxel). $p<0.05$ The data in panels A-B are from wild type (WT) and triple IP3R knockout (TKO) DT40 cells. C) Fold increase in caspase-3 activity in cells treated with 500nM paclitaxel in DT40 WT cells (black) and DT40 TKO cells (red). Caspase-3-like activity was assessed by measuring the linear rate of cleavage of a fluorescent substrate (Ac-DEVD-R110).

BRCA1 BRCT Has a Putative Lipid Binding Domain

We identified a potential lipid binding domain within the C-terminal tandem BRCT domain of BRCA1 using Adaptive-BLAST (183). This method used a position specific scoring matrix (PSSM) to compare the BRCA1 protein sequence to the fatty acid binding domain of over 1100 fatty acid binding proteins. Each residue in a sequence of BRCA1 with identity or similarity to a fatty acid binding domain received a score of 1(similarity) or 2 (identity). The positional score of each BRCA1 residue was calculated using the number of similar or identical sequences it was a part of. Specifically, we found that amino acid residues 1664-1696 within the first BRCT repeat have a strong potential for lipid binding based upon homology to fatty acid binding proteins (Fig. 4-2A; see methods). Examination of high scoring residues within this sequence identified a basic region with several threonine residues common to lipid binding pockets, including phosphatidic acid binding proteins (Fig. 4-2B; (184,185)). When mapped onto the structure of the tandem BRCT domains, these residues were solvent accessible and thus potentially able to participate in lipid binding (Fig. 4-2C).

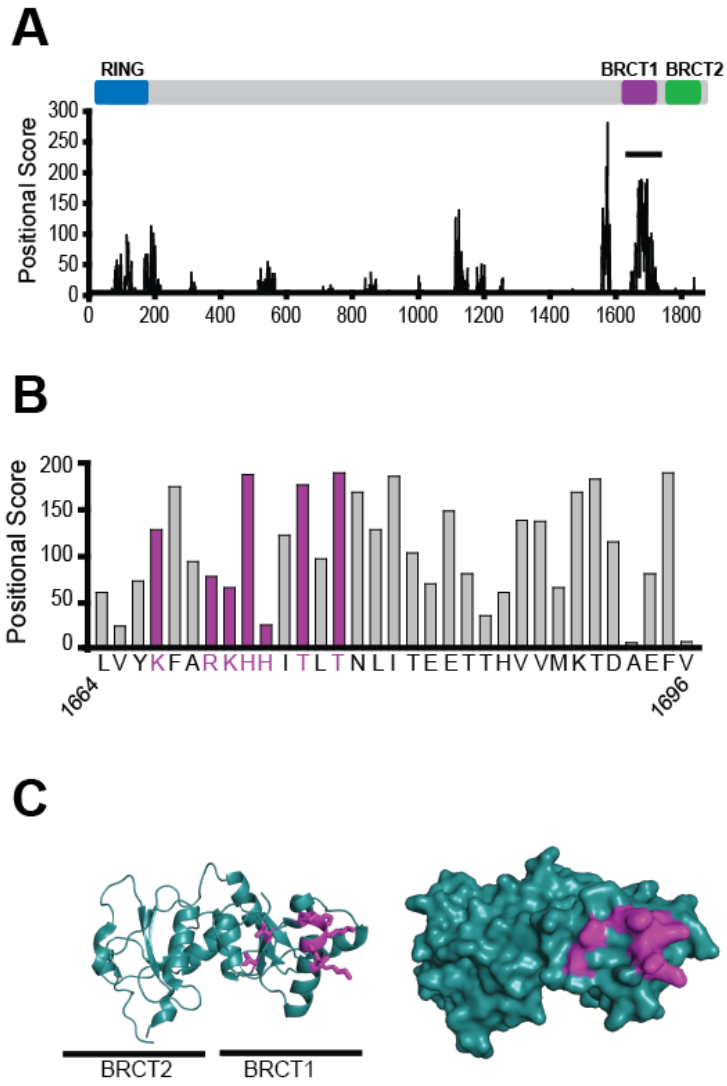

Figure 4-2. BRCA1 BRCT domain has a putative lipid binding domain. A) Positional score of amino acid residues in BRCA1 which have homology to 1185 fatty acid binding protein-specific Position Specific Scoring Matrix (PSSM) libraries (see methods). A high scoring region is present in the first BRCT repeat encompassing amino acids 1664-1696 (indicated by a solid line). Relative positions of domains in BRCA1 are shown above the graph as a schematic. B) Amino acids encompassing the putative lipid binding motif. Basic residues and adjacent threonine residues found in some lipid binding pockets (184,185) are highlighted in purple. C) Ribbon diagram (left) and space fill diagram (right) of the tandem BRCT domains of BRCA1. Purple residues correspond to the highlighted residues labeled in panel B.

BRCA1 BRCT Domain has Lipid Binding Activity

To experimentally test this hypothesis, we purified recombinant GST-BRCT and performed a lipid strip overlay experiment. GST-RING domain was used as a negative control. GST- BRCT was capable of binding several lipids including phosphatidic acid (PA), phosphatidyl inositol-3,5-P_2 and phosphatidyl inositol-4,5-P_2, whereas GST-RING was unable to bind to any lipids (Fig. 4-3). Thus, the BRCT domain of BRCA1 has a previously uncharacterized lipid binding activity which may mediate constitutive localization at ER membranes. Constitutive BRCA1 association with the ER may allow for rapid recruitment to the IP_3R upon apoptotic induction. Of note, clinically relevant mutations of residues within the BRCT domain alter the subcellular distribution of BRCA1, which may be related to the lipid binding activity of the BRCT domain (131). It is also possible that the BRCA1 lipid binding domain may interact with other cellular membranes in addition to the ER. While this may indeed be the case and may have functional relevance, with respect to this study we have shown that BRCA1 is recruited to the IP_3R on ER membranes during apoptosis to mediate apoptotic calcium release (Fig. 3-5), and further that BRCA1 retention at the ER is reduced in IP3R null cells (Fig. 4-1A-B). Thus, at least some of the pro-apoptotic activities of BRCA1 are mediated by ER localization and binding to IP_3R channels.

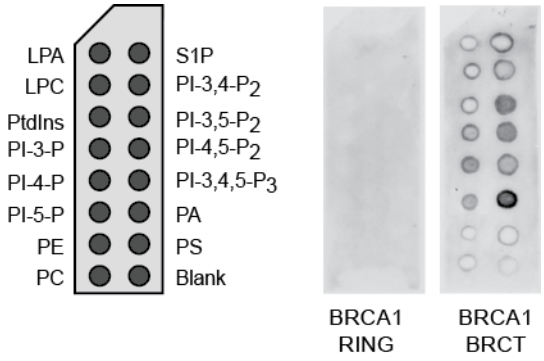

Figure 4-3. BRCA1 BRCT domain has lipid binding activity.
Lipid binding of GST-RING and GST-BRCT to PIP strips. Lipid spots present on the PIP strips are indicated in the schematic.

Phosphatidic Acid Production by PERK Increases at the ER During Apoptosis.

We have now shown that the BRCA1 BRCT domain has lipid binding activity and binds the strongest to phosphatidic acid (PA). PA is synthesized by several enzymes that produce PA by modifying other membrane lipids. PA producing pathways include acyl transferase, phospholipase D (PLD), and diacylglycerol kinase (DGK) (reviewed in (186)). PLD and DGK are considered the primary PA producing enzymes and deregulation of these enzymes have been associated with various disease phenotypes including cancer, neurodegeneration, diabetes, bipolar disorder, and seizure (187,188). PA is present at all cellular membranes and contributes to several signaling pathways (reviewed in (185,186)). We hypothesized that PA production at the ER may have an effect on BRCA1 association and recruitment to the ER. In order to monitor PA production we used the PA sensor PASS (Phosphatidic Acid biosensor with Superior Sensitivity). The PASS construct combines the *S. cerevisiea* Spo20 PA binding domain, a nuclear export signal, and a fluorescent protein. Under resting conditions, PASS cellular distribution is primarily diffuse across the cell. After PA production caused by activation of a PA production pathway, PASS accumulates at the site of PA accumulation and can visualized by fluorescent microscopy (152). We examined the production of PA at the ER by measuring FRET between GFP-PASS and the fluorescent ER-marker DsRed-ER. By monitoring changes in FRET we found that PA is produced at the ER during paclitaxel-induced apoptosis (Fig. 4-4 A-B). PA production at the ER may be a mechanism for not only BRCA1 ER association but also for BRCA1 recruitment to the ER during apoptosis.

Recent studies have shown that PA is produced by the ER stress-activated protein PERK (180). Studies have also shown that paclitaxel treatment can induce ER stress very

rapidly after treatment before any markers of mitochondrial-associated apoptosis can be detected (179). In order to determine if paclitaxel-induced PA production was dependent on PERK activity we used a PERK inhibitor to block PERK-dependent PA production. Pre-treatment of cells with PERK inhibitor prior to paclitaxel treatment inhibited PA production at the ER (Fig. 4-4 C). This suggests that PERK is responsible for paclitaxel-induced production of PA.

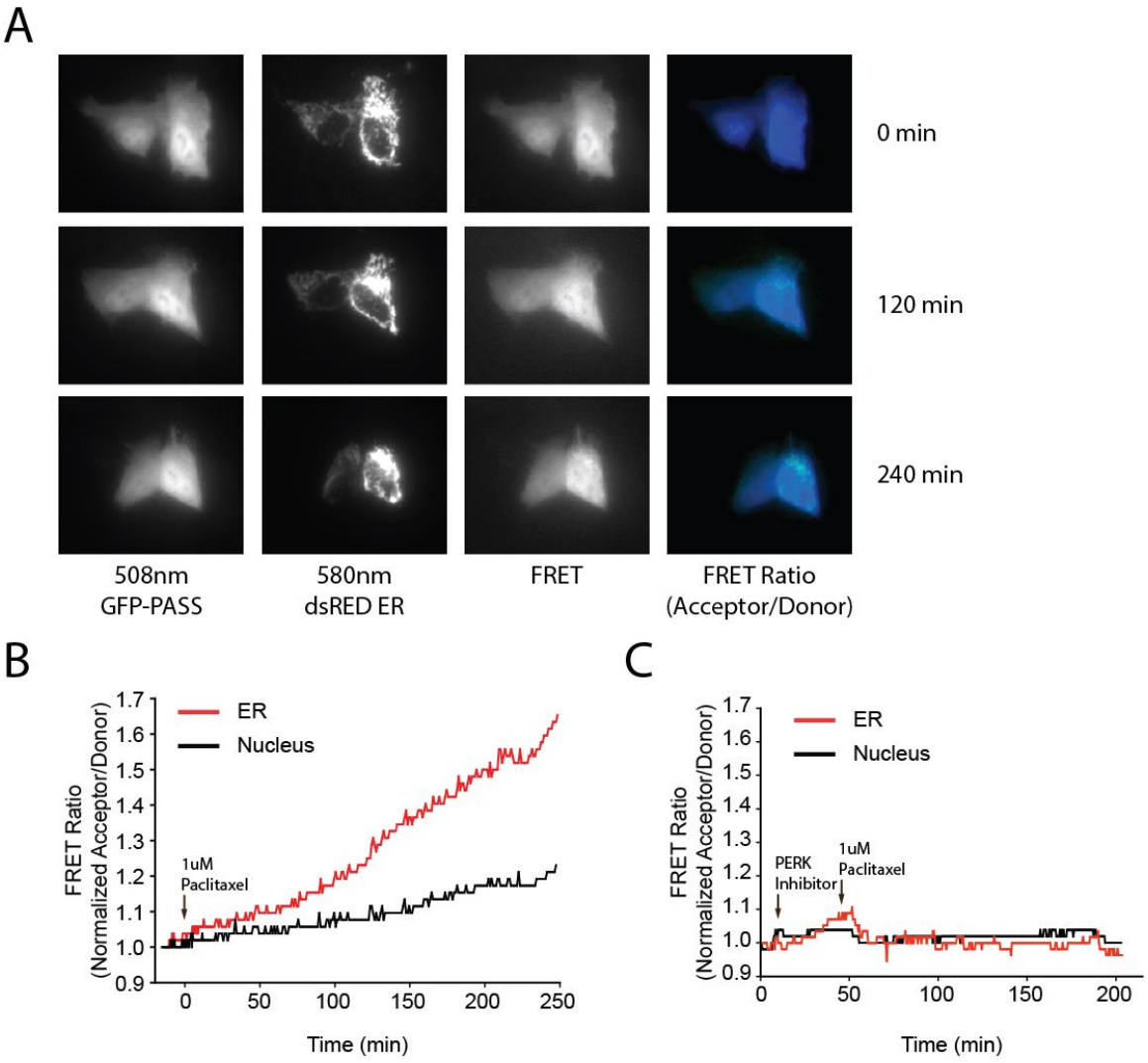

Figure 4-4. Phosphatidic Acid increases at the ER during paclitaxel-induced apoptosis.

A) Representative images of GFP-PASS, dsRED-ER, the FRET signal (GFP excitation, dsRED emission; see methods), and the FRET ratio (dsRED/GFP). Images from 0, 2, and 4 hours after treatment with 1uM paclitaxel. B) Live cell imaging of FRET dynamics in HeLa cells expressing GFP-PASS and dsRED-ER. Cells were treated with 1uM paclitaxel at the time indicated. Data from FRET ratio of cytosolic and nuclear regions of interest (normalized acceptor/donor) is reported on the x-axis. C) Live cell imaging of FRET dynamics in HeLa cells expressing GFP-PASS and dsRED-ER. Cells were pre-treated with PERK inhibitor (GSK 2606414) at the time indicated prior to paclitaxel treatment. Data from FRET ratio of cytosolic and nuclear regions of interest (normalized acceptor/donor) is reported on the x-axis.

DISCUSSION

We have previously found that BRCA1 associates with the ER and interacts physically with the IP_3R at the ER. In this study we show that BRCA1 is recruited to the ER during paclitaxel-induced apoptosis. We show that BRCA1 recruitment to the ER is dependent on IP_3R expression, however a pool of BRCA1 is associated with the ER independent of IP_3R expression. We have identified a putative lipid binding domain in the BRCA1 BRCT domain and found that the BRCA1 BRCT domain has lipid binding activity. Lastly, we found that PA is produced at the ER during paclitaxel-induced apoptosis, suggesting a mechanism for BRCA1 ER association and ER recruitment during apoptosis. Recent studies have shown that paclitaxel treatment induces ER stress including activation of PERK, and that PERK has a lipid kinase activity that converts diacylglycerol (DAG) into PA (179,180). Paclitaxel was shown to cause ER stress by 1-3 hours after treatment and cause apoptotic effects at the mitochondria 6-9 hours after treatment (179).This time course matches our experiments in that we found that PA production at the ER begins about one hour after paclitaxel treatment. These findings suggest a potential mechanism for recruitment of BRCA1 to the ER after paclitaxel treatment which includes ER stress, PA production, BRCA1 recruitment, and increased IP_3R calcium transfer to the mitochondria.

Chapter 5: Conclusions and Future Directions

BRCA1 tumor suppressor activity can be partially explained by its nuclear role in homologous recombination and DNA repair (66,189). However, non-nuclear BRCA1 has a pro-apoptotic function that may also contribute to tumor suppression that is poorly understood. Fully understanding all roles of BRCA1 will allow for rational design of therapeutic strategies to treat BRCA1-null or down regulated cancers as well as BRCA1-positive cancers. In this study we aimed to examine the role of BRCA1 on IP_3R function. While much is known about IP_3R and BRCA1 separately, the interaction between IP_3R and BRCA1 as investigated in our studies in the previous chapters is completely novel.

THE IP_3R-BRCA1 PHYSICAL INTERACTION

In Chapter Three we examined the physical interaction between IP_3R and BRCA1. We examined and confirmed the interaction with multiple methods that included *in vitro* binding of recombinant proteins, co-immunoprecipitation of endogenous proteins and FRET analysis of tagged proteins in live cells undergoing apoptosis. *In vitro* binding experiments using recombinant proteins combined with the FRET analysis confirmed a direct interaction between IP_3R and BRCA1 (Fig. 3-1A, 3-5A). *In vitro* binding experiments exclude any other proteins as necessary for binding, while FRET signals require very close proximity. *In vitro* binding also narrowed down the domains of BRCA1 and IP_3R involved in the interaction between the two proteins. We found that the interaction is mediated by the BRCA1 RING domain and the IP_3R carboxy-terminal domain (Fig. 3-1A). The co-immunoprecipitation experiments and the FRET analysis confirmed that this interaction occurs between endogenous full-length BRCA1 and IP_3R (Fig 3-1B, 3-5A). Furthermore, using single channel recordings we found that BRCA1 has

a direct effect on IP_3R activity which further suggests a direct functional interaction between the two proteins.

We also confirmed using subcellular fractionation and FRET that BRCA1 is associated with the ER in non-apoptotic and apoptotic cells. Future studies should examine the subcellular localization of BRCA1 more closely, including potential plasma membrane and MAM association. Future experiments should also focus on determining the specific binding sites of BRCA1 and IP_3R required for their interaction using truncated recombinant protein in *in vitro* binding experiments.

EFFECT OF BRCA1 ON IP_3R CALCIUM SIGNALING

After identifying a physical interaction between BRCA1 and IP_3R we examined the functional interaction between the two proteins by examining the effect of BRCA1 on agonist-induced calcium release. Extracellular addition of histamine activates the histamine receptor. The histamine receptor is a G-protein coupled receptor and activates phospholipase C through activation of the G-protein. Phospholipase C converts PIP_2 in to diacylglycerol (DAG) and IP_3 which therefore activates the IP_3R. We found that BRCA1 overexpression increases agonist-induced calcium release at subsaturating doses of histamine (Fig. 3-2B-C). We also found that BRCA1 increases calcium oscillation frequency at saturating doses of histamine (Fig. 3-2H). To confirm these results, siRNA knockdown of BRCA1 resulted in a decrease in agonist-induced calcium release and oscillation frequency (Fig. 3-3B-F). This confirms that BRCA1 increases agonist induced calcium release, however it does not suggest a direct effect of BRCA1 on IP_3R function as there are several steps between histidine binding to the histidine receptor and IP_3 binding

to the IP_3R. We next aimed to rule out the possibility that the effect of BRCA1 on IP_3R activity was due to an effect of BRCA1 any of the intermediate steps.

To further investigate the direct effect of BRCA1 on IP_3R function we used permeabilized HeLa cells. This approach allows for direct addition of IP_3R and therefore direct activation of IP_3R. This approach bypasses the intermediate signaling and enzymatic steps between agonist addition and IP_3R activation. This experiment removes any potential upstream or off target effects of BRCA1 that could confound the interpretation of our results. Confirmatory of our previous findings we found that with direct addition of IP_3, overexpression of BRCA1 caused increased calcium release when compared to wild type cells (Fig3-2J-K). This suggests that BRCA1 has a direct effect on IP_3R activity and that BRCA1 does not require any upstream signaling to modulate IP_3R function. BRCA1 increases the sensitivity of the IP_3R to IP_3 to increase calcium release. Further experiments could be performed to investigate the effect of cancer-associated mutations on BRCA1-relguation of IP_3R function. As we found in Figure 3-1A, the RING domain of BRCA1 interacts with the IP_3R. As discussed in the Introduction, many cancer-related mutations occur in this region. Several of these mutations inhibit BRCA1 function in DNA repair and ubiquitination, it is possible that RING mutations may also have an effect on BRCA1 modulation of IP_3R activity. Interestingly, the IP_3R can also be regulated by ubiquitination (190). Further studies should investigate possibility of BRCA1 regulation of the IP_3R through ubiquitination.

BRCA1 RING DOMAIN DESTABILIZES THE IP_3R CLOSED CONFORMATION

While we have shown that BRCA1 has a direct effect on IP_3R calcium release and increases IP_3R sensitivity to IP_3, we aimed to further confirm that a direct interaction

between BRCA1 and IP_3R causes this effect. Other proteins (e.g. PML) are protein scaffolds that recruit other proteins that directly regulate IP_3R activity. Since BRCA1 acts as a scaffold in HR it is possible that BRCA1 simply acts as a scaffold to modulate IP_3R activity by recruiting another protein into the proximity of the IP_3R. We have previously determined that the BRCA1 RING domain mediates an interaction with the carboxy-terminal domain of IP_3R, therefore we used recombinant BRCA1 RING domain in a nuclear patch clamp experiment to determine the effect of BRCA1 on IP_3R gating and conductance. Addition of BRCA1 RING domain to an IP_3R isolated by an on-nucleus patch clamp caused an increase in the IP_3R open probability which was caused by destabilization of the closed state of the IP_3R (Fig. 3-4B-C). This experiment confirms that a direct interaction between the BRCA1 RING domain and IP_3R causes the BRCA1-mediated increase in calcium signaling that we have seen in both agonist-induced and apoptotic calcium release. This experiment further confirms that BRCA1 has a direct effect on IP_3R function. These experiments were carried out at optimal levels of calcium and ATP and a subsaturating concentration of IP_3. As reviewed previously, IP_3R function is affected greatly by changes in IP_3, calcium, and ATP concentrations. Future studies should further examine the effects of BRCA1 regulation of IP_3R function in relation to changing IP_3R ligand concentrations. Additionally, as mentioned in the previous section, cancer-related BRCA1 RING mutations should be tested in regards to IP_3R activity.

EFFECT OF BRCA1 ON APOPTOTIC CALCIUM RELEASE

Since IP_3Rs have a strong role in apoptotic calcium signaling and we have shown both a physical and functional interaction between BRCA1 and IP_3R, we next examined the effect of BRCA1 on apoptotic calcium release. Not only did we find increased

apoptotic calcium release in BRCA1-expressing cells when compared to BRCA1-null cells, we found that BRCA1 expression was required for apoptotic calcium release with paclitaxel treatment. This suggests a potential non-nuclear and pro-apoptotic mechanism for BRCA1. This also suggests that non-nuclear BRCA1 may act as a pro-apoptotic protein by increasing IP_3R apoptotic calcium release.

Other groups have shown that BRCA1 expression affects the sensitivity of cells to various stimuli depending on the type of stimuli (176). BRCA1 protects cells from DNA-damaging agents, but sensitizes cells to other types of drugs like paclitaxel. Future directions should include experiments to determine if BRCA1-dependent apoptotic calcium release is involved in other types of apoptotic stimuli (e.g. UV, cisplatin, staurosporine).

BRCA1 Recruitment to the ER is Dependent on IP_3R

To examine the subcellular location of BRCA1 during apoptosis we used FRET imaging and subcellular fractionation after treatment with paclitaxel. Using CFP-IP_3R and YFP-BRCA1 as a FRET pair, we found that the interaction between the two proteins increases during paclitaxel-induced apoptosis (Fig. 3-5B). This could be due to recruitment of BRCA1 from the nucleus to the ER where it interacts with IP_3Rs or due to recruitment of BRCA1 already associated with the ER that binds to IP_3Rs during paclitaxel-induced apoptosis. We further examined BRCA1 localization during apoptosis using subcellular fractionation. We found that a pool of BRCA1 is associated with the ER during non-apoptotic conditions and this pool increases during paclitaxel-induced apoptosis (Fig. 4-1B). This suggests recruitment of BRCA1 from other organelles during apoptosis. We also found that IP_3R knock-out cells show no increase in ER-associated BRCA1 during

apoptosis, suggesting that apoptotic recruitment of BRCA1 to the ER is IP_3R-dependent. Lastly, we found that a pool of BRCA1 is associated with the ER independent of IP_3R expression in non-apoptotic cells. This suggests that an additional mechanism may be responsible in part for BRCA1 ER-association. We hypothesize and further discuss in Chapter Four that BRCA1 associates with the ER through a lipid binding domain. In summary, these experiments suggest that BRCA1 is recruited to the ER in an IP_3R dependent manner during paclitaxel-induced apoptosis, however a pool of BRCA1 is also associated with the ER during non-apoptotic conditions independent of IP_3R expression.

We have determined that BRCA1 is recruited to the ER during apoptosis. However, we have not determined if BRCA1 is recruited from other organelles to interact with IP_3R or if BRCA1. In order to determine if BRCA1 is recruited from the nucleus leptomycin B or NES mutated BRCA1 should be used to block nuclear export of BRCA1 during apoptosis. This would determine if BRCA1 nuclear export is required for recruitment of BRCA1 to the ER during apoptosis or if BRCA1 is recruited from other non-nuclear pools to the ER.

BRCA1 BRCT DOMAIN HAS LIPID BINDING ACTIVITY

In order to explain the IP_3R-independent BRCA1 association with the ER we examined the lipid-binding potential of BRCA1. We found that BRCA1 has a putative lipid binding domain within the BRCT region with a bioinformatics approach (Fig. 4-2). We tested this using PIP strips and found that the BRCT domain of BRCA1 indeed has lipid binding activity. We found that the BRCT domain binds to the lipids PA, phosphatidyl inositol-3,5-P_2 and phosphatidyl inositol-4,5-P_2 (Fig 4-3). We have now identified an IP_3R binding domain in the BRCA1 RING domain and a putative lipid

binding domain in the BRCA1 BRCT domain. This suggests that the BRCT domain mediates ER association of BRCA1 while the RING domain mediates IP_3R interaction (Ill 5-1). Interestingly, other studies have linked BRCA1 to lipid synthesis through an interaction with and inhibition of the Acetyl-CoA Carboxylase alpha (ACCA) resulting in inhibited fatty acid production (191). However, the link between those findings and our findings are unclear at this time.

While we did not test a fully exhaustive array of lipids, this experiment gave us a good starting point and confirmed that the BRCT domain has lipid binding activity. Further experiments could confirm the lipid binding activity in a more physiological setting. These experiments could include lipid bead pulldown experiments and synthetic liposome pulldown experiments. A liposome pulldown experiment would be especially useful since the lipid composition of the vesicles can be varied to resemble that of a cellular membrane.

An important additional future direction would be deletion of the BRCT domain of BRCA1 and repetition of the FRET analysis and subcellular localization experiments described earlier (Fig. 3-5 and 3-1). This set of experiments would determine if the BRCA1 BRCT domain is necessary and sufficient for ER-association as well as BRCA1 recruitment to the ER during apoptosis. Using the fatty acid binding protein prediction data in Figure 4-2 and logical comparison of the BRCA1 BRCT domain to other PA binding proteins we can identify residues of potentially physiological importance in terms of lipid binding (184,185). We have identified the basic residues in the sequence 1670RKHH1673 and the threonine residues in the sequences 1675TLT1677 and 1681TEETT1685 as potentially important residues. Charge reversal of the basic residues or mutation of the threonine residues to alanine may have an effect on lipid binding. The

mutations could be made in either YFP-BRCA1 or GST-BRCT constructs. Mutated YFP-BRCA1 could be used in FRET imaging experiments similar to Figure 3-5 with paclitaxel treatment to determine the effect of these potential lipid binding regions on ER association and apoptotic recruitment of BRCA1 to the ER. Mutated GST-BRCT could be used in *in vitro* experiments to determine the effect of these potential lipid binding regions on lipid binding using PIP strip experiments similar to Figure 4-2.

PHOSPHATIDIC ACID PRODUCTION INCREASES AT THE ER DURING APOPTOSIS

We have shown that PA production by PERK increases exclusively at the ER during apoptosis using FRET imaging of the FRET pair GFP-PASS and dsRED-ER. This suggests a possible mechanism for BRCA1 recruitment to the ER during apoptosis, as we have also shown that BRCA1 has PA binding activity (Fig. 4-3). Recently, a group found that the ER-stress activated protein, PERK, has a lipid kinase activity and produces PA (180). Since PERK is a transmembrane protein retained in the ER, this suggests a potential mechanism for ER specific PA production. Additionally, paclitaxel has been shown to activate markers of ER stress including PERK (however, only the protein kinase activity was tested) (179). This study found that paclitaxel-induced ER-stress occurs at 1-3 hours after paclitaxel treatment, while mitochondrial effects appear after 6-9 hours suggesting that ER stress precedes mitochondrial effects and may be the cause of apoptotic cell death due to paclitaxel treatment. This time course agrees with our findings as we found increased PA production at the ER after 1 hour of paclitaxel treatment (Fig. 4-4).

We hypothesize that paclitaxel treatment induces ER stress, PERK activation and PA production, which causes recruitment of BRCA1 to the ER where it interacts with IP_3Rs

to increase apoptotic calcium release (Ill. 5-1). Further examination of this hypothesis is a major potential future direction for this work. The mechanism of action for paclitaxel is not clearly understood and may be cell cycle-dependent. Specifically, the mechanism for induction of ER-stress and the resulting apoptotic cell death is not well characterized (192). However it is well established that paclitaxel treatment causes rapid activation of the kinase JNK (175). JNK regulates several proteins including members of the Bcl-2 family and when activated, inhibits the anti-apoptotic effects of Bcl-2 by phosphorylation which could link paclitaxel induced ER-stress to apoptotic cell death (reviewed in (193)). By continuing to examine this hypothesis, it is possible that BRCA1 may be a secondary link between paclitaxel-induced ER stress and apoptosis.

GENERAL CONCLUSIONS AND SIGNIFICANCE

BRCA1 localization has been a debated topic for many years (162,194). Non-nuclear roles of BRCA1 have been identified at the centrosome, the mitochondria and now the ER. BRCA1 localization has also been linked to its pro-apoptotic potential and its tumor suppressor function (130,194). Similarly, the effect of different classes of chemotherapeutics can differ based on the BRCA1-status of a cell (176). We have found that BRCA1 increases IP_3R-mediated calcium release at the ER through a direct interaction independent of any other cellular effects of BRCA1. We have also found that BRCA1 is recruited to the ER during apoptosis and that BRCA1 expression is required for apoptotic calcium release after paclitaxel treatment and that BRCA1 expression sensitizes cells to paclitaxel treatment. Other groups have found that BRCA1 protects cells from DNA damaging agents (e.g. Cisplatin, UV damage) through the DNA-repair function of BRCA1. It is possible then that the chemotherapeutic efficacy of different classes of

chemotherapeutics may differ based on the location of the mechanism (e.g. ER stress or DNA damage in the nucleus) of apoptotic-induction in regards to BRCA1 localization as depicted in our current working model (Ill. 5-1).

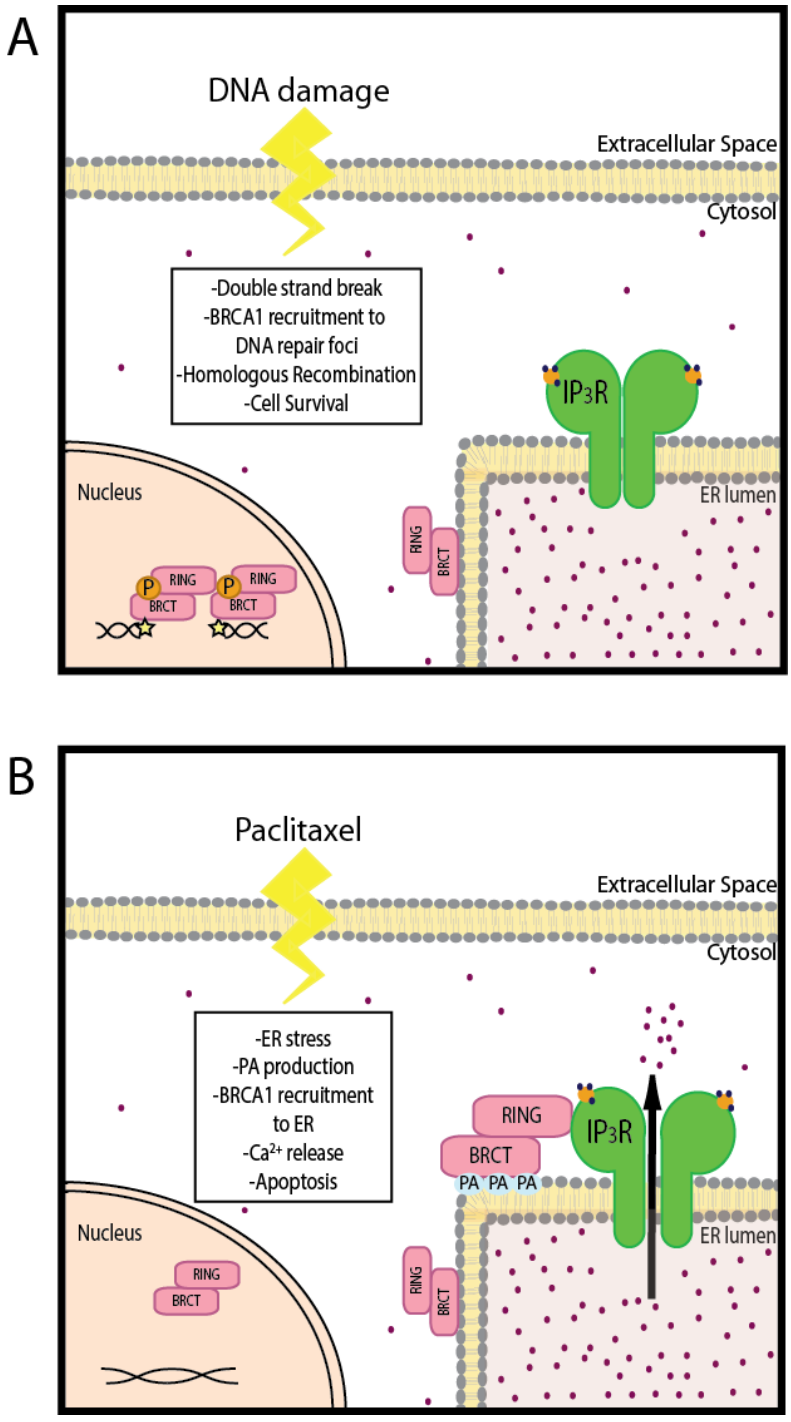

Illustration 5-1. Working model of BRCA1 involvement in two types of apoptotic stimuli.

DNA damage (A) and paclitaxel (B) cause opposite effects in cells that express BRCA1. DNA damage causes nuclear retention of BRCA1 at nuclear foci. Paclitaxel treatment causes ER stress and recruitment of BRCA1 to the ER where it causes increased calcium release from the IP_3R and apoptosis. BRCA1 is depicted by its two domains "RING" and "BRCT".

DNA damage-inducing agents (e.g. Cisplatin, UV) cause DNA damage in the nucleus. DNA double strand breaks can be repaired by BRCA1-dependent HR. DNA damage-induced signaling causes BRCA1 recruitment to nuclear DNA damage repair foci, therefore preventing nuclear export of BRCA1. Nuclear retention of BRCA1 would prevent recruitment of pro-apoptotic BRCA1 to the ER and prevent increased BRCA1 interaction with IP_3R and increased pro-apoptotic calcium release (Ill. 5-1). This agrees with the finding that BRCA1 expression protects cells from apoptosis after cisplatin treatment (176).

Microtubule stabilizing agents (e.g. paclitaxel, vinorelbine) induce cell death through stabilization of microtubules and paclitaxel may induce apoptosis through ER stress, both non-nuclear mechanisms. In these situations, BRCA1 is not sequestered in the nucleus, as in the previous example, and BRCA1 is available to be recruited to the ER to promote apoptotic IP_3R calcium release. This agrees with the finding that BRCA1 expression sensitizes cells to microtubule stabilizing agents ((176), and Fig 3-5D-F).

Future directions should include analysis of the role of BRCA1 in response to apoptotic stimuli with various mechanisms of triggering apoptosis. Experiments should examine recruitment of BRCA1 to the ER in response to various stimuli. If BRCA1 is not recruited to the ER by DNA-damaging agents, nuclear retention of BRCA1 may explain why an apoptotic pathway is not activated. Other ER-stress inducing agents may also cause BRCA1 recruitment to the ER. An interesting experiment would be to examine the efficacy of an ER-stress inducing agent in BRCA1 knock-down cells. It is also possible that BRCA1 nuclear export and association with the ER is a common phenomenon across all types of apoptosis. This would suggest that BRCA1 is exported from the nucleus as a result

of overwhelming cellular stress, independent of the type of stress. This would suggest a "switch or "sensor" function of BRCA1. In this case BRCA1 may act as a sensor that determines if the level of DNA damage is too overwhelming to be repaired by DNA repair machinery. This could possibly be associated with the cell cycle checkpoint function of BRCA1 that has been previously established. It is also possible that the pool of BRCA1 associated with the ER under non-apoptotic conditions may be sufficient to activate IP_3Rs under stress conditions regardless of the type of stress. This could be examined using leptomycin B to inhibit nuclear export of BRCA1. NES mutant BRCA1 should not be used here since NES mutant BRCA1 localizes primarily to the nucleus which would result in much lower amounts of ER-associated BRCA1 when compared to wild type BRCA1.

The ability of BRCA1 to be exported from the nucleus to interact with the IP_3R may be critical for the ability of an agent to induce cell death. As testing the BRCA1 status of a patient or a tumor biopsy becomes easier and more cost effective, it is essential that we continue to investigate the role of BRCA1 in modulating the effect and efficacy of chemotherapeutic strategies. The findings presented here offer a novel pathway through which BRCA1 functions outside of the nucleus and a potential pathway that may affect the efficacy of chemotherapeutics in cancer patients.

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Mason Guest Scholar Award, 2013
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PEER REVEIWED ARTICLES:

Serena C. Hedgepeth, M. Iveth Garcia, Larry E. Wagner II , Ana M. Rodriguez, Sree V. Chintapalli, Russell R. Snyder, Gary D.V. Hankins, Beric R. Henderson, Kirsty M. Brodie, David I. Yule, Damian B. van Rossum, and Darren Boehning; The BRCA1 Tumor Suppressor Binds To Inositol-1,4,5-Trisphophate Receptors To Stimulate Apoptotic Calcium Release. *Journal of Biological Chemistry* (2015)In press.

Serena L. Clark, Ana M. Rodriguez, Russell R. Snyder, Gary D.V. Hankins, Darren Boehning; Structure-Function of the tumor suppressor BRCA1. *Computational and Structural Biotechnology Journal* (2012).

ABSTRACTS:

"BRCA1 is localized to the ER and directly influences apoptotic calcium signaling"

9/23/10 -- Neuroscience and Cell Biology Research Retreat

Poster Presentation

Galveston, TX

"BRCA1 is localized to the ER and directly influences inositol-1,4,5-trisphosphate receptor- mediated apoptotic calcium signaling"

3/9/11 – Biophysical Society National Meeting

Poster Presentation

Baltimore, Maryland

"BRCA1 Interacts with IP_3R and Phosphatidic Acid at the ER During Apoptosis"

2/27/2014 – UT Health Biochemistry Graduate Program Retreat

Oral Presentation

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3/4/2014 – Cell Biology Graduate Symposium

Oral Presentation

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