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Identifying Substrate and E2 Interactions of the BRCA1/BARD1 Ubiquitin Ligase

Devin Eugene Christensen

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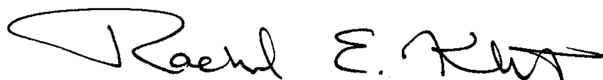

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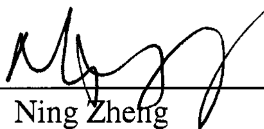

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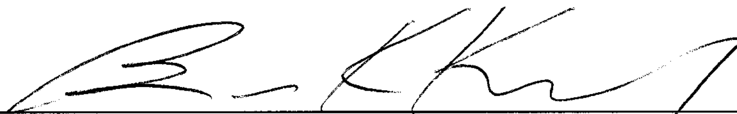

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Abstract

Identifying Substrate and E2 Interactions of the BRCA1/BARD1 Ubiquitin Ligase

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My thesis project addresses the need to understand the function of the Breast Cancer Susceptibility Protein, BRCA1, during the process of protein ubiquitination. A significant number of known cancer predisposing mutations are found within BRCA1's RING domain which, when associated with BARD1, functions as a ubiquitin ligase. Cancer associated RING mutations of BRCA1 eliminate ligase activity *in vitro*. Substrates of the BRCA1/BARD1 ubiquitin ligase are likely to be involved in important cellular processes related to the function of BRCA1 in tumor suppression, and provide a direct link between BRCA1's ligase function and the development of cancer.

A ubiquitin ligase interacts with both a substrate and a ubiquitin conjugating enzyme (E2) to mediate the transfer of ubiquitin from the E2 to the substrate. To properly identify substrates, the E2s that interact and function with BRCA1 must also be determined. Using a "structure-based" yeast two-hybrid strategy, six new BRCA1-

E2 interactions were identified for a total of ten E2s that bind to the BRCA1 RING. I found that for BRCA1 the E2 determines the type of ubiquitination product: depending on the E2 present during a ubiquitination assay different ubiquitination products are synthesized on a protein substrate including, mono-ubiquitin, Lys6-, Lys48-, or Lys63-linked poly-ubiquitin chains.

I have identified two proteins that interact with BARD1, BABS and ZO-2. I also discovered that BABS is ubiquitinated by BRCA1/BARD1, *in vitro*, using a subset of the BRCA1-interacting E2s. Ubiquitin transfer is dictated by substrate-E2 interactions, as E2s that transfer ubiquitin to BABS share the common ability to interact with BABS. Though not tested as a substrate, ZO-2 may provide a link between BRCA1 and the tissue specific nature of cancer associated mutations. ZO-2 is a suspected tumor suppressor that has been linked to estrogen signaling and breast tumorigenesis. The identification of BRCA1/BARD1 substrates, and the fate conferred upon them by the ubiquitin tag, will lead to a better understanding of the association between loss-of-function mutations in BRCA1 and breast cancer.

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DEDICATION

For my beautiful wife Shaundeen, and our sons Garrison and Logan

Chapter 1. Introduction to BRCA1 and the Ubiquitination Pathway

BRCA1 and Breast and Ovarian Cancer Susceptibility

Inherited susceptibility to breast cancer accounts for approximately 10% of all breast cancer cases and is largely attributable to germ line mutations in either *BRCA1* or *BRCA2*. A woman's risk of developing breast cancer is greatly increased based upon family history of the disease. The number of occurrences, a young age at onset of disease, and a history of ovarian cancer within the immediate and extended family greatly increase a woman's risk. A strong genetic link to breast cancer lead to a proposal in 1990 of a gene encoding inherited susceptibly to breast cancer (Hall et al., 1990). Four years later the breast cancer susceptibility gene, *BRCA1*, was identified (Miki et al., 1994). Mutations in this tumor suppressor gene predispose to both breast and ovarian cancer with a lifetime risk of over 80% and over 40% respectively (Zhang and Powell, 2005).

Since its discovery, BRCA1 has been intensely investigated to define its normal cellular function(s), which will lead to an understanding of why inherited loss-of-function mutations lead to breast and ovarian tumorigenesis. Multiple cellular functions have been suggested for BRCA1 that may contribute to its tumor suppressor activity: cell cycle control, DNA damage repair, apoptosis, transcription, chromosomal segregation, and protein ubiquitination (reviewed in Daniel, 2002; Kubista et al., 2002b; Lacroix and Leclercq, 2005). The function of BRCA1 during the process of DNA damage repair has received the most attention as being a critical determinate of tumor suppression. Cells deficient in BRCA1 exhibit an increased level of chromosomal

aberrations and have an increased sensitivity to DNA damaging agents (Deng, 2006). Three different DNA damage repair pathways are deficient in cells lacking BRCA1: homologous recombination repair (HHR), non-homologous end joining (NHEJ), and mismatch repair (MMR). Several reports have also found BRCA1 associated directly or indirectly with several proteins known to be involved during the process of DNA damage repair including RAD51, MRE11-RAD50-NBS1 complex, ATM, BLM, and H2AX (reviewed in Deng and Wang, 2003). The precise function of BRCA1 during DNA damage repair is unknown. However, cellular localization studies have found that in response to DNA damaging agents, such as ionizing radiation, BRCA1 diffuses from S-phase specific nuclear foci and reaccumulates at DNA-damage specific foci (Scully et al., 1997). The nature, composition, and function of either foci is unknown. The only reported difference between the two foci is the colocalization of PCNA with BRCA1 at DNA-damage specific foci and not at S-phase foci.

Although there has been no report of a direct interaction between BRCA1 and PCNA, an understanding of PCNA function may allow a better understanding of the nature of BRCA1-containing foci that form in response to DNA damage. PCNA plays a key role in both DNA replication and repair by acting as a sliding clamp ensuring the processivity of DNA polymerases and recruiting proteins involved in DNA damage repair. Actively growing cells need to fully replicate their DNA prior to exiting S-phase of the cell cycle. Damaged DNA results in stalled replication forks delaying the process of DNA replication. Post-replication repair encompasses the mechanisms that a cell uses to deal with stalled replication forks and utilizes both error-prone and error-free processes. PCNA has a key role in determining how the cell chooses between error-

prone and error-free pathways in dealing with a stalled replication fork (reviewed in (Watts, 2006). Mono-ubiquitination of PCNA at a stalled replication fork leads to recruitment of translesion polymerases, which are error-prone leading to reduced fidelity. In contrast, poly-ubiquitination of PCNA leads to error-free pathways of DNA damage repair believed to involve template switching, although the nature of this pathway is still unclear. The Rad6-Rad18 complex in responsible for mono-ubiquitination of PCNA and the Rad5-Ubc13-Mms2 complex is responsible for poly-ubiquitination of mono-ubiquitinated PCNA. Most of the research into PCNA function at stalled replication forks has been conducted in yeast PCNA also functions in a similar and perhaps more complex manner in human cells (Unk et al., 2006). Interestingly, human cells deficient for Ubc13 are also compromised for homologous recombination repair and display a phenotype highly similar to BRCA1 deficient cells (Zhao et al., 2007).

The reported association of BRCA1 with several DNA damage repair pathways and the sensitivity to DNA damaging agents in BRCA1-deficient cells has resulted in an increased emphasis on BRCA1's potential function in DNA damage repair as being a critical determinate of tumor suppression (Durant and Nickoloff, 2005; Jasin, 2002; Venkitaraman, 2001). A proposed mechanism by which loss of BRCA1 function allows for tumor progression suggests BRCA1 deficiency might result in the accumulation of DNA damage, and the inability to respond appropriately to genomic damage leads to tumorigenesis (Zhang and Powell, 2005). However, an interesting paradox with BRCA1 is that although it is expressed in most tissues in the body, mutations of BRCA1 predispose primarily to epithelial tumors of the breast and ovarian

tissues. Loss of a critical role in DNA damage repair may not explain the tissue specificity of BRCA1 cancer associated mutations.

A possible mechanism for the tissue specificity of BRCA1 loss-of-function mutations and cancer is a potential connection to the estrogen receptor (ERα). Breast cancer has long been established as a hormone based disease, as inappropriate responses to the mitogenic actions of estrogens occur in the majority of malignant breast tumors (McDonnell and Norris, 2002). The ovarian hormone estrogen has a primary role in the establishment and maintenance of reproductive function (McDonnell and Norris, 2002) and regulates the growth and development of breast and ovarian epithelial cells. Tissue development requires two basic steps; first, cellular proliferation to generate more cells, followed by, differentiation of those cells to a specific cell type. During differentiation the processes underlying proliferation are slowly inhibited until cells are completely differentiated. While estrogen acts on ER to promote the proliferation of breast epithelial cells, BRCA1 seems to play a role in control of proliferation and differentiation. Evidence in support of a role of BRCA1 in cellular proliferation and development is found in the developing mouse where BRCA1 is highly expressed in all tissues, particularly those that are rapidly proliferating and undergoing differentiation (Marquis et al., 1995). Also, inhibition of BRCA1 expression triggers accelerated proliferation rates and over-expression of BRCA1 causes proliferation arrest suggesting an important role in controlling the process of cellular growth and expansion (reviewed in (Kubista et al., 2002b). Furthermore, BRCA1 may function in a tissue specific manner in controlling the process of differentiation as its expression is upregulated in breast epithelial cells induced to differentiate *in vitro* (Rajan et al., 1996), while forced

reduction of BRCA1 expression attenuates the *in vitro* differentiation of breast epithelial cells, but not muscle or neuronal cells (Kubista et al., 2002a). A strong case for the tissue specificity of BRCA1 mutations may be provided by the ability of BRCA1 to inhibit estrogen receptor (ERα) signaling (Fan et al., 1999). Loss of the inhibitory effect of BRCA1 on ERα could result in uncontrolled proliferation of estrogen responsive breast epithelial cells promoting the development of breast cancer. Additionally, loss of the DNA damage repair functions of BRCA1 could also be a factor in this model as uncontrolled rapid proliferation would result in the multiplication of non-corrected DNA damage further promoting the development of breast cancer. A potential link between BRCA1 and estrogen signaling is a very interesting prospective as both breast and ovarian tissues are estrogen responsive, however no link has been observed between BRCA1 and cancers found in two other estrogen responsive tissues the endometrium and cervix.

Despite proposed mechanisms of breast tumor formation by *BRCA1* loss-of-functions mutations, the precise cellular function of BRCA1 is unknown. Thus, a better understanding of BRCA1 function(s) and the tissue specificity of cancer associated mutations will come from an investigation of its biochemical properties. *BRCA1* encodes a large protein of 1863 amino acid residues which contains two highly conserved domains. At the amino-terminus of BRCA1 is a RING domain which functions in the process of ubiquitin ligation (Brzovic et al., 2003; Lorick et al., 1999) and at the C-terminus are two BRCT repeats reported to bind to phosphorylated peptides (Yu et al., 2003) (Fig. 1.1). Cancer associated mutations of BRCA1 are located throughout the protein, however, a significant number ~20% lie with in the

RING domain which encodes only about 5% of the protein (Brzovic et al., 2001). Hence, a closer inspection of the function of the BRCA1 RING domain may provide the best opportunity to investigate why loss-of-function mutations lead to breast cancer susceptibility.

A major advancement in understanding BRCA1 function came through the discovery that RING domains can function as ubiquitin protein ligases (Lorick et al., 1999). The ubiquitin ligase activity of the RING domain of BRCA1 requires the tight association of another protein BARD1 (BRCA1 Associated RING Domain) (Hashizume et al., 2001). BARD1 was one of the first proteins found to associate with BRCA1 (Wu et al., 1996) and contains similar architecture to BRCA1 in that it also has an amino-terminal RING domain and C-terminal BRCT repeats. Most of the BRCA1 protein found within a cell is associated with BARD1 (Yu and Baer, 2000). Structural investigation into the binding between BRCA1 and BARD1 has determined that their interaction is mediated by hydrophobic residues that flank their respective RING domains, which form a four-helix bundle (Fig. 1.1) (Brzovic et al., 2001). The RING:RING heterodimer of BRCA1 /BARD1 functions as a ubiquitin ligase by direct interaction between the RING of BRCA1 and the ubiquitin conjugation enzyme UbcH5 (Brzovic et al., 2003). Although BARD1 does not play a direct role in the ubiquitin ligase activity of BRCA1, its presence is required to hold BRCA1 in the appropriate conformation necessary for activity. Thus, BRCA1/BARD1 form an obligate heterodimer and are very likely to act as a single unit within the cell.

Ubiquitination

Ubiquitin is a small 76 amino acid protein that can be covalently attached to substrate proteins in eukaryotic cells. This attachment results in the formation of an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of a substrate lysine residue. The process of ubiquitination is mediated by a cascade of three enzymes. First, in an ATP dependent manner the carboxyl group of the C-terminal glycine of ubiquitin is activated and attached via a thiol-ester bond to the active-site cysteine of the ubiquitin activating enzyme, UBA1 (E1). Second, the activated ubiquitin is transferred from the E1 to the active-site cysteine of a ubiquitin conjugation enzyme (E2). The last step is conducted by a ubiquitin ligase (E3) which associates with both an activated E2 and a substrate mediating the transfer of ubiquitin from the E2 to a lysine residue on the substrate (Fig. 1.2).

Ubiquitination is a diverse signaling mechanism, as this single protein can be attached to a substrate in several different forms. Mono-ubiquitination is the attachment of a single ubiquitin molecule to a lysine residue on a protein substrate and in some cases to more than one lysine per target protein. Poly-ubiquitination is the synthesis of a ubiquitin chain attached to a single lysine on the substrate, where a ubiquitin attached during one round of the ubiquitin cascade becomes the substrate for attachment of the next ubiquitin. There are seven surface exposed lysines in ubiquitin, five (Lys6, Lys11, Lys29, Lys48, and Lys63) (Arnason and Ellison, 1994; Baboshina and Haas, 1996) have been reported to be utilized in poly-ubiquitin chains. The two most documented ubiquitin chain linkages are conjugated through Lys48 or Lys63. A poly-ubiquitin chain linked through Lys48 of ubiquitin targets the attached substrate for proteasome

mediated degradation (Pickart, 1997). The ubiquitin proteasome system is the principle mechanism for the turnover of short lived proteins. Substrates destined for proteasome mediated degradation include abnormal, damaged, mis-localized and regulatory proteins whose functions are no longer needed or could be toxic to the cell. Lys63-linked poly-ubiquitin chains have been demonstrated to be involved in error-free DNA damage repair (Hofmann and Pickart, 1999) and in NF-κB activation (Deng et al., 2000). As mentioned earlier the poly-ubiquitin chain linkages found on PCNA that promote error-free DNA damage repair mechanisms are Lys63-linked. Ubiquitin modification of a substrate can affect the activity, interactions, localization, or stability of the modified substrate. Hence, each of the different types of ubiquitination events that can occur on a substrate has the potential to function as a different signaling mechanism in controlling the fate and function of the attached substrate.

There are potentially hundreds of ubiquitin ligases in the human genome characterized by three different domains found to possess ubiquitin ligase activity; HECT, RING, and U-box. The HECT domain functions in a fundamentally different manner than the RING or U-box in ubiquitin transfer to substrate. Similar to the E2 and E1 enzymes, the HECT domain has an active-site cysteine and forms a thiol-ester intermediate with ubiquitin prior to transfer to the substrate. RING and U-box E3s do not possess an active-site cysteine for formation of a thiol-ester intermediate, but rather are believed to function by recruiting the ubiquitin-activated E2 into close proximity to the E3-bound substrate.

E3s can also be subdivided based upon whether the ligase is comprised of a single amino acid chain or multiple protein subunits. A single chain E3 possesses the

RING domain and substrate binding site within the same polypeptide. A good example of single chain E3s are the TRIM/RBCC family members as they consist of a RING domain, one or two B-box motifs, a coiled-coil region and a variable C-terminus. As with other E3s the RING domain interacts with E2s, and substrates appear to be recognized by the B-box and coiled-coil motifs (Meroni and Diez-Roux, 2005). The cullin-RING ligases are a good representative of multisubunit E3s. There are seven different cullins expressed in human cells each with the capacity to form a scaffold for assembly of an active ligase by recruiting RING domain containing proteins, adaptors, and substrate receptors (Petroski and Deshaies, 2005). For example, CUL1 contains a long ridged architecture which binds to Rbx1 at the C-terminus and to SKP1 at the N-terminus (Zheng et al., 2002). Rbx1 contains a RING domain for recruiting an E2 for ubiquitin transfer, while SKP1 is an adaptor protein that recruits substrate receptors. Adaptor proteins such as SKP1 and elongin C bind to cullins through a motif structurally related to the BTB-domain. For substrate recognition SKP1 binds to F-box containing proteins that in turn are involved in substrate selectivity, and elongin C binds to SOCS-box containing substrate receptor proteins. In the human genome there are hundreds of different F-box, SOCS-box, and BTB domain containing proteins, thus there is the potential that a single cullin could be used by a variety of adaptor and substrate receptors to create a cullin-RING ligase for recognition and ubiquitination of a multitude of different substrates.

The human genome encodes over 30 different E2s characterized by a core ~150 amino acid Ubc domain. E2s are divided into different classes based upon whether they possess additional amino acid extensions from the core Ubc domain. Class I E2s

encode only the core Ubc domain, Class II E2s contain additional C-terminal residues, Class III E2s contain additional N-terminal residues, and Class IV E2s have both N- and C-terminal extensions from the core Ubc domain. Another subgroup of E2s are the UEVs or ubiquitin E2 variants which contain the core Ubc domain but lack the critical active-site cysteine necessary for ubiquitin transfer. The significant number of different E2s suggests a more diverse role than to simply transfer ubiquitin form the E1 to the E3 bound substrate for E3 directed ubiquitination. Some E2s have been identified to synthesize specific ubiquitin chain linkages. Ubc13, a class I E2, forms a heterodimer with Mms2, a UEV, for the synthesis of Lys63 linked poly-ubiquitin chains (Hofmann and Pickart, 1999). The Ubc13/Mms2 heterodimer has two different ubiquitin binding sites, a donor ubiquitin binds to the active site of Ubc13 and an acceptor ubiquitin binds non-covalently to Mms2. A recent crystal structure of Mms2/Ubc13~ubiquitin (ubiquitin covalently bound to the active-site of Ubc13) has provided much insight into the potential mechanism of Ubc13/Mms2 directed synthesis of Lys63-linked ubiquitin chains (Eddins et al., 2006). In the structure, the donor ubiquitin of one Mms2/Ubc13~ubiquitin complex was bound to the acceptor-binding site of an adjacent Mms2/Ubc13~ubiquitin complex. This arrangement allowed for the visualization of the unique manner that Mms2 positions Lys63 of the acceptor ubiquitin within 5Å of the C-terminal Gly76 of the Ubc13 bound donor ubiquitin. The close proximity of Lys63 to Gly76 provides the mechanistic insight where by Ubc13/Mms2 synthesizes exclusively Lys63-linked poly-ubiquitin chains.

Another example of E2 directed poly-ubiquitination is the class II E2 Ube2k, which possesses a C-terminal ubiquitin binding domain (UBA) and exclusively

synthesizes Lys48-linked poly-ubiquitin chains (Haldeman et al., 1997). The UBA domain is required for Ube2k's poly-ubiquitination activity and presumably binds to an acceptor ubiquitin for transfer of a donor ubiquitin from the active-site in a similar manner to Ubc13/Mms2. UbcH5 also binds ubiquitin non-covalently (Brzovic et al., 2006) as well as at the active-site cysteine and can synthesis poly-ubiquitin chains. Although, poly-ubiquitination activity depends on its ability to bind ubiquitin non-covalently a single specific ubiquitin linkage type has not been associated with UbcH5. Depending on the E3 with which it associates, UbcH5 can synthesize different types of poly-ubiquitin chain linkages. With BRCA1, UbcH5 synthesizes Lys6-linked poly-ubiquitin chains (Nishikawa et al., 2004; Wu-Baer et al., 2003). UbcH5 can also assemble Lys29- and Lys48-linked chains using partially purified E3s form rabbit reticulocyte lysate (Mastrandrea et al., 1999). It is also unclear whether the non-covalently bound ubiquitin of UbcH5 functions as an acceptor ubiquitin or if the poly-ubiquitination activity of UbcH5 functions by a different mechanism. A common feature of each of these E2s capable of poly-ubiquitination is the ability to bind to ubiquitin non-covalently. However, specific ubiquitin lysine chain linkages are dictated by the unique positioning of the acceptor ubiquitin by Mms2/Ubc13 and presumably by Ube2k.

Another possible function of E2s is assistance of the E3 in substrate selection. This assistance may be manifest by a weak but direct association between the E2 and substrate, and may allow for selection of substrate lysine residues for ubiquitin attachment. Evidence in support of substrate-E2 interactions arises from the ubiquitin-like pathway of sumoylation. Analogous to the ubiquitin pathway SUMO is also

covalently conjugated to substrate proteins using its own E1, E2, and E3 enzymes. Currently only one E2, Ubc9, has been identified for conjugation of SUMO to substrate lysine residues. *In vitro* sumoylation of IκBα (Desterro et al., 1999; Okuma et al., 1999), as well as other SUMO substrates, does not require an E3 for SUMO modification, suggesting specificity for substrate selection is conferred at least in part by elements contained within Ubc9. Both *in vivo* and *in vitro* sumoylation of IκBα occurs on the same lysine residue indicating that E3-independent recognition of IκBα by Ubc9 is biologically relevant. Additionally, a sumoylation consensus site has been identified, ψKXE (Johnson and Blobel, 1999) (where ψ represents a large hydrophobic amino acid and X represents any amino acid), as a common but not necessarily an exclusive site for SUMO attachment to substrate lysine resides. A structural basis for E2-substrate interactions arises from a co-crystal structure of Ubc9 with RanGAP1, a SUMO substrate (Bernier-Villamor et al., 2002). In the structure Ubc9 directly interacts with the SUMO consensus site of RanGAP1 bringing Lys526 within 3.5 Å of the active-site of Ubc9. Additional contacts between RanGAP1 and a C-terminal helix of Ubc9 are also observed (Fig. 1.3). These additional contacts between substrate and E2 provide a structural basis for *in vitro* sumoylation of RanGAP1 as well as other substrates in the absence of an E3. However, this does not rule out the importance of SUMO E3s as sumoylation of RanGAP1 is further stimulated by RanBP2, a SUMO E3.

As seen with Ubc9 and RanGAP1, ubiquitin E2s may also show some interaction with a substrate to assist the E3 in ubiquitination. However, a consensus site

for ubiquitination has not been identified for the collective group of ubiquitin E2s, though, this does not rule out the possibility of consensus motifs for individual E2s. Also, substrate-E2 interactions have not been identified for the ubiquitin pathway. One of the mysteries of ubiquitination is the inability to identify a single modified substrate lysine from *in vitro* ubiquitination assays. Often, mutation of lysine residues identified to be ubiquitinated by mass spectrometry fails to eliminate ubiquitination of the substrate during *in vitro* assays, suggesting against specific E2-substrate interactions. However, a lack in specificity for distinct ubiquitin attachment sites does not rule out the possibility of substrate-E2 interactions. Depending on the type of signal elicited by ubiquitination it may not matter if the same lysine residue is uniformly modified. For example, if the poly-ubiquitin signal is for proteasome-mediated degradation it would not matter which and how many substrate lysines are modified. Another possibility for lack of specificity for substrate lysine residues during ubiquitin attachment *in vitro* may be an inherent property unique to UbcH5, the most common E2 used in these assays. The mechanism utilized by UbcH5 during the process of poly-ubiquitination is unclear though it appears different than that used by Ubc13/Mms2 and Ube2k. UbcH5 appears to be highly promiscuous, and it sole use in most *in vitro* assays may be obscuring the ability to properly define universal mechanisms of E2 functions. For specificity, UbcH5 may require additional unidentified factors to assist in substrate and lysine selectivity not found in most *in vitro* assays.

Interactions between E2s and E3s are thought to be highly selective and are often identified by a substrate independent auto-ubiquitination assay, GST-pull downs, or yeast two-hybrid screens. Since, known E2/E3 interactions are weak and E2s

possess different properties of poly-ubiquitination it is not surprising that usually only one E2 (most often UbcH5) is found to interact with a given E3. The mechanisms underlying E2 selection by an E3 are an important area of investigation in the field of ubiquitination. Common E2 surfaces utilized when interacting with an E3 are helix-1, loop L1, and loop L2 (Fig. 1.4) of the Ubc core domain. An obvious limit to the structural diversity of ubiquitin E2s is the fact that they must all interact with the E1 for transfer of activated ubiquitin. Interestingly, helix-1 of the Ubc core domain is utilized for both E1 and E3 binding (Huang et al., 2005; Zheng et al., 2000). While this overlap in binding-sites ensures that ubiquitin pathway enzymes are not used by ubiquitin-like proteins such as SUMO, this overlap reduces the opportunity for specificity of E2/E3 interactions. Hence, there is the possibility that more than one E2 may bind to a single E3. A few E3s (CHIP, c-Cbl, BRCA1, and the Kaposi's sarcoma-associated herpesvirus K3) have already been shown to bind to at least two different E2s. CHIP (Zhang et al., 2005b) and K3 (Dodd et al., 2004) intact with both UbcH5 and UbcH13, where as c-Cbl (Zheng et al., 2000) and BRCA1 (Brzovic et al., 2003) interact with both UbcH5 and UbcH7. The use of multiple E2s by a single E3 is a very interesting prospective as a much more diverse role may be played by the E3. Depending on the E2 used an E3 may synthesize different poly-ubiquitin chain linkages or ubiquitinate a different subset of substrates. Hence, identifying all the E2s that can bind to a given E3 will provide the critical subset of E2s that should be used when investigating the ubiquitin ligase properties of an E3.

Objectives

The specific goals of my thesis project are:

1) Identify at least one substrate of the BRCA1/BARD1 ubiquitin ligase.
2) Evaluate the need for other protein factors (such as E2s) for BRCA1 mediated ubiquitination of putative substrates.

The function of BRCA1 as a ubiquitin ligase along with breast cancer associated mutations that disrupt this function (Brzovic et al., 2003) begs the question, what are the cellular targets of BRCA1/BARD1 mediated ubiquitination? Cancer associated mutations that result in loss of the ubiquitin ligase activity of BRCA1 would also eliminate the critical ubiquitin modification of one or more substrates allowing for tumor progression. Hence, knowing the identity of a substrate will provide an understanding of the important mechanism utilized by BRCA1 to protect against breast tumorigenesis. As BRCA1 has been suggested to be involved in many different cellular processes there are likely to be multiple substrates ubiquitinated by BRCA1/BARD1. However, a link between BRCA1 RING mutations and cancer tissue specificity may be limited to one or a few substrates. Mutations of the *BRCA1* or *BRCA2* genes only account for ~40% of hereditary breast cancers, half of the remaining "mystery families" still show linkage for inherited susceptibility to breast cancer to *BRCA1* or *BRCA2* (Tom Walsh, personal communication). The remaining ~30% of all cases of inherited susceptibility to breast cancer are likely due to mutations in other additional unidentified genes rather than *BRCA1* or *BRCA2*. It is very likely that one or more substrates of BRCA1 may be linked to inherited susceptibility to breast cancer in non-BRCA1/BRCA2 mutant carriers.

A few candidate substrates for BRCA1 have been reported since the beginning of my thesis project; NPM (Sato et al., 2004), ERα (Eakin et al., 2007), γ-tubulin (Starita et al., 2004), RNA pol II (Kleiman et al., 2005), p53 (Dong et al., 2003), histone H2AX (Chen et al., 2002), and CtIP (Yu et al., 2006). However, none of these putative substrates explain a direct connection between BRCA1 ligase-inactive cancer associated mutations to tissue specific susceptibility for breast and ovarian cancers. NPM, γ-tubulin, RNA pol II, p53, and CtIP are each involved in basic cellular processes such as chromosomal segregation, transcription, apoptosis, and DNA damage sensing and repair which, are not unique to breast or ovarian epithelial cells. Hence, loss of a critical ubiquitination event in NPM, γ-tubulin, RNA pol II, p53, or CtIP would likely be equally destructive to most tissue types and not unique to breast and ovarian tissues. ERα as a putative substrate has the potential of directly linking the tissue specific nature of cancer predisposing BRCA1 mutations to the breast and ovaries, however, these are not the only estrogen responsive tissues.

Attempting to identify a substrate of a ubiquitin ligase is a difficult task as there is not a standardized method. The most common approach for identifying substrates is to test proteins already known to associate with the ubiquitin ligase in an *in vitro* ubiquitination assay. However, as BRCA1 is highly investigated my assumption is that most proteins already known to interact with BRCA1 have already been tested. Therefore, my objective is to identify unexpected substrates of BRCA1, and to search for them in a tissue specific manner. Based upon the architecture of BRCA1/BARD1 (Fig. 1.1) it is unclear where possible substrates may bind. As the RING domain plays

a central role in ubiquitination by interacting with E2s, a likely substrate binding site would be closely situated to the RING of BRCA1. However, there are no conserved domains found in the amino acid sequences of BRCA1 or BARD1 C-terminal to their RING domains with the exception of the distant BRCT and ANKRYN domains. For BRCA1 the BRCT domain is separated from the RING by ~1550 amino acids and for BARD1 there are ~300 and ~440 amino acids between the RING domain and the ANKRYN and BRCT domains respectively. As the 3D structure of the entire BRCA1/BARD1 complex is unknown one can not determine if any of the known conserved domains of BRCA1 or BARD1 are situated closely to the RING of BRCA1 such that they could function in substrate recognition.

All E3 ubiquitin ligases must interact with both an E2 and either directly or indirectly with a substrate. The human genome encodes 30 to 40 E2s and hundreds of potential E3s. Thus, each E2 must interact with more than one E3, however, emerging evidence suggests that a single E3 can interact with more than one E2 (Dodd et al., 2004; Plans et al., 2006; Zhang et al., 2005b). A significant hindrance in the ability to identify substrates of any E3 may be the use of only a single E2, most often UbcH5, for *in vitro* ubiquitination experiments. There is the possibility that while certain E2s can interact with the same E3 only a subset of these E2s may actually transfer ubiquitin to a specific substrate. Hence, different E2s in combination with the same E3 may transfer ubiquitin to different E3-bound substrates. Therefore, when attempting to identify substrates of a given E3 one should first define the complement of E2s that can interact directly with the E3. Thus, during the process of identifying substrates of

BRCA1/BARD1 I have also set out to determine the E2s that interact with the RING of BRCA1.

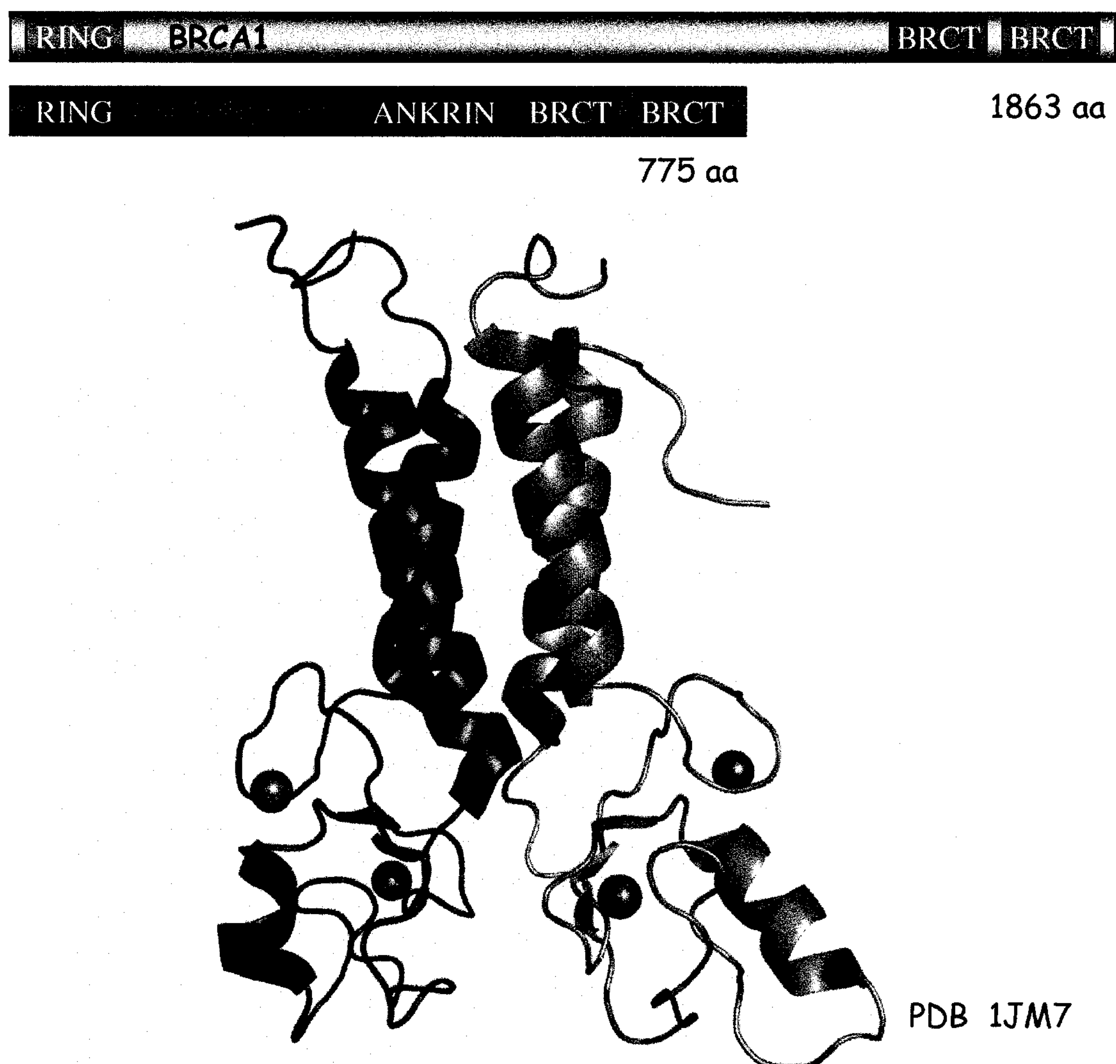


Figure 1.1 Domain Architecture of BRCA1 and BARD1.

BRCA1 and BARD1 are composed of similar domain architecture as they both have an N-terminal RING domain and C-terminal BRCT repeats. BARD1 also possesses ANKRIN repeats, which just precede the BRCT domains. These are the only conserved domains found in both proteins. Also shown is the NMR structure of the RING:RING heterodimer of BRCA1/BARD1. The structure is represented by the first ~100 amino acids of each protein which comprises their respective RING domains, with BARD1 in blue and BRCA1 in gray. Each RING domain contains two zinc atoms shown in magenta.

Figure 1.2 Ubiquitination Cascade.

Diagram of the enzymatic process of ubiquitination illustrating the ATP-dependent activation of ubiquitin by the ubiquitin-activating enzyme (E1). Next the E1 transfers the activated ubiquitin to a ubiquitin-conjugating enzyme (E2). During the first two steps of the ubiquitination cascade the C-terminal Gly76 of ubiquitin is linked *via* a thiol ester bond to the active-site cysteines of the E1 and E2. The ubiquitin charged E2 then associates with an E3 (for this diagram the E3 is of RING or U-box type). Current models of ubiquitination place substrate recognition solely on the E3, which brings the ubiquitin-charged E2 in close proximity to the E3-bound substrate mediating transfer of ubiquitin from E2 to substrate. The ubiquitin is covalently linked to substrate lysine residues *via* an isopeptide bond. Through multiple rounds or the cascade a poly-ubiquitin chain can be synthesized on the substrate. When the signal elicited by the ubiquitin tag is no longer needed, the isopeptide bond between the substrate and ubiquitin is hydrolyzed by a class of deubiquitination enzymes (DUBs).

Figure 1.3 Structure of RanGAP1-SUMO1/Ubc9, i.e. A Substrate-E2 Interaction.

Shown in blue is a surface representation of RanGAP1 with SUMO1 (green) covalently bound to Lys526 in yellow. Ubc9 is shown in red with the arrow pointing to an interaction surface between Ubc9 and RanGAP1 that is not directly involved with recognition of Lys526 for sumoylation.

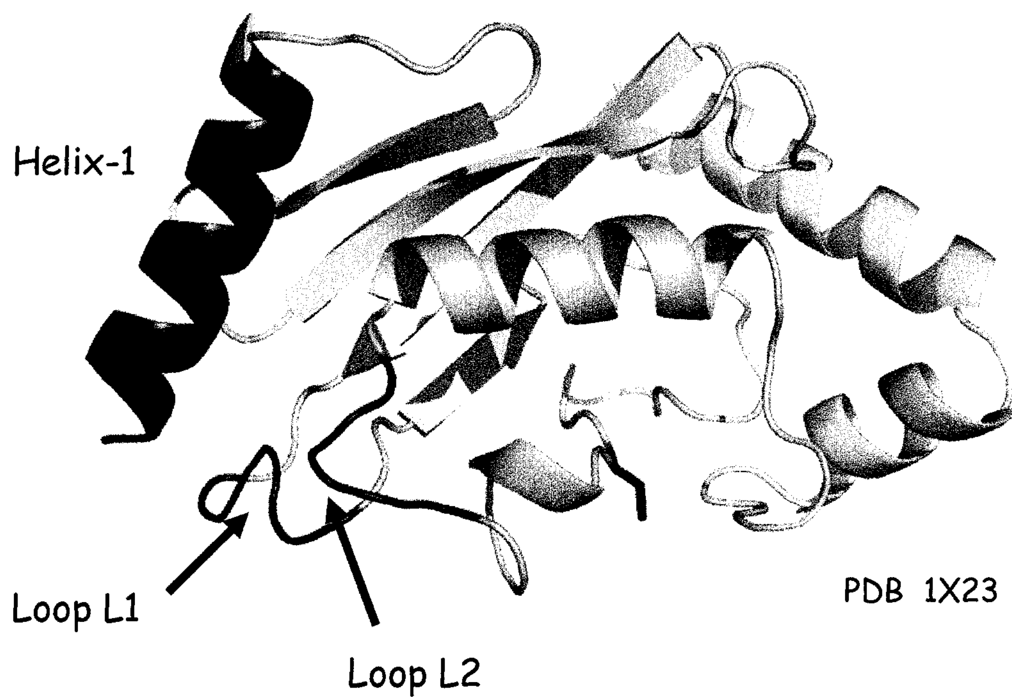


Figure 1.4 E2 Surfaces Used for Recognition by E3s.

Colored in blue on the structure of UbcH5c are three surfaces recognized in general by RING or U-box E3s for interacting with E2s.

Chapter 2. Novel E2:BRCA1 Interactions Dictate the Synthesis of Either Mono- or Specific Poly-Ubiquitin Chain Linkages

Introduction

Inherited germ-line mutations of the breast and ovarian cancer susceptibility protein, BRCA1, predisposes an individual to early onset of breast and ovarian tumors. Since its discovery, BRCA1 has been intensely investigated to define its normal cellular function so as to understand why loss-of-function mutations lead to breast and ovarian tumorigenesis. Many cellular functions have been suggested for BRCA1 including DNA damage repair, cell cycle control, apoptosis, chromosomal segregation, and transcriptional regulation, however, the role of BRCA1 during any of these processes remains unknown. *BRCA1* encodes a large protein of 1863 amino acids, with two highly conserved domains, an N-terminal RING domain and C-terminal BRCT repeats. The only biochemical activity associated with BRCA1 is found at its RING domain, which functions as a ubiquitin ligase. A significant number of cancer-associated BRCA1 missense mutations are found within the RING domain, and these eliminate ubiquitin ligase activity (Brzovic et al., 2003). Hence, loss of ubiquitin ligase activity in the RING domain of BRCA1 is closely associated with breast cancer susceptibility. Therefore, it is imperative to fully understand the function of this BRCA1 domain.

A ubiquitin ligase (E3) functions at the crossroad between activation of ubiquitin and its covalent attachment to substrate proteins. The process is initiated by the ubiquitin-activating enzyme (E1), which functions to create an ATP-dependent thiol ester bond between its active-site cysteine and the C-terminal Gly76 of ubiquitin. Activated ubiquitin is transferred from E1 to the active-site cysteine of a ubiquitin-

conjugation enzyme (E2). Specificity for ubiquitination is believed to be mediated primarily by an E3, which interacts directly with both an E2 and a substrate to meditate the transfer of ubiquitin from E2 to a lysine residue (*via* an isopeptide bond) on the E3-bound substrate.

Ubiquitination of substrate proteins is a diverse signaling mechanism as this single protein molecule can be attached to a substrate in a variety of forms, resulting in targeting of the modified substrate to different cellular processes. Mono-ubiquitination is the attachment of a single ubiquitin to a substrate lysine residue. Multiple lysine residues may be modified within a single substrate. Mono-ubiquitination is involved in at least three distinct cellular functions: histone regulation, endocytosis, and the budding of retroviruses from the plasma membrane (reviewed in Hicke, 2001). Poly-ubiquitination is the synthesis of a multi-ubiquitin chain on the substrate, where a ubiquitin attached during one round of the ubiquitination cascade becomes the substrate for the next ubiquitin transfer. There are seven surface exposed lysines in ubiquitin, each with the potential to be utilized for poly-ubiquitin chain linkage sites. The two most reported poly-ubiquitin chain linkages are formed *via* Lys48 or Lys63 of ubiquitin. A chain of at least four ubiquitin subunits linked at Lys48 is the primary signal for proteasome mediated degradation (Pickart, 1997). Lys63-linked chains target a substrate for non-proteasomal processes such as involvement in post-replicative repair of DNA damage (Hoege et al., 2002) and in activation of NF-κB (Deng et al., 2000).

A major goal when investigating the function of an E3 is to identify its substrates. Knowing the substrates and the type of ubiquitin modification (mono vs. specific poly-ubiquitin linkages) that an E3 synthesizes on a substrate will greatly aid in

the ability to investigate the function of the E3. However, identifying substrates of an E3 is not an easy task, as the mechanisms underlying substrate specificity, E2-E3 interactions, and selection of poly-ubiquitin linkage types are not well understood. Some of these processes might be determined by the E2. Some E2s have been found to function in the synthesis of specific ubiquitin chain linkages, such as the Ubc13/Mms2 heterodimer (Hofmann and Pickart, 1999), and Ube2k (Haldeman et al., 1997), which exclusively direct the synthesis of Lys63 or Lys48 poly-ubiquitin chains, respectively. Although there are likely to be cases of E2-independent mechanisms of poly-ubiquitin chain selection, Ube2k and Ubc13/Mms2 represent a mechanism whereby poly-ubiquitin chain selection is determined by the E2. There are over thirty E2s encoded in the human genome and similar to Ube2k and Ubc13/Mms2 each has the potential to play a unique role during the process of ubiquitination. The type of ubiquitin modification that can occur on the substrate therefore may depend on the E2 with which the E3 associates. Therefore, an important process in understanding an E3's function is to identify the E2s with which it interacts.

Interactions between E2s and E3s are weak and of modest affinity, making them difficult to detect. Current practice to identify functional E2-E3 pairings is to test a limited panel of E2s with the E3 of interest in a substrate-independent ubiquitination assay (monitors formation of poly-ubiquitin chains) or in an auto-ubiquitination assay (monitors ubiquitination of the E3 itself). However, there are limitations to these methods. The first approach detects formation of poly-ubiquitin chains and will fail to identify E2-E3 pairs responsible for mono-ubiquitination. Furthermore, one of the most often used E2s, UbcH5 (and its family members) is highly active in substrate-

independent assays with most E3s, an activity that may just be an inherent property of UbcH5 not found in other E2s. The second strategy will only identify those pairings that transfer ubiquitin to the E3 itself. However, it is possible that certain E2-E3 pairings are active only with specific substrates, and will therefore not yield a positive result in either of the two widely-used protocols. Additionally, there are at least eleven ubiquitin-like proteins (Ubl) (Huang et al., 2004) in the human genome that are also covalently attached to substrate proteins in a similar manner to ubiquitin using their own dedicated E1, E2, and E3 enzymes. Currently, there are no criteria for identifying a Ubl-E3 ligase and given the structural similarity between known Ubl E2s (Ubc9 and UbcH12) and ubiquitin E2s, it is expected that the ligases for Ubls will use similar structural motifs (i.e. RINGs, etc) to the ubiquitin E3s. Thus, an activity assay will not detect E2-E3 interactions of a Ubl pathway unless the specific Ubl and its E1 are included.

An absolute requirement for a functional E2-E3 pair in any context is a direct physical interaction between them. Many reports suggest that E2-E3 interactions are highly selective and that only a single E2 functions with a given E3. In contrast are a growing number of reports of an E3 interacting with more than one E2 (Dodd et al., 2004; Plans et al., 2006; Zhang et al., 2005b). Additionally, common E2 structural elements utilized for known E2-E3 pairs are highly conserved among the family of E2s. Of note is helix-1, which is also recognized by the E1 limiting the diversity of this surface of ubiquitin E2s. A high level of conservation within the E2s' surface used to interact with E3s suggests the possibility that more than one E2 can interact with a given E3. Defining the complement of E2s that can interact with a given E3 may

provide a significant advantage for identifying putative substrates of that E3, as well as the type of ubiquitin modification that can be attached to the substrate. Many E3s are likely to have more that one substrate and there is no guarantee that each E2 capable of interaction with the E3 will transfer ubiquitin to every substrate. Therefore, a strategy capable of identifying all interacting E2-E3 pairs based on binding would provide the critical subset of all possible E2s that should be considered in future studies of that E3. Common approaches for identifying protein-protein interactions such as co-immunoprecipitation or pull-downs may not be effective for evaluating all E2-E3 pairings, as E2-E3 interactions are transient and of modest affinity.

To overcome the difficulties in identifying weakly associated E2-E3 pairings we designed a "structure-based" yeast two-hybrid strategy based on the 3D structure of the heterodimeric RING E3 BRCA1/BARD1 and demonstrate that it is capable of identifying novel E2-E3 interactions. Specifically, a "bait" construct designed from the heterodimeric RING E3, BRCA1/BARD1, was used to screen a library of human E2 "prey" constructs. The screen identified not only the known interacting E2 partners for BRCA1/BARD1, UbcH5 and UbcH7 (Brzovic et al., 2003), but also six new E2 partners for BRCA1. When paired with different E2s, BRCA1-mediated ubiquitination yield differential products ranging from mono-ubiquitination to select poly-ubiquitin chains. Comparison of these functional E2s define residues important for recognition by BRCA1, and provide a mechanistic rationale that distinguishes BRCA1-interacting E2s that build poly-ubiquitin chains from those that transfer a single ubiquitin. This work also demonstrates that E2 proteins can function synergistically to build specific chain linkages, as E2s such as the Ubc13/Mms2 heterodimer can selectively build

Lys63-linked chains on a mono-ubiquitinated substrate in a BRCA1-dependent fashion. These results not only suggest a potential "division of labor" among the various E2 proteins, but also provide a direct functional link for BRCA1 in the process of DNA repair.

Results

Design of the Structure-Based BRCA1/BARD1 "Bait"

Structural studies of E2-E3 complexes have revealed the E2-interacting regions for RING, U-box, and HECT-domain E3s (Huang et al., 1999; Zhang et al., 2005b; Zheng et al., 2000). In the case of the RING heterodimer BRCA1/BARD1, the RING motif of BRCA1 interacts directly with the E2s UbcH5c and UbcH7, although only UbcH5c shows activity in either substrate-independent or auto-ubiquitination assays (Brzovic et al., 2003). No physical interactions between UbcH5c or UbcH7 and the BARD1 RING were detected, but the N-terminal portion of BARD1 is required for BRCA1's E3 ligase activity (Hashizume et al., 2001), indicating that it is the heterodimeric structure that imparts functional activity. Furthermore, a directed yeast two-hybrid experiment in which only the BRCA1 subunit is used as bait does not result in a positive interaction with a UbcH5c prey, as determined by yeast growth on selective media (Fig. 2.1A), consistent with the idea that structural integrity of the heterodimeric RING is critical. We therefore designed a fused version of the BRCA1/BARD1 RING heterodimer aimed at presenting a structurally relevant "bait" molecule in the context of a yeast two-hybrid experiment.

Design of the fusion protein was based on the BRCA1/BARD1 RING:RING heterodimer structure, comprised of the first ~110 residues of each subunit (Brzovic et al., 2001). In the solution structure, the C-terminal end of the BARD1 RING domain is within 10 Å of the N-terminal end of the BRCA1 subunit. A five-residue linker, Gly_2-Ser-Gly_2, was used to link the RING domain of BARD1 (residues 26-126) to the RING domain of BRCA1 (residues 2-109) to create the fusion protein $BD_{126}FBC_{109}$ (Fig. 2.1B). To confirm that the fusion protein retains both structure and function, it was expressed in bacteria, purified, and tested in a substrate-independent assay with UbcH5c. $BD_{126}FBC_{109}$ has identical ubiquitin ligase activity to its non-fused counterpart BC_{112}/BD_{140} (BRCA1 residues 1-112 and BARD1 residues 26-140; data not shown). The ^{1}H, ^{15}N-HSQC NMR spectrum of the fusion is virtually identical to the spectrum of the non-fused heterodimer, with the exception of additional peaks that arise from the linker, confirming the structural integrity of the fused "heterodimer" (data not shown). $BD_{126}FBC_{109}$ was therefore fused to the DNA-binding domain of Gal4 to generate our structure-based yeast two-hybrid bait.

As proof-of-principle, $BD_{126}FBC_{109}$ was tested for interaction with UbcH5c in a directed yeast two-hybrid screen. The $BD_{126}FBC_{109}$ bait showed an interaction with the UbcH5c prey while a bait comprised of the identical BRCA1 residues alone was unable to support growth (Fig. 2.1A). The BRCA1-alone bait supports growth in the presence of a BARD1 prey, confirming that this bait molecule is capable of protein-protein interactions. Inclusion of the ligase inactive BRCA1 RING mutation Ile26Ala, which disrupts the interaction with UbcH5c and UbcH7 (Brzovic et al., 2003), in the context of the fused bait ($BD_{126}FBC_{109}$-I26A), abrogates the two-hybrid interaction

with UbcH5c (Fig. 2.1A). Together, these results confirm that the structure-based bait design recapitulates the salient features of the BRCA1/BARD1 E3 interaction with the E2, UbcH5c, namely that the interaction requires the intact heterodimeric structure and that it can be disrupted by the Ile26Ala mutation.

Identification of BRCA1-Interacting E2s

BD126FBC109 bait was used in a directed yeast two-hybrid screen against twenty-four human E2s and six human E2 variants (UEVs) as prey to identify additional human E2s that can interact with BRCA1/BARD1. Eleven E2s supported growth of yeast cells on selective media in the presence of BD126FBC109 above the background level of the empty vector controls: UbcH5a (Ube2d1), UbcH5b (Ube2d2), UbcH5c (Ube2d3), UbcH6 (Ube2e1), Ube2e2, UbcM2 (Ube2e3), Ube2k (E2-25K), UbcH7 (Ube2l3), Ubc13 (Ube2n1), Ubc9 (Ube2i), and Ube2w (Fig. 2.1C and Fig. 2.2). These E2s were subsequently tested against the BRCA1 RING mutant BD126FBC109-I26A. All E2s with the exception of Ubc9 lost the ability to interact with BD126FBC109-I26A. By analogy with UbcH5c, these results suggest that UbcH6, Ube2e2, UbcM2, Ube2k, Ubc13, and Ube2w bind directly to the RING motif of BRCA1.

Interactions inferred from a yeast two-hybrid experiment must be confirmed by another method capable of detecting direct interactions. As mentioned earlier, E2-E3 interactions are challenging to detect using traditional methods such as pull-downs and gel filtration, as the interaction is of fairly modest affinity. We therefore used 2D ^{1}H, ^{15}N -TROSY-HSQC NMR to detect direct protein-protein interactions by collecting

spectra of ^{15}N-labeled BC112/BD140 (non-fused) in the presence and absence of each purified E2. HSQC spectra are sensitive indicators of molecular interactions because the resonance position of each peak in the spectrum is exquisitely sensitive to the chemical environment of its associated amide group (Zuiderweg, 2002). We have previously used this approach to map the interactions between BRCA1/BARD1 and the E2s UbcH5c and UbcH7 (Brzovic et al., 2003). NMR spectra were acquired in the presence of UbcH6, Ube2e2, UbcM2, Ube2k, Ubc13, Ubc9, and Ube2w (representative spectra are shown in Fig. 2.3). In every case except Ubc9, addition of a purified E2 resulted in significant perturbations to the BRCA1/BARD1 NMR spectrum, confirming a direct interaction with the BRCA1/BARD1 heterodimer. As further corroboration of the yeast two-hybrid results and to ensure that the direct binding observed in the NMR experiments cannot be attributed solely to high protein concentrations, HSQC spectra were collected in the presence of an E2 or UEV that did not support growth in the two-hybrid context. Addition of Rad6, Ube2v1, or Mms2 caused no detectable perturbations in the NMR spectrum of BRCA1/BARD1 (data not shown), confirming that the observed spectral effects are not due to non-specific interactions at high NMR concentrations.

In addition to providing direct confirmation of a protein-protein interaction, HSQC NMR experiments reveal information on the nature of the E2-E3 interaction. Resonances that arise from groups on the binding surface are expected to be perturbed by small (sub-stoichiometric) amounts of E2. As shown in Figure 2.3, perturbations elicited by small amounts of each E2 are remarkably similar to each other and to the perturbations previously reported for UbcH5c (Brzovic et al., 2003). In particular, the

first peak affected upon addition of each E2 is Ile26 of BRCA1, confirming its central role in E2 binding, as suggested from the directed yeast two-hybrid screens with the Ile26Ala mutant bait. Similar magnitudes of perturbations in the BRCA1-RING peaks are observed for each E2 at early titration points. Thus, although there are small differences in the details of each E2 interaction, we conclude that UbcH6, Ube2e2, UbcM2, Ube2w, Ube2k, and Ubc13 all bind with similar affinities to the same surface of the BRCA1 RING as does UbcH5c.

Ubc9 is the only E2 that showed a positive yeast two-hybrid interaction but failed to interact with BRCA1/BARD1 as judged by NMR. Ubc9 is also the only E2 that retains its ability to interact with the mutant BD126FBC109-I26A in the yeast two-hybrid assay. These results suggest that if there is indeed an interaction, Ubc9 recognizes a different site on the BRCA1/BARD1 heterodimer and this potential interaction is not direct.

E2 selection by BRCA1

The ten E2s (eight counting UbcH5 isoforms a, b, and c as the same) found to interact with the RING of BRCA1 represent a diverse group of E2s. UbcH5, UbcH7, Ubc13, and Ube2w are class I E2s as they possess only the Ubc core domain. Ube2k is a class II E2 and possesses a C-terminal extension from the Ubc core domain. UbcH6, Ube2e2, and UbcM2 are class III E2s possessing unique N-terminal extensions from their nearly identical core Ubc domains. To understand how this diverse group of E2s all interact with the same surface on the RING of BRCA1, we looked for unifying features among the set of BRCA1-interacting E2s that are not shared with other (non-

interacting) E2s. A sequence alignment of the BRCA1-interacting E2s was compared with a non-interacting set (Fig. 2.4). There are many conserved features among all E2s as they share the same core domain and the ability to interact with the E1. We used NMR chemical shift mapping to identify the surface on UbcH5c to which BRCA1 binds. 2D HSQC-TROSY spectra of ^{15}N-labeled UbcH5c with increasing amounts of (unlabeled) BC112/BD140 were collected and compared, and the UbcH5c resonances most perturbed by BRCA1 were identified. Two structural elements of UbcH5c affected most are helix-1 (residues Arg5, Ile6, Lys8, Asp12, and Leu13) and loop L2 (residues Ala96, Thr98, Ile99, Ser100, and Val102) (Fig. 2.5), consistent with these elements comprising the major interaction site for the BRCA1 RING. The surface exposed residues of the BRCA1-interaction site are highlighted in the multiple sequence alignment in Figure 2.4. Three of these positions are strongly conserved within the BRCA1-interacting group of E2s: UbcH5c residues Arg5, Lys8 (in helix-1), and Ala96 (in loop L2). Alignment of non-interacting human E2 sequences reveals no cases that contain all three conserved residues.

The ability of the BRCA1/BARD1 heterodimer to auto-ubiquitinate itself (Brzovic et al., 2006; Hashizume et al., 2001) was used to test the functional importance of the putative E2 binding determinants. For the auto-ubiquitination assays a heterodimer comprised of the first 304 amino acids of BRCA1 (BC304) and amino acids 26-327 of BARD1 (BD327) was used. UbcH5 residues Arg5 and Lys8 are not good candidates for mutational analysis of E2-E3 interactions as they are on helix-1, which is also recognized by the E1 (Huang et al., 2005) and is therefore necessary for ubiquitin-E2 thiol ester formation. To assess the role of Ala96 in BRCA1-mediated E2

recognition we mutated Ala96 of UbcH5c to Ser and Asp and tested the mutant E2's ability to facilitate auto-ubiquitination of BRCA1. Serine and aspartic acid were chosen because similar amino acids are found at the analogous position in two of the non-BRCA1 interacting E2s. As shown in Figure 2.6, UbcH5c-A96S exhibits only weak activity with BRCA1, and UbcH5c-A96D has no detectable activity. Both UbcH5c mutants retain the ability to form a thiol ester with ubiquitin comparable to wild-type UbcH5c (Fig. 2.7) consistent with their proper folding. Furthermore, a comparison of the HSQC spectra of wild-type and UbcH5c-A96D reveals no major structural changes in UbcH5c by the Ala96Asp mutation (data not shown). The 2D HSQC-TROSY spectrum of ^{15}N-labeled UbcH5c-A96D shows no detectable shifts after addition of (unlabeled) BC112/BD140 (data not shown). Hence, the observed loss of BRCA1 auto-ubiquitination activity by UbcH5c-A96D can be attributed entirely to a loss in its ability to interact with the RING of BRCA1. Together, these results demonstrate that Ala96 of UbcH5c is a critical determinant for binding to BRCA1 and we propose that the analogous Ala residues in the BRCA1-interacting E2s play a similar role. Based on this analysis, we conclude it unlikely that any of the remaining human E2s tested in our directed yeast two-hybrid screen will bind directly to the RING of BRCA1.

Characterization of BRCA1 Auto-Ubiquitination by its Interacting E2s

The new BRCA1-interacting E2s identified by the yeast two-hybrid strategy and confirmed by NMR were tested for activity with the BRCA1/BARD1 E3. Figure 2.8A shows that the BRCA1 subunit is efficiently poly-ubiquitinated when UbcH5c is used as the E2. UbcH6, Ube2e2, UbcM2, and Ube2w also transfer ubiquitin to BRCA1, but the

predominant products are mono-ubiquitinated BRCA1. The core Ubc domains of the Class III E2s UbcH6, Ube2e2, and UbcM2 are over 95% identical to each other, which may explain why each of these E2s displays similar properties with BRCA1. The Ubc domain of these three E2s also possess the highest level of identity with UbcH5c at ~64 %.

Given the high degree of similarity with UbcH5c it is notable that the class III E2s do not poly-ubiquitinate BRCA1. The ability of UbcH5c to poly-ubiquitinate BRCA1 depends upon a non-covalent interaction between ubiquitin and the β-sheet surface of UbcH5c (Brzovic et al., 2006). We have previously shown that mutation of UbcH5c (Ser22Arg) in the β-sheet surface disrupts the ability of ubiquitin to bind non-covalently and results in an E2 that transfers only a single ubiquitin to BRCA1 similar to what is observed for UbcH6, Ube2e2, UbcM2, and Ube2w. We therefore tested UbcM2 and Ube2w, E2s that mono-ubiquitinate BRCA1, for their ability to bind to ubiquitin non-covalently. As determined by HSQC-NMR binding experiments UbcM2 (data not shown) and Ube2w (D.M. Wenzel, D.E.C., and R.E.K., unpublished data) do not bind to free ubiquitin. UbcH6 and Ube2e2 are also unlikely to bind free ubiquitin based on sequence comparison with the Ubc domain of UbcM2. By analogy with UbcH5c and the β-sheet mutant, UbcH5c-S22R, the inability of UbcH6, Ube2e2, UbcM2, and Ube2w to build poly-ubiquitin chains with BRCA1/BARD1 correlates with the inability to interact with ubiquitin in a non-covalent manner.

Three E2s UbcH7, Ube2k, and Ubc13, failed to transfer ubiquitin to BRCA1 (Fig. 2.8A) despite their ability to bind to the same surface on BRCA1 as the active E2s. *In vitro*, the Ubc13/Mms2 heterodimer can synthesize unanchored Lys63-linked poly-

ubiquitin chains. The chain building activity of Ubc13/Mms2 depends on the ability of Mms2, a UEV, to bind to an acceptor ubiquitin non-covalently (McKenna et al., 2001). As a subset of BRCA1-interacting E2s mono-ubiquitinate BRCA1, we tested whether the mono-ubiquitin attached to BRCA1 can serve as an acceptor ubiquitin for Ubc13/Mms2-mediated synthesis of poly-ubiquitin chains. To test this hypothesis, auto-ubiquitination assays with BRCA1/BARD1 in the combined presence of equimolar amounts of Ube2w, Ubc13, and Mms2 were performed. As shown in Fig. 2.8B, the combination of Ube2w with Ubc13/Mms2 produces a ladder of ubiquitinated-BRCA1 bands. The addition of multiple ubiquitins to BRCA1 requires the presence of all three E2s, as any combination of two of the three E2s results only in mono-ubiquitination when Ube2w is present or no ubiquitination with only Ubc13/Mms2 (Fig. 2.9). Based on these results it appears that BRCA1 is first mono-ubiquitinated by Ube2w, then BRCA1-ubiquitin becomes a substrate for Ubc13/Mms2. Lys63Arg mutant ubiquitin was used to determine whether the multi-ubiquitinated BRCA1 is due to multiple mono-ubiquitination events or poly-ubiquitin chain synthesis. Use of ubiquitin-K63R results only in mono-ubiquitination of BRCA1 with the mixture of Ube2w, Ubc13, and Mms2 (Fig. 2.8B), indicating that this combination of E2s results in the synthesis of a Lys63-linked poly-ubiquitin chain attached to BRCA1.

As Ubc13/Mms2 can build unanchored Lys63-linked poly-ubiquitin chains in the absence of an E3, we tested whether the chains attached to BRCA1 required a direct E2-E3 interaction between BRCA1 and Ubc13. By analogy with our results with UbcH5c-A96D, we mutated the conserved loop L2 Ala98 in Ubc13 to aspartic acid. The mutant Ubc13-A98D/Mms2 complex is unaffected in its ability to create E3-

independent unanchored poly-ubiquitin chains (Fig. 2.10). However, Ubc13-A98D/Mms2 is unable to synthesize a poly-ubiquitin chain attached to mono-ubiquitinated BRCA1 (Fig. 2.8B). These results demonstrate that poly-ubiquitination of BRCA1 by Ubc13/Mms2 strictly depends on a functional RING-E2 interaction between BRCA1 and Ubc13, and not just on the attachment of a ubiquitin to BRCA1.

The C-terminal tail of Ube2k has been defined as a ubiquitin-associated domain (UBA) based upon sequence homology. This tail is required for the activity of Ube2k and the synthesis of exclusively Lys48-linked poly-ubiquitin chains *in vitro* (Haldeman et al., 1997). Though it has been implied that Ube2k can bind to free ubiquitin (Merkley and Shaw, 2004), we confirmed this experimentally by HSQC-NMR (D.M. Wenzel, D.E.C., and R.E.K., unpublished data). We tested whether Ube2k can also recognize mono-ubiquitinated BRCA1 as an acceptor ubiquitin for poly-ubiquitin chain elongation similar to Ubc13/Mms2. When mixed with Ube2w in a BRCA1 auto-ubiquitination assay, Ube2k extends a poly-ubiquitin chain from a mono-ubiquitinated BRCA1 (Fig. 2.8C). The poly-ubiquitin chain synthesized on BRCA1 is linked *via* Lys48, as ubiquitin-K48R abrogates product formation. Chain building is dependent on the RING-E2 interaction between Ube2k and BRCA1 as the Ube2k loop L2 mutant Ala103Asp also abolishes product formation (Fig. 2.8C).

Discussion

We have identified six new RING-E2 interactions for the BRCA1/BARD1 heterodimeric ubiquitin ligase. Critical determinants of E2-BRCA1 RING interactions are Ile26 of BRCA1 and a conserved alanine in loop L2 of the E2, as mutation at either

site abrogates both binding and activity. Based upon the E2 with which it associates, BRCA1 can facilitate either mono-ubiquitination or poly-ubiquitination though Lys6 (Nishikawa et al., 2004; Wu-Baer et al., 2003), Lys48 or Lys63 of ubiquitin. Our studies allow us to draw some mechanistic conclusions about how different E2s function with BRCA1/BARD1 as the ubiquitin E3 ligase. Ubc13/Mms2, Ube2k, and UbcH5c each build poly-ubiquitin chains on BRCA1 and share a common ability to bind ubiquitin not only at their active-site cysteine but also non-covalently at a distal site. The nature and location of the non-covalent ubiquitin-binding sites are different for UbcH5c, Ubc13/Mms2, and Ube2k, and presumably the difference in the ubiquitin binding geometry is a major determinant in the different ubiquitin chain linkages that each E2 produces with, and on, BRCA1. UbcH6, Ube2e2, UbcM2, and Ube2w do not poly-ubiquitinate BRCA1, but rather transfer a single ubiquitin to BRCA1. UbcM2 and Ube2w do not bind ubiquitin non-covalently, and due to their high level of sequence homology to the core Ubc domain of UbcM2 it is unlikely that UbcH6 and Ube2e2 bind ubiquitin. These results are consistent with our previous report that a single-site mutation of UbcH5c, Ser22Arg, abrogates the non-covalent ubiquitin-UbcH5 interaction and yields only mono-ubiquitinated BRCA1 as its product. Thus, for the BRCA1/BARD1 ubiquitin ligase, there is a clear correlation between an E2's ability to bind ubiquitin at a site other than its active-site and its ability to build poly-ubiquitin chains with BRCA1.

Neither Ubc13/Mms2 nor Ube2k can transfer ubiquitin directly to BRCA1; they both require BRCA1-ubiquitin as their substrate. We therefore classify Ubc13/Mms2 and Ube2k as ubiquitin-specific E2s with BRCA1/BARD1, as they conjugate ubiquitin

to another ubiquitin in our assay system. In contrast, we classify UbcH6, Ube2e2, UbcM2, and Ube2w as substrate-specific E2s with BRCA1 as they transfer ubiquitin directly to BRCA1 as a substrate (to yield mono-ubiquitinated BRCA1) but not ubiquitin itself (no chains are produced). Among the BRCA1-interacting E2s, UbcH5c is unique in its ability to function as both a substrate-specific and ubiquitin-specific E2 (i.e., "non"-specific) by transferring the first ubiquitin to BRCA1 and extending the chain. The salient features of the three classes of E2s are shown schematically in Figure 2.11. At this point UbcH7 can not be classified with any of the other BRCA1-interacting E2s, as it does not transfer ubiquitin to BRCA1 under any assay conditions tested. However, UbcH7 may select for a different type of substrate not present in our auto-ubiquitination assay or may function by a different mechanism. By comparison, in the absence of Mms2, Ubc13 displays the same properties as UbcH7. Therefore, UbcH7 may require additional unidentified factors necessary for activity with BRCA1/BARD1.

A major question that arises from this work is how an E3 selects the E2s with which it can interact and function. The structural elements that BRCA1 recognizes on an E2 seems to differ somewhat from that reported for other E3s. Common features of the E2 recognized by an E3 for interaction are helix-1, loop L1, and loop L2. Structural and mutational studies of RING-E2 interactions between c-Cbl-UbcH7 (Zheng et al., 2000), CHIP-Ubc13/Mms2 (Zhang et al., 2005b), and CNOT4-UbcH5b (Dominguez et al., 2004) indicate that all three E2 structural elements are utilized for E3 binding, with loop L1 playing a major role. Specifically, loop L1 residues Phe63 of UbcH7, Met64 of Ubc13, and Lys63 of UbcH5b are strongly implicated in interactions with c-Cbl, CHIP,

and CNOT4, respectively. For example, mutation of Lys63 in UbcH5b to aspartic acid abrogates the interaction with CNOT4 (Winkler et al., 2004). The UbcH5b/CNOT4 interaction can be regained by a compensatory mutation of Glu49 to lysine in the RING of CNOT4. However, the position analogous to Lys63 in UbcH5 is not conserved among the BRCA1-interacting set of E2s and in particular Ube2w has aspartic acid at this position, suggesting this is not a determinant of E2 binding to BRCA1. Consistent with this, our NMR mapping studies of BRCA1 and UbcH5c, do not identify loop L1 as a structural element that is highly perturbed upon binding to the RING of BRCA1 (Fig. 2.5). Instead, loop L2 exhibits large chemical shift perturbations. Among the UbcH5c loop L2 residues that exhibit large chemical shifts upon BRCA1 binding, only Ala96 is strictly conserved in the interacting set of E2s. BRCA1 and each of the other structurally characterized E3s utilize the residue corresponding to Ala96 of UbcH5c when interacting with an E2. This alanine is strictly conserved among the BRCA1-interacting E2s and poorly conserved among the E2s that do not interact with BRCA1. The ability of c-Cbl, CHIP, and CNOT4 to discriminate elements of loop L1 may allow them to select a subset of the BRCA1-interacing E2s. It is known, for example, that CHIP interacts with both UbcH5 and Ubc13 and that c-Cbl interacts with UbcH5 and UbcH7. Given CNOT4's strong preference for a lysine at the position corresponding to UbcH5 Lys63, we would predict that other potential CNOT4-interacting E2s could include UbcH6, Ube2e2, UbcM2, and UbcH7, but not Ube2w.

How BRCA1 chooses among its interacting set of E2s for ubiquitination of specific substrates is not yet known. Additional determinants that guide E2-E3 pairings may arise from other protein components that associate with the E3. BRCA1 has been

found in several different multi-protein complexes in the cell (Chiba and Parvin, 2001). Different E2s among the BRCA1-interacting set could be preferred depending on the particular BRCA1/BARD1-containing complex and substrate to be modified. Even without structural knowledge of any of the BRCA1 complexes, we expect that a substrate comes in close proximity to the E2. Therefore, a substrate itself may contribute to which E2 is used for its BRCA1-dependent ubiquitination. Two reported substrates of BRCA1-dependent ubiquitination, estrogen receptor-alpha (ERα) (Eakin et al., 2007) and γ-tubulin (Starita et al., 2004), are good cases in point. Despite the strong poly-ubiquitination activity of BRCA1 with UbcH5 in auto-ubiquitination, both substrates are primarily mono-ubiquitinated with this E2-E3 pair. The only difference between poly-ubiquitination and mono-ubiquitination activities of BRCA1 with UbcH5c is the nature of the substrate. Thus, possible elements within the substrate dictate the nature of the ubiquitination event mediated by BRCA1-UbcH5c. If the substrate can contribute to the nature of the ubiquitin product generated by BRCA1-UbcH5c, it may also be possible that a substrate can contribute to which E2 an E3 selects for specific substrate ubiquitination. Other factors that have been suggested to contribute to E2 selection by an E3 are the sub-cellular localization or tissue distribution of the E2. BRCA1, UbcH5, UbcH7, Ubc13, UbcM2, and Ube2w are all found in the nucleus (Anan et al., 1998; Plafker et al., 2004; Plans et al., 2006; Scully et al., 1997; Yin et al., 2006). However, their sub-nuclear localization has not been rigorously investigated so it is unclear if sub-cellular localization effects E2 selection for BRCA1. It is also unlikely that BRCA1 itself prefers a given E2 over other interacting E2s, as the simultaneous presence of Ube2w with Ubc13/Mms2 does not adversely effect the

ability of Ube2w to mono-ubiquitinate BRCA1 or the ability of Ubc13/Mms2 to build a chain on BRCA1. In this regard, Ubc13/Mms2 is also able to efficiently recognize mono-ubiquitinated BRCA1 generated by UbcM2 and UbcH5c-S22R when these E2s are present at equimolar concentrations (data not shown). Thus, although each E2 binds to the same surface of BRCA1, the presence of another BRCA1-interacting E2 at equal concentration does not negatively effect its activity, and both E2s are able to independently access the BRCA1-RING to carry out their respective functions.

The ability of BRCA1 to synthesize different types of ubiquitination products depending on the E2 with which it associates implies that it can target different substrates for different fates. It has been reported that BRCA1 can synthesize Lys6-linked ubiquitin chains when paired with UbcH5. Although the functional consequence of Lys6-linked poly-ubiquitin chains is unknown, UbcH5 has been found associated with BRCA1/BARD1 complexes in response to DNA damage (Polanowska et al., 2006) suggesting that this E2-E3 pairing is physiologically important. Several investigations into the function of BRCA1/BARD1 strongly implicate the complex to be involved in multiple DNA damage repair pathways. Of particular importance, BRCA1 seems to play a significant yet undefined role during the process of DNA double-strand break (DSB) repair by homologous recombination (HR). In this context, our discovery that BRCA1 both binds to and is active with the E2 heterodimer Ubc13/Mms2 is particularly intriguing. Genetic studies in yeast have identified a role for Ubc13, Mms2, and Lys63-linked poly-ubiquitin chains in DNA damage repair (Hofmann and Pickart, 1999). In yeast, error-free post-replication repair is mediated by Lys63-linked poly-ubiquitination of mono-ubiquitinated PCNA by Ubc13/Mms2 and the E3, Rad5 (Hoege

et al., 2002). Recently, investigation of Ubc13 function in higher eukaryotes has suggested an additional role for Ubc13 in DSB repair by HR (Zhao et al., 2007). Chicken DT40 cells deficient for Ubc13 or human cells with depleted levels of Ubc13 are deficient in their ability to promote HR-mediated DSB repair, a defect similar to that seen in cells lacking BRCA1. Notably, ionizing radiation-induced ubiquitination activity associated with BRCA1 immunoprecipitates is severely reduced in Ubc13 siRNA-treated cells. Zhao *et al.* note that the simplest interpretation of their observations is that BRCA1 cooperates with Ubc13 to promote ubiquitination at sites of DNA damage. However, they were unsuccessful in attempts to detect an interaction between Ubc13 and BRCA1, as E2-E3 interactions are notoriously hard to identify due to their weak affinities. Our studies provide direct evidence that BRCA1 and Ubc13/Mms2 can both physically and functionally interact to promote Lys63-linked poly-ubiquitin chains on a mono-ubiquitinated substrate. Taking our discovery into account with the results of Zhao *et al.* we propose that Ubc13 functionally interacts with BRCA1 in response to DNA DSB leading to ubiquitination events necessary for efficient repair of DNA DSB by HR.

BRCA1 is also implicated in transcription-coupled repair (TCR) of DNA damage. As part of the TCR pathway following UV irradiation, a fraction of the RNA polymerase II (RNA pol II) large subunit is ubiquitinated and degraded by the proteasome. *In vitro*, BRCA1 can ubiquitinate the large subunit of RNA pol II (Kleiman et al., 2005; Starita et al., 2005). Depletion of BRCA1 and BARD1 by siRNA prevents proteasomal-mediated degradation of RNA pol II (Kleiman et al., 2005). The ability of BRCA1 to promote Lys48-linked poly-ubiquitin chains with Ube2k, a signal

that targets a substrate to the proteasome, is consistent with its ability to both ubiquitinate RNA pol II and promote its degradation.

Binding to both a substrate and an E2 is a necessary function for all E3 ligase complexes. Standard models for the ubiquitin transfer cascade suggest all E2s perform the same general function by simply mediating transfer of activated ubiquitin from the E1 and concluding with E3-directed ubiquitin attachment to putative substrates. With over thirty E2s in the human genome we felt that E2s may serve more specialized and distinct functions that may not be detected in the standard substrate-independent ubiquitination assay. We therefore set out to identify E2-E3 interactions based upon binding as a prerequisite to activity. Directed yeast two-hybrid screens between our structure-based BRCA1/BARD1 bait and thirty human E2 prey constructs identified ten E2s that can interact with the RING of BRCA1, with six of these E2-E3 interactions being previously unknown. Such a large number of E2s functioning with a single E3 was unexpected as prevailing models assume a high degree of specificity in the E2-E3 interaction. However, the ability of multiple E2s to function with a single E3 may allow an E3 to ubiquitinate unique substrates differentially or to build ubiquitin chains with different topologies, leading to the possibility of different fates for different substrates. As new substrates of BRCA1 are identified it will be important to determine which E2(s) possesses the ability to transfer ubiquitin to that substrate, making it prudent to include all BRCA1-interacting E2s in assays of potential substrates of BRCA1/BARD1. Ultimately, the identification of all possible E2s that can interact with a given E3 will lead to a more complete understanding of substrate ubiquitination.

Experimental Procedures

Bacterial Expression Plasmids, Protein Expression, and Purification

To generate N-terminal 6X His tagged proteins the primers TAATGCACCATCATCATCATCA and ATACTACTACTACTACCACGTA were annealed and ligated into pET24a+ (Novagen) utilizing the Nde I restriction site generating the plasmid pET24H. To create a DNA construct encoding BD126FBC109 the following primers were used to amplify the respective BARD1 and BRCA1 RING domains: GGCTGGCCATATGGAACCGGATGGTCGC, CGCGGATCCGCCATCTTCTTTCAAATCTGACAGCT, CGCGGATCCGGCGGCGATTTATCTGCTCTTCGCGTTG, and CGACGCGTCGACTTATTTTGCAAAATTATAGCTGTTTGC. The resulting PCR products were digested with Nde I, BamH I, and Sal I (New England Biolabs) and sequentially ligated into pET24H. Genes encoding Ube2k, Ubc13, and Rad6b were cloned into pET24H. Genes encoding UbcH6, Ube2e2, Ube2w, Mms2, and Uev1a were cloned into pET24a+. The gene encoding Ubc9 was cloned into pET28a (Novagen).

All proteins used for NMR or ubiquitination assays were expressed in BL21 Star (DE3) (Invitrogen), except UbcH7, Mms2, and Uev1a which were expressed in Rosetta (DE3) cells (Novagen). Bacteria were grown in either rich LB media or minimal MOPS medium supplemented with [^{15}N] ammonium chloride (Cambridge Isotope Labs). Plasmid constructs, expression, and purification of BC112/BD140 and Flag-BC304/BD302 were performed as described previously (Brzovic et al., 2006). His_6-Ube2k, His_6-Ubc13, and His_6-Rad6b were purified by Ni^{2+}-affinity chromatography,

according to the manufacturers instructions (Sigma) followed by size exclusion chromatography. UbcH5c, UbcH6, Ube2e2, UbcM2, Ube2w, UbcH7, Ubc9, Mms2, and Uev1a were purified by cation exchange (SP Sepharose) eluted with a 0-0.5 M NaCl gradient in 30 mM MES, 1 mM EDTA, pH 6.0. E2 rich fractions were pooled and purified further by size exclusion chromatography in 25 mM sodium phosphate buffer pH 7.0 and 0.15 M NaCl. pET28N-His$_6$-Uba1 (E1) was purified by Ni^{2+}-affinity and anion exchange (Poros HQ) eluted with a 0-3 M NaCl gradient. Ubiquitin was purified as described by Pickart and Raasi (Pickart and Raasi, 2005).

Directed Yeast Two-Hybrid Screens

DNA encoding BD126FBC109 was sub-cloned into pGBKT7 (Clontech) using Nde I and Sal I restriction sites. The I26A mutation was introduced by Quikchange (Stratagene) and confirmed by DNA sequencing (UW, Biochemistry Sequencing Facility). Genes encoding each of the E2s in Fig. 2.1C were inserted into pENTR-D-Topo (Invitrogen) according to the manufacturer's instructions. We thank the following for their generosity in sharing plasmids encoding the genes for various human E2s: Dr. Tomohiko Ohta for Rad6b, UbcH10, and Ube2k; Dr. Brenda Schulman for UbcH5b and UbcH12; Dr. Kevin Loric for Ube2h, UbcH8, Ubc13, and Cdc34; Dr. Stanley Lin for Uev1a; Dr. Wei Xiao for Mms2. UbcH6, Ube2e2, UbcM2, Ubc9, Ube2m3, Ube2s, Ube2t, and Ube2w were PCR amplified from Matchmaker Human Ovary cDNA library (Clonetech). Genes encoding Ube2g1, Ube2u, Ube2z, Ube2v2, Ube2v3, Ube2q2, Ft1, and TSG101 were purchased from Open Biosystems. Using the Gateway Vector Conversion System (Invitrogen) the gateway cassette was inserted into pACT2

(Clontech). Recombinational cloning was performed with the LR clonase kit (Invitrogen) resulting in a pACT2 vector encoding each E2.

Respective “bait” (pGBKT7) and “prey” (pACT2) plasmids were co-transformed into the yeast strain AH109 (Clontech). Positive transformants were selected on minimal SD -Leu/-Trp media (Clontech). A single colony for each bait/prey combination was suspended in 100 μL of sterile water in a 96 well plate. Using a replica plater (Sigma) yeast cells from the single colony were spotted onto selective media (SD -His/-Leu/-Trp with 0, 1, 2.5, 5, and 10 mM 3AT (Sigma) and non-selective control media SD -Leu/-Trp. Yeast were incubated at 30°C for 7 days and photographed.

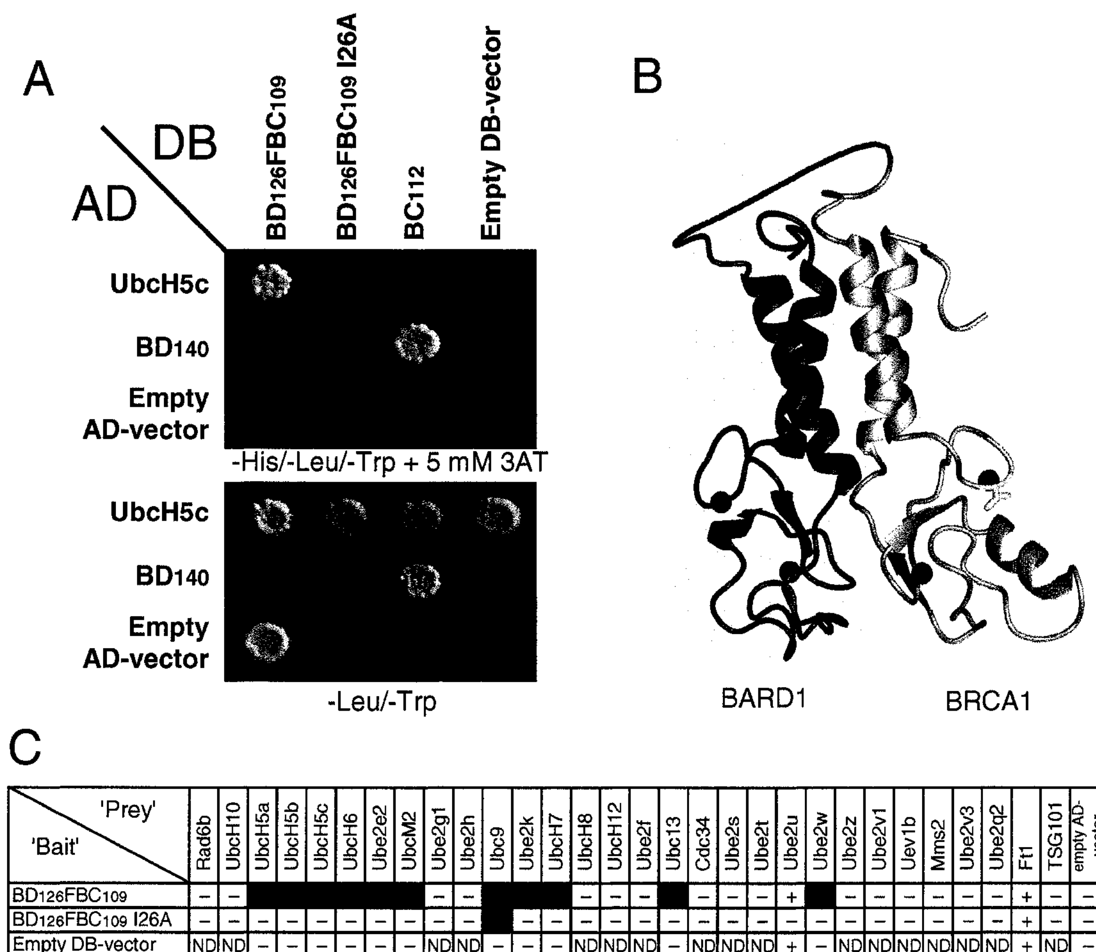
NMR Spectroscopy

Titration of uniformly ^{15}N-labeled BRCA1/BARD1 (BC112/BD140) heterodimer (initial concentration 0.3 mM) with each unlabeled E2 noted in the text were performed at 35°C in 25 mM sodium phosphate buffer pH 7.0, 0.15 M NaCl, and 10% D_2O. Sequential addition of each E2 to 0, 0.125, 0.25, 0.5, and 1 equivalents of E2 to BRCA1/BARD1 were made. ^{1}H, ^{15}N-TROSY spectra were recorded on a Bruker DMX 500 MHz spectrometer. Spectra were processed and analyzed using NMRPipe (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994).

Auto-Ubiquitination Activity Assays

150 μL reaction mixtures for BRCA1-directed ubiquitination assays included 1.6 μM Flag-BC304/BD302 heterodimer, 1.2 μM specified E2, 20 μM ubiquitin, and 0.5

μM wheat Uba1. All reactions with Ube2k were assayed with 0.2 μM human E1 (BioMol). Reactions were initiated by addition of 5 mM ATP and 10 mM $MgCl_2$. Samples were collected at 0, 30, 60, and 120 minutes after addition of ATP. Reaction products were resolved on a NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen) and transferred onto PVDF membranes (Biorad). The membranes were probed with mouse anti-Flag (Sigma) antibody, followed by goat anti-mouse secondary antibody conjugated to Alexa Fluor 680 (Molecular Probes). Blotted proteins were detected by using an Odyssey infrared imaging system (Licor).



'Prey' / 'Bait'	Rad6b	UbcH10	UbcH5a	UbcH5b	UbcH5c	UbcH6	Ube2e2	UbcM2	Ube2g1	Ube2h	Ubc9	Ube2k	UbcH7	UbcH8	UbcH12	Ube2f	Ubc13	Cdc34	Ube2s	Ube2t	Ube2u	Ube2w	Ube2z	Ube2v1	Uev1b	Mms2	Ube2v3	Ube2q2	Ft1	TSG101	empty AD-vector
BD126FBC109	–	–							–	–				–	–	–		–	–	–	+		–	–	–	–	–	–	+	–	–
BD126FBC109 I26A	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Empty DB-vector	ND	ND	–	–	–	–	–	–	ND	ND	–	–	–	ND	ND	ND	–	ND	ND	ND	+	–	ND	ND	ND	ND	ND	ND	+	ND	–

Figure 2.1. A "Structure Based" Fused BRCA1/BARD1 Construct Interacts with Multiple Different E2s in a Yeast Two-Hybrid Analysis.

A) Proof-of-principle screen demonstrating that UbcH5c interacts with the RING:RING fusion BD126FBC109 but not with the Ile26Ala RING mutant of BRCA1 or the RING of BRCA1 alone (BC112). Yeast that can grow under selective conditions (upper panel) indicate an interaction between the respective bait (Gal4 DNA-binding domain (DB)) and prey (Gal4 activation domain (AD)) fusions. Lower panel shows that the yeast are viable under control conditions. B) Model of BD126FBC109. The model is based on the structure of the RING:RING heterodimer of BRCA1/BARD1 (PDB 1JM7) with a five amino acid linker sequence shown schematically in red connecting the C-terminal residue of the BARD1 RING to the N-terminal residue of BRCA1. Ile26 of the BRCA1 RING is highlighted in yellow. C) Summary of directed yeast-two hybrid screens with the respective "bait" DNA-binding (DB) fusions and "prey" activation domain (AD) fusions. The symbols + or – indicate the presence or absence of growth on selective media, and ND is not determined. Positive interactions specific for the "bait" BD126FBC109 and not empty vector controls are highlighted in red.

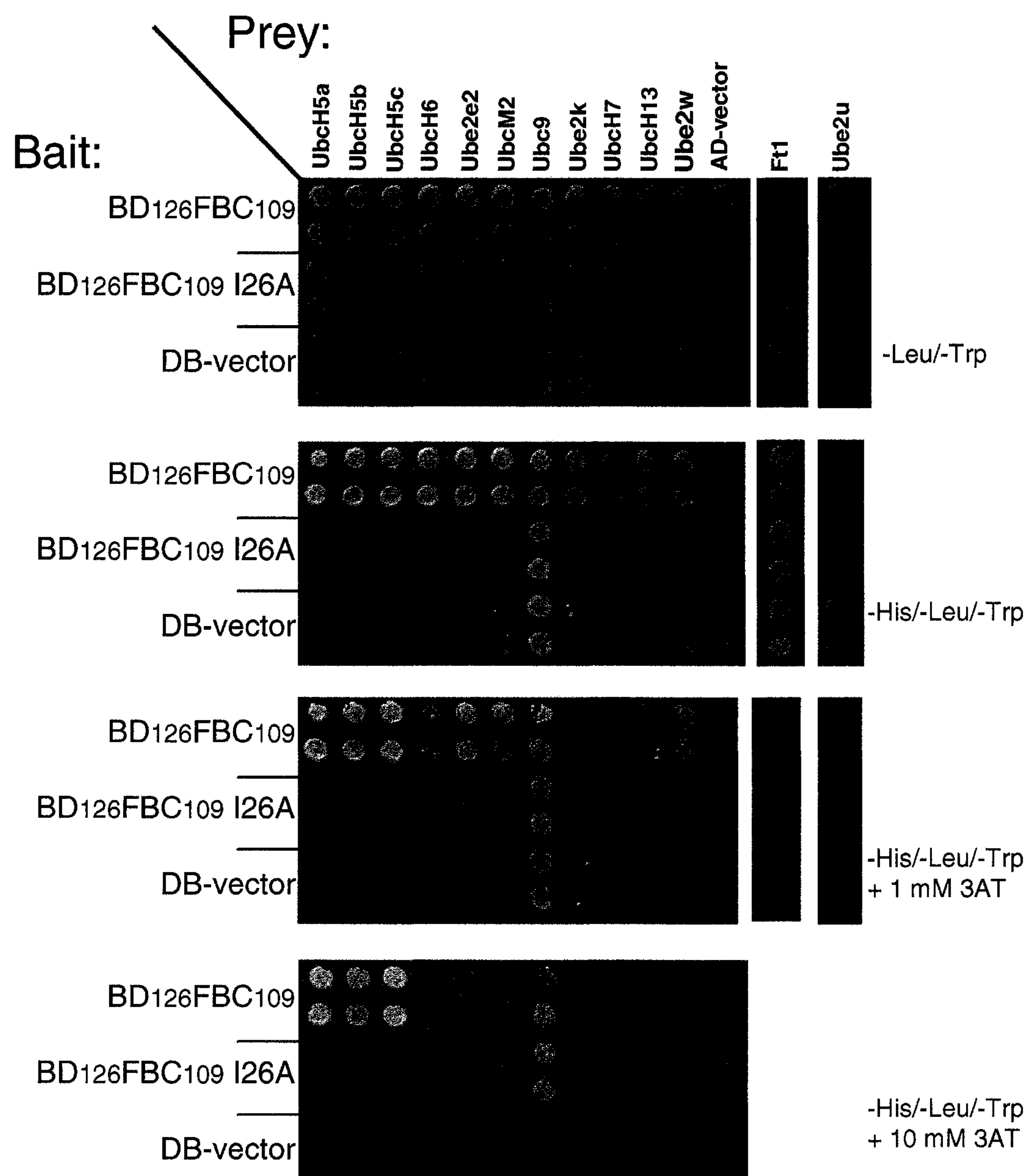


Figure 2.2 Structure-Based E2 BRCA1/BARD1 Screen.

Directed yeast two-hybrid screens with E2s found to interact with BD126FBC109. Plasmids for each "bait" and "prey" pair were co-transformed together and with GAL4 DNA binding (DB) and GAL4 activation domain (AD) empty vector controls into the yeast strain AH109. Positive transformants were selected on minimal synthetic media lacking leucine and tryptophan (-Leu/-Trp). Two colonies for each "bait" and "prey" were replica plated on selective (-His/-Leu/-Trp ± 3AT) and non-selective media (-Leu/-Trp). Each E2 was also screened against BD126FBC109 I26A to test for interaction with the BRCA1 RING.

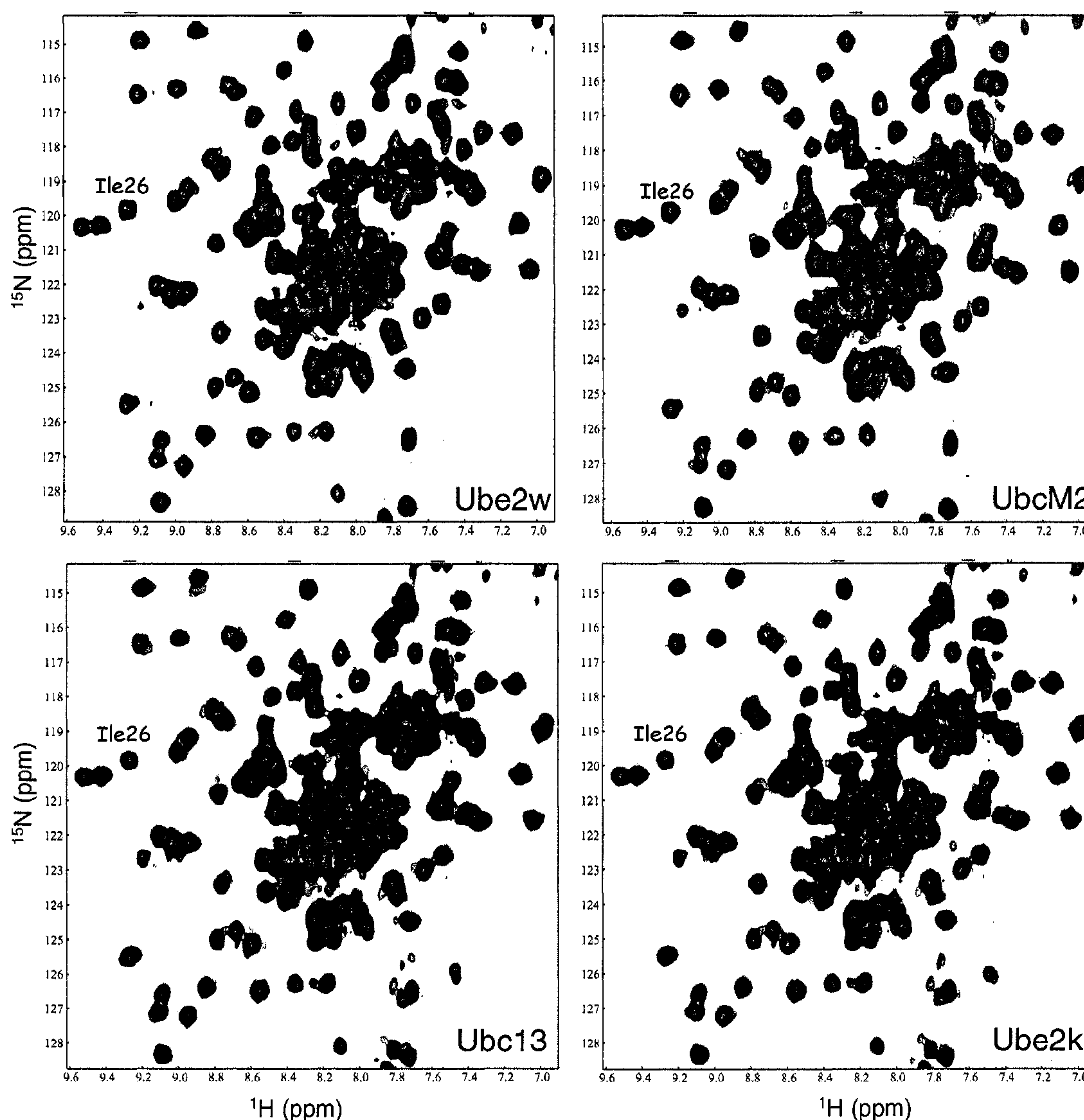


Figure 2.3. Confirmation of Direct E2-BRCA1/BARD1 Interactions by 2D HSQC NMR.

^{1}H, ^{15}N -TROSY-HSQC titrations of E2 binding to the BRCA1/BARD1 RING/RING heterodimer. Overlay of an expanded region of the TROSY spectrum of ^{15}N-BC112/BD140 alone (black) and with the addition of 0.5 mol equivalent of an unlabeled E2 (red) is shown. The resonance peak for Ile26 of BRCA1 is labeled. Spectra collected with the addition of UbcH6 and Ube2e2 are not shown as their effects on ^{15}N-BC112/BD140 are indistinguishable to UbcM2. Spectra collected with the addition of Ubc9 are not shown as Ubc9 had no effect on the ^{1}H, ^{15}N -TROSY-HSQC spectrum of ^{15}N-BC112/BD140 (black and red spectrum are identical).

		Helix-1		Loop L1		Loop L2
		* * *				* * *
UbcH5a	1	MALKRIQKELSDLQRD	:57	PTDYPFKPP	:92	QWSPALTVSKVLL
UbcH5b	1	MALKRIHKELNDLARD	:57	PTDYPFKPP	:92	QWSPALTISKVLL
UbcH5c	1	MALKRINKELSDLARD	:57	PTDYPFKPP	:92	QWSPALTISKVLL
UbcH6	47	TSAKRIQKELADITLD	:103	TPEYPFKPP	:138	NWSPALTISKVLL
Ube2e2	55	TSAKRIQKELAEITLD	:111	SPDYPFKPP	:146	NWSPALTISKVLL
UbcM2	61	TSAKRIQKELAEITLD	:117	SSDYPFKPP	:152	NWSPALTISKVLL
Ube2k	7	QRIKREFKEVLKSEET	:63	PETYPFNPP	:99	QWAAAMTLRTVLL
UbcH7	2	AASRRLMKELEEIRKC	:58	PAEYPFKPP	:94	NWKPATKTDQVIQ
Ubc13	3	GLPRRIIKETQRLLAE	:59	PEEYPMAAP	:94	KWSPALQIRTVLL
Ube2w	14	WFPKRLQKELLALQND	:72	SSRYPFDSP	:109	DWSPALSVQSVCL
Rad6b	4	PARRRLMRDFKRLQED	:60	SEEYPNKPP	:95	RWSPTYDVSSILT
UbcH10	30	PVGKRLQQELMTLMMS	:86	PSGYPYNAP	:121	KWSALYDVRTILL
Ube2g1	8	LLLRRQLAELNKNPVE	:62	PKDYPLRPP	:110	RWLPIHTVETIMI
Ube2h	6	PGKRRMDTDVVKLIES	:58	PDKYPFKSP	:94	TWTALYDLTNIFE
Ubc9	10	AQERKAWRKDHPFGFV	:65	KDDYPSSPP	:102	DWRPAITIKQILL
UbcH8	2	MASMRVVKELEDLQKK	:58	PPEYPFKPP	:94	NWKPCTKTCQVLE
UbcH12	29	AAQLRIQKDINELNLP	:83	GQGYPHDPP	:118	DWKPVLTINSIIY
Ube2f	32	VRDKLLVKEVAELEAN	:88	PDAYNMVPP	:129	GWAPTRTLKDVVW
cdc34	8	SSQKALLLELKGLQEE	:65	PIDYPYSPP	:113	RWNPTQNVRTILL
Ube2s	11	HIIRLVYKEVTTLTAD	:67	GKDFPASPP	:102	DWTAELGIRHVLL
Ube2t	2	QRASRLKRELHMLATE	:58	PERYPFEPP	:97	AWRPSLNIATVLT
Ube2u	4	RAYLLLHRDFCDLKEN	:60	TSEYNYAPP	:98	KWNTNYTLSSILL
Ube2z	1	MSIYKEPPPGMFV	:47	PPDYPPIHP	:91	AWSPAQSISSVLI

Figure 2.4. Sequence Alignment of Helix-1, Loop L1, and Loop L2 of BRCA1-Interacting and Non-Interacting E2s.

The majority of BRCA1 interacting residues reside in helix-1 and loop L2 of UbcH5c (see Figure 2.5). Each E2 was aligned relative to the sequence of UbcH5c. The first group of E2s are those that directly interact with the RING of BRCA1. The lower group of E2s were present in the yeast two-hybrid screen but do not interact with BRCA1. Surface exposed residues of UbcH5c that are involved in the direct interaction with BRCA1 are marked (*). Shaded are residues aligned with Arg5, Lys8, and Ala96 of UbcH5c which are strictly conserved among the BRCA1-interacting set and poorly conserved among the non-interacting E2s.

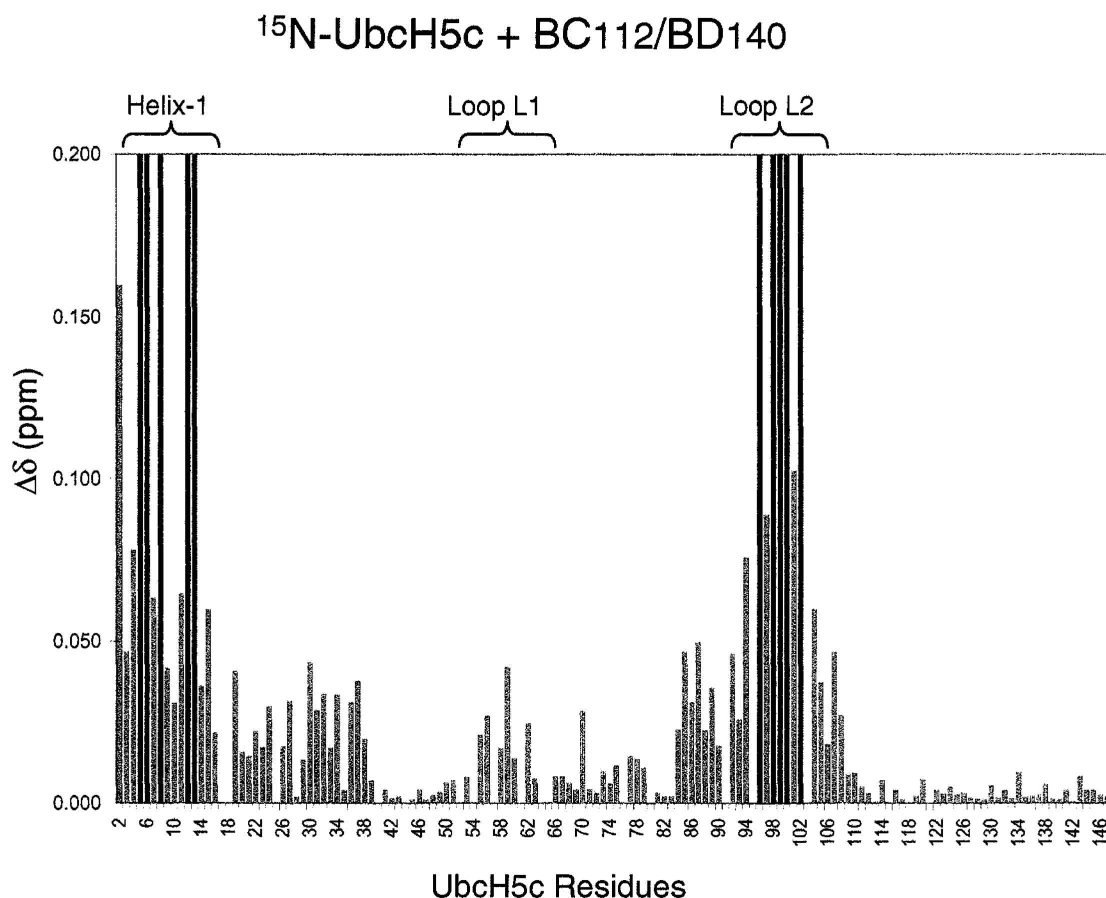


Figure 2.5. Chemical Shift Perturbations on ^{15}N-UbcH5c due to binding of BC112/BD140.

Chemical shift perturbations of ^{15}N-UbcH5c residues (350 μM) upon addition of BC112/BD140 (1.0 eq). Combined chemical shift differences were calculated using the equation $\Delta\delta=[(\delta_{HN})^2 + (\delta_N/6.51)^2]^{1/2}$. Red bars correspond to resonances of UbcH5c that are completely broadened upon addition of BC112/BD140. The average Δδ of all UbcH5c resonances observed in the presence of BC112/BD140 is 0.03 ppm. E2 structural elements involved in other reported E2-E3 interactions are noted. Data was collected and processed by Dr. Peter Brzovic

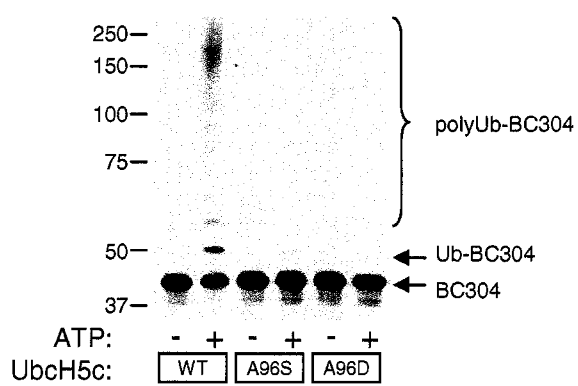


Figure 2.6. Mutation of Conserved Alanine in Loop L2 of UbcH5c Eliminates Auto-Ubiquitination of BRCA1.

Purified wheat E1, ubiquitin, and Flag-BC304/BD327 were mixed with the indicated mutant or wild-type UbcH5c. Time points for ubiquitination were taken at 0 (-) and 60 min. (+) after addition of ATP. Reaction products are visualized by α-Flag western blot of Flag-BC304. Slower migrating bands above the main Flag-BC304 band are ubiquitinated forms of BC304.

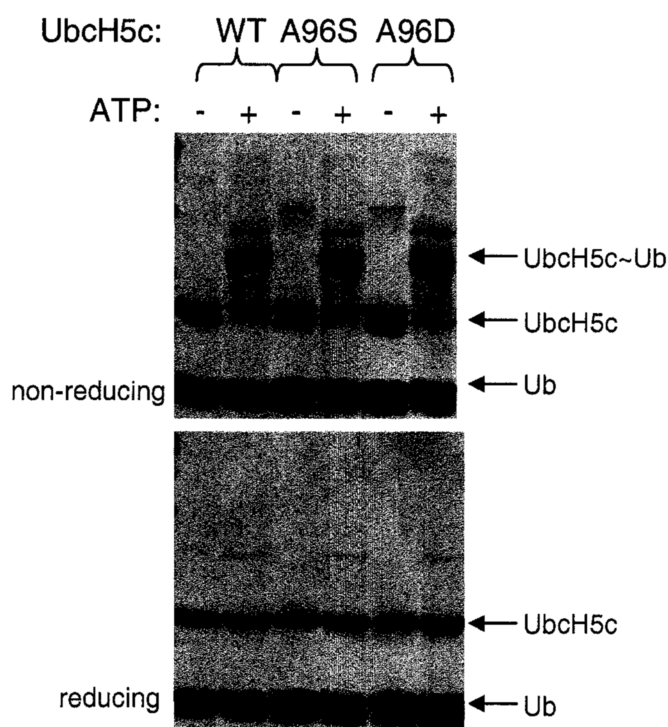
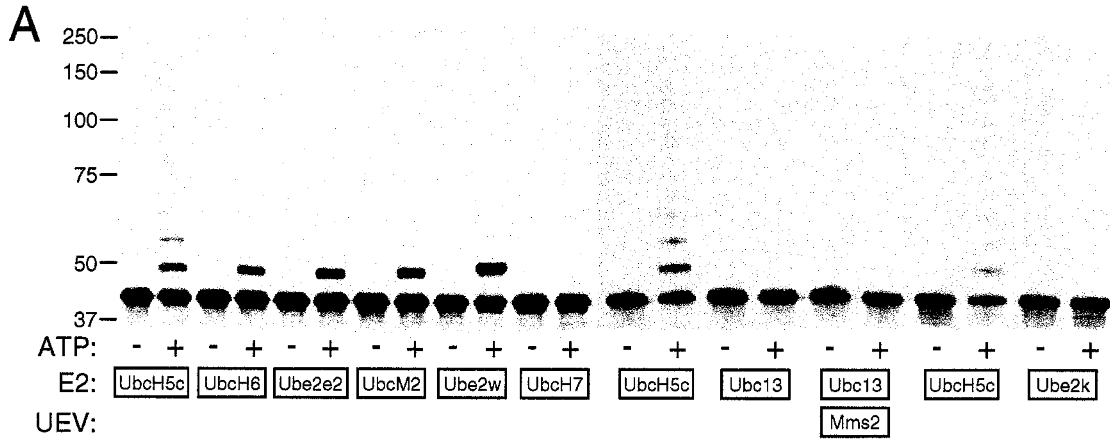
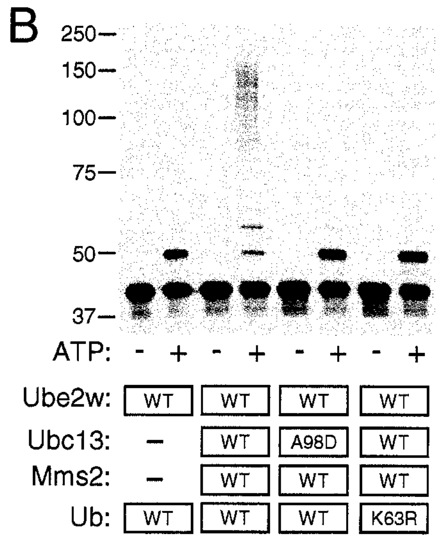
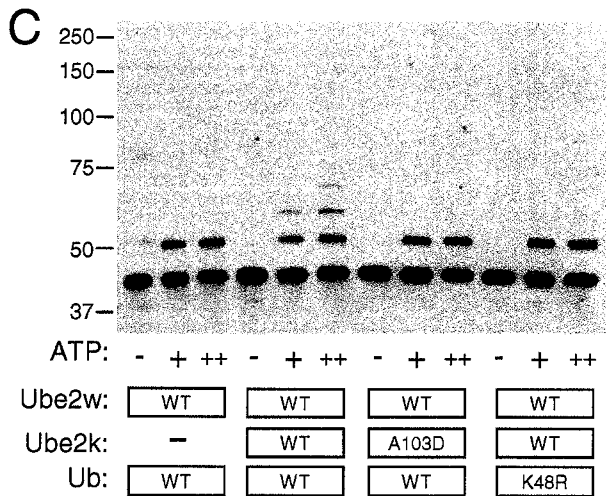


Figure 2.7. UbcH5c Ala96 Mutants Form a Thiol Ester with Ubiquitin as Efficiently as Wild-Type E2.

Coomassie stained SDS-PAGE gels showing E2~ubiquitin thiol ester formation with mutant and wild-type UbcH5c. Assays contained 10 μM E2, 0.5 μM E1, 20 μM Ubiquitin (Ub), and either 0 (-) or 5 mM ATP with 10 mM $MgCl_2$ (+) and were incubated at 37°C for 30 minutes.


A
250
150
100
75
50
37
ATP: - + - + - + - + - + - + - + - + - + - + - +
E2: UbcH5c UbcH6 Ube2e2 UbcM2 Ube2w UbcH7 UbcH5c Ubc13 Ubc13 UbcH5c Ube2k
UEV: Mms2


B
250
150
100
75
50
37
ATP: - + - + - + - +
Ube2w: WT WT WT WT
Ubc13: – WT A98D WT
Mms2: – WT WT WT
Ub: WT WT WT K63R


C
250
150
100
75
50
37
ATP: - + ++ - + ++ - + ++ - + ++
Ube2w: WT WT WT WT
Ube2k: – WT A103D WT
Ub: WT WT WT K48R

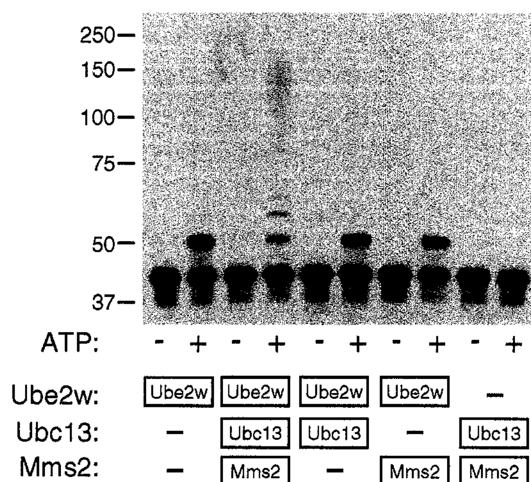


Figure 2.9. Poly-Ubiquitination of BRCA1 by Ubc13/Mms2 and Ube2w.

α-Flag western blot showing auto-ubiquitination of Flag-BC304/BD327 with equal concentration of each indicated E2. Only the combination of Ubc13, Mms2 and Ube2w results in poly-ubiquitinated BRCA1. Omission of any one of the E2s results in either mono-ubiquitination or no activity. Ubc13/Mms2 can also poly-ubiquitinate BRCA1 in combination with the other mono-ubiquitinating E2s, UbcM2 and UbcH5c-S22R (data not shown).

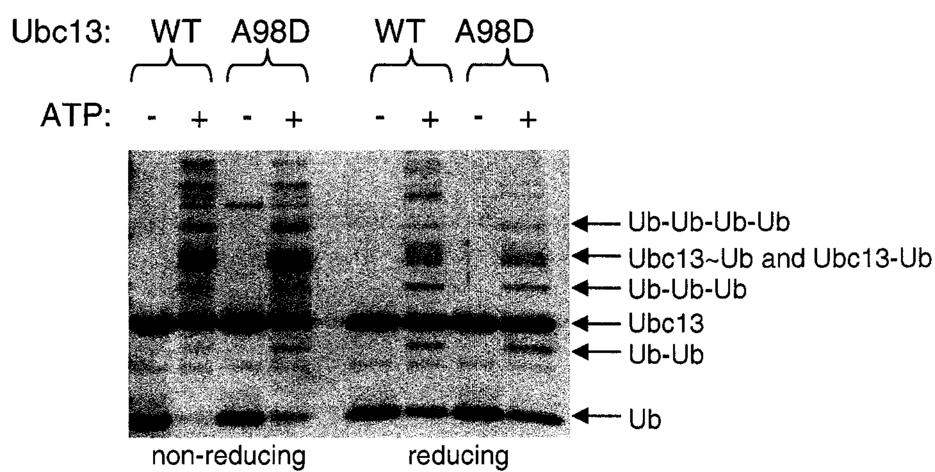


Figure 2.10. Ubc13-A98D is Charged with Ubiquitin and Synthesizes Poly-Ubiquitin Chains as Efficiently as Wild-Type.

Coomassie stained SDS-PAGE gels showing E2~ubiquitin thiol ester formation with mutant and wild-type Ubc13 and poly-ubiquitin chain formation. Assays contained 10 μM E2, 10 μM Mms2, 0.5 μM E1, 20 μM Ubiquitin (Ub), and either 0 (-) or 5 mM ATP with 10 mM $MgCl_2$ (+) and were incubated at 37°C for 30 minutes. Thiol ester bonds are sensitive to reducing agents and are indicated by (~) and isopeptide bonds are not sensitive to reducing agents and are indicated by (-).

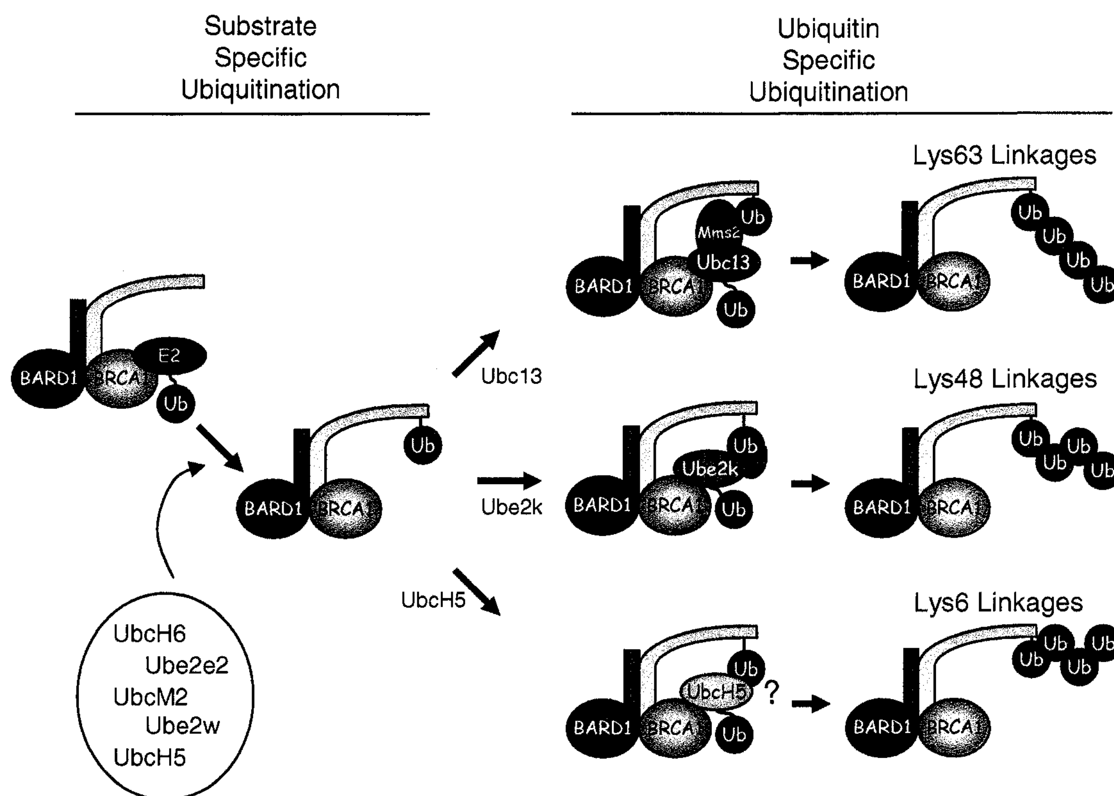


Figure 2.11. Model of BRCA1/BARD1 Auto-Ubiquitination Mechanisms.

BRCA1-interacting E2s are classified based on what they recognize for ubiquitin transfer. The "substrate-specific" E2s UbcH6, Ube2e2, UbcM2, and Ube2w all transfer ubiquitin directly to BRCA1. "Ubiquitin-specific" E2s, Ubc13/Mms2 and Ube2k, bind ubiquitin non-covalently and only recognize BRCA1-ubiquitin for synthesis of Lys63 or Lys48-linked poly-ubiquitin chains. UbcH5 does not show specificity as it can recognize BRCA1 for ubiquitin transfer as well as extend a poly-ubiquitin chain. UbcH7 is not shown as it does not auto-ubiquitinate BRCA1. A non-covalent interaction with ubiquitin is a common property among the "ubiquitin-specific" E2s and is likely necessary for poly-ubiquitin chain elongation.

Chapter 3. Substrate-E2 Interaction Dictates BRCA1 E2 Selection for Ubiquitin Transfer to a BARD1-Associated BRCA1 Substrate

Introduction

The breast and ovarian cancer susceptibility gene, *BRCA1*, was first identified based upon linkage analysis of families with high incidence of breast or ovarian cancer (Hall et al., 1990; Miki et al., 1994). Since its discovery BRCA1 has been intensely investigated to determine its normal cellular function so as to understand why mutations lead to breast and ovarian cancers. Several functions have been suggested for BRCA1, most of which involve maintenance of genomic integrity: DNA damage repair (Zhang and Powell, 2005), centrosome duplication (Deng, 2002), chromatin remodeling (Bochar et al., 2000), and transcription (Mullan et al., 2006). However, the biochemical function of BRCA1 in any of these processes is unknown.

BRCA1 encodes a large protein of 1863 amino acid residues with two highly conserved domains, a RING domain at its N-terminus and two BRCT repeats at its extreme C-terminus. The only biochemical activity identified for BRCA1 is its ability to function as a ubiquitin ligase (Hashizume et al., 2001). The ubiquitin ligase activity of BRCA1 is mediated by its RING domain (Brzovic et al., 2003) and requires heterodimerization with another RING containing protein BARD1 (Hashizume et al., 2001). From the three-dimensional structure of the BRCA1/BARD1 RING:RING heterodimer (Brzovic et al., 2001) it is apparent that their association is required to maintain the appropriate structural conformation of their respective RING domains. Not only does the ubiquitin ligase activity of BRCA1 require dimerization with BARD1, the two proteins appear to function as a single complex *in vivo* as most of the

BRCA1 protein in the cell is found associated with BARD1 (Yu and Baer, 2000), and mice deficient for either BRCA1 or BARD1 display very similar phenotypes (McCarthy et al., 2003).

A ubiquitin ligase (E3) functions at the cross road between activation of ubiquitin and its attachment to a lysine residue on a substrate. The process of ubiquitination begins with the ubiquitin-activating enzyme (E1) which forms an ATP-dependent thiol ester bond with the C-terminal Gly76 of ubiquitin. The activated ubiquitin is transferred from the E1 to the active-site cysteine of a ubiquitin-conjugating enzyme (E2). By interacting with both a substrate and an activated E2, the E3 mediates the transfer of ubiquitin from the E2 active-site to a substrate lysine residue, *via* an unknown mechanism. The coordinated action of the ubiquitin cascade results in a mono-ubiquitinated substrate or the synthesis of a poly-ubiquitin chain on the substrate where the ubiquitin attached during one round of the cascade becomes the substrate for the next ubiquitin transfer. There are seven lysine residues in ubiquitin, all with the potential to be utilized for poly-ubiquitin chains. Protein ubiquitination is a diverse signaling mechanism used to regulate a host of different cellular processes including protein degradation (Pickart, 1997), DNA damage repair (Hofmann and Pickart, 1999), transcription (Dhananjayan et al., 2005), signal transduction (Deng et al., 2000), and endocytosis (Hicke, 2001). The type of ubiquitination product synthesized on a substrate can have a profound effect on its function. For example, Lys48-linked poly-ubiquitin chains target the attached substrate for degradation by the proteasome.

The standard model of ubiquitination implies pair wise interactions between substrate-E3 and E3-E2. Common assumptions of this model are: 1) the E3 is the

primary determinate of substrate specificity and 2) an E3 functions with a single E2. In contrast to this model we have recently reported that BRCA1 can interact with eight different human E2s (Christensen et al., 2007) and BRCA1 synthesizes different ubiquitination products ranging from mono-ubiquitin to specific poly-ubiquitin chain linkages depending on the E2 used in an auto-ubiquitination assay. How BRCA1 selects among the E2s, with which it interacts, for ubiquitin transfer to a particular substrate is not known. One possibility is that the substrate may help select the E2 *via* a direct E2-substrate interaction. Evidence in support of a direct interaction between an E2 and substrate is found in the ubiquitin-like pathway of sumoylation. Many SUMO targets are sumoylated *in vitro* in the absence of an E3 (Seeler and Dejean, 2003), suggesting that the SUMO E2, Ubc9, plays a significant role in substrate selection. A substrate binding site was identified on Ubc9 using NMR interaction studies between the E2 and two SUMO targets, p53 and c-Jun (Lin et al., 2002). Direct evidence for substrate-E2 interactions in the SUMO pathway is provided by a crystal structure where Ubc9 directly interacts with RanGAP1 (Bernier-Villamor et al., 2002), a sumoylated substrate.

Well defined protein interaction domains within the E3 represent likely substrate binding sites. For example, many E3s of the TRIM/RBCC family recognize their substrates using conserved B-box 1, B-box 2, or coiled-coil domains (Meroni and Diez-Roux, 2005). Many of the reported substrates ubiquitinated by BRCA1/BARD1 appear to bind to the BRCA1 subunit of the complex (Eakin et al., 2007; Starita et al., 2005; Starita et al., 2004; Yu et al., 2006), although the location of a specific substrate binding site is unknown for many of these substrates. Outside of its RING domain, which is

responsible for E2 binding, the only identifiable domain in BRCA1 is its BRCT repeats; as the remainder of the large protein does not contain clear domains as candidates for substrate recognition. The distal BRCTs serve as a binding site for one reported substrate, CtIP (Yu et al., 2006).
However, BRCA1-dependent ubiquitination of ERα (Eakin et al., 2007) and NPM (Sato et al., 2004) does not require the BRCT domain, suggesting that additional binding sites for ubiquitination targets exist within BRCA1/BARD1.

Though ubiquitin ligase activity is mediated by the direct association of the BRCA1 RING with any of several different E2s, the BARD1 subunit is required for activity and represents another polypeptide with the potential to bind substrates. To date, BARD1 has been largely ignored in reported searches for cellular targets of the BRCA1/BARD1 E3. As BARD1 is an integral member of the BRCA1 complex we set out to identify potential substrates that bind primarily to the BARD1 subunit and to assess whether any identified putative substrate can be ubiquitinated by BRCA1/BARD1 with one or more of the recently identified set of BRCA1-interacting E2s.

There are no standardized methods to identifying substrates of an E3 and the mechanisms utilized for substrate selection are not well characterized. The process is further complicated for BRCA1/BARD1 as the E3 is comprised of two proteins forming a heterodimeric complex. To overcome the complexities associated with identifying proteins that interact with a heterodimeric E3 we designed a novel fusion of BRCA1/BARD1 for use as "bait" in a yeast two-hybrid screen. Our design maintains the structural integrity of the RING domains and incorporates a bias toward selection of

BARD1-associated substrates. We report the identification of a BARD1-Associated BRCA1 Substrate, BABS (KIAA0701) a previously uncharacterized protein, from a human ovarian cDNA library. BRCA1/BARD1 mediates transfer of ubiquitin to BABS using the E2s UbcH5c, Ube2w, Ubc13, and Ube2k, *in vitro*. Unexpectedly, we also found that BABS binds to this subset of the BRCA1-interacting E2s. The ubiquitination of BABS not only requires substrate-E3 and E3-E2 interactions, but a substrate-E2 interaction as well, as disruption of any of these interactions eliminates ubiquitination. This work demonstrates that 1) substrates can bind to the BARD1 subunit of the heterodimeric complex, 2) E2s other than UbcH5 can function with BRCA1 for substrate ubiquitination, and 3) ubiquitination of BABS requires a three-way interaction among the E3, E2, and substrate, in contrast to prevailing models.

Results

Identification of BABS by Yeast Two-Hybrid Screen with a BRCA1/BARD1 Fusion "Bait"

BRCA1 and BARD1 form a obligate heterodimer mediated by α-helices formed by residues that flank their respective RING domains (Brzovic et al., 2001). Direct interaction is required to maintain their respective RING domains in the appropriate structural conformation. We designed a fused version of BRCA1/BARD1 to include both subunits as bait in a yeast two-hybrid screen. Design of the fusion was based on the three dimensional structure of the BRCA1/BARD1 RING:RING heterodimer in which the N- and C-terminal residues are relatively close together and unstructured (Brzovic et al., 2001). A six-residue linker, Gly_3-Ser-Gly_2, was used to fuse the RING

domain of BRCA1 (residues 1-98) to the N-terminus of BARD1. The main principles behind the design of this fusion are the same as a similar BRCA1/BARD1 RING:RING fusion reported previously (Christensen et al., 2007), however, the arrangement allows for inclusion of additional BARD1 residues beyond its RING domain. We reasoned that substrate binding sites might be located proximal to the (E2-binding) RING domains and therefore included BARD1 residues 29-327 to create the fusion BC98FBD327 (Fig. 3.1A). The BRCA1 ligase-inactive mutation Ile26Ala (Brzovic et al., 2003) was incorporated into our bait (BC98(I26A)FBD327) to eliminate any potentially negative effects associated with using an active E3 in the presence of the ubiquitin pathway components within the yeast cell. For example, an active ligase could target a substrate for degradation without sufficiently activating the reporter cassette, thus, eliminating the potential to identify substrates of the E3.

As mutations in BRCA1 lead primarily to breast and ovarian cancers, we screened BC98(I26A)FBD327 against a human ovarian cDNA library. From the screen we identified a previously uncharacterized protein KIAA0701 (accession numbers NP_055869 and EAW97619) that interacts with the BC98(I26A)FBD327 bait but not with the empty DNA-binding domain (DB) vector (Fig. 3.1B, columns 1 and 8), referred to as BABS for BARD1-Associated BRCA1 Substrate. BABS encodes a large protein of 1464 residues with no recognizable conserved domains, except a predicted coiled-coil region encompassing the last ~50 residues. The BABS construct recovered from the two-hybrid screen encodes residues 933-1464.

To identify the regions of BRCA1 or BARD1 important for interaction, directed two-hybrid screens between BABS and various mutations of the BC98(I26A)FBD327 bait

were conducted, as illustrated in Figure 3.1A. Truncation of BARD1 just beyond the RING domain (BC98(I26A)FBD126, Fig. 3.1B, column 4) eliminates the interaction with BABS, suggesting BABS binds to BARD1 residues beyond the RING domain. BABS supports growth of yeast with BC98(I26A)FBD170 and BD126-327 (BARD1 residues 126-327, Fig. 3.1B columns 3 and 5), consistent with an interaction with BARD1 residues beyond the RING domain. This analysis indicates that BABS recognizes BARD1 residues 126-170 as its primary binding-site. To evaluate the effect of the ligase inactive BRCA1 mutation (Ile26Ala) on the ability to detect the BABS interaction, we rescreened BABS against bait constructs with wild-type BRCA1 (Fig. 3.1B, columns 6 and 7). Unexpectedly, the short construct BC98FBD126 supports growth in contrast to BC98(I26A)FBD126 (Fig. 3.1B, column 4 vs. 7). Thus, wild-type BRCA1 RING appears to facilitate an interaction with BABS. Previous work has established that the Ile26Ala mutation of BRCA1 specifically disrupts its ability to interact with E2s (Christensen et al., 2007; Brzovic et al., 2006). There are at least three E2s in yeast that are close homologs to BRCA1-interacting E2s UbcH5c (yUbc4 and yUbc5) and Ubc13 (yUbc13). Thus, the ability of BC98FBD126, but not BC98(I26A)FBD126, to support yeast growth with BABS may be due to a bridging interaction mediated by endogenous yeast E2s.

BABS Interacts with and is Ubiquitinated by a Subset of BRCA1-Interacting E2s

To address the possibility that an E2 can bridge the interaction between the RING of BRCA1 and BABS, directed yeast two-hybrid screens were conducted between BABS and the BRCA1-interacting E2s: UbcH5c, UbcH6, Ube2e2, UbcM2,

Ube2k, UbcH7, Ubc13, and Ube2w. The UEV Mms2 was also included as it forms a heterodimer with Ubc13. UbcH5c, Ubc13, and Ube2w all support yeast growth with BABS suggesting an interaction with each of these E2s (Fig. 3.2). Ube2k also appears to interact with BABS as this pair supports yeast growth slightly better than the BD-vector control (Fig. 3.2). Thus, the two-hybrid results indicate that BABS can bind both to BARD1, in a region proximal to its RING domain, and to the E2s UbcH5, Ubc13, Ube2w, and Ube2k, which in turn bind to the BRCA1 RING (Christensen et al., 2007).

Additional directed two-hybrid screens map the BARD1 and E2 binding sites to within BABS residues 1145 to 1402 (data not shown). For *in vitro* ubiquitination assays we expressed and purified BABS (residues 1145 to 1402) from *E. coli*. Ubiquitination of BABS was assayed using a BRCA1/BARD1 heterodimer comprised of approximately the first 300 residues of each subunit (BC304/BD327) as the E3 with each of the BRCA1-interacting E2s. As shown in Figure 3.3A, UbcH5c and Ube2w transfer ubiquitin to BABS in the presence of BRCA1/BARD1 with the primary product being mono-ubiquitinated BABS. None of the remaining BRCA1-interacting E2s transfer ubiquitin to BABS, with the exception of a small amount of transfer observed with UbcH6, Ube2e2, and UbcM2 (Fig. 3.3A).

We have classified the BRCA1-interacting E2s based on their ability to auto-ubiquitinate BRCA1 (Christensen et al., 2007). Ube2w, UbcH6, Ube2e2, UbcM2, and UbcH5 were classified as "substrate-specific" E2s because they transfer ubiquitin directly to BRCA1 as the substrate. Ubc13/Mms2 and Ube2k were classified as "ubiquitin-specific" E2s because they do not transfer ubiquitin directly to BRCA1, but rather recognize a mono-ubiquitin attached to BRCA1 as their substrate for poly-

ubiquitin chain synthesis. We tested whether the mono-ubiquitinated BABS product generated by Ube2w can function as a substrate for the chain-building E2s, Ubc13/Mms2 and Ube2k. Equal molar amounts of Ube2w and Ubc13/Mms2 or Ube2w and Ube2k were included in a BRCA1/BARD1-dependent ubiquitination assay of BABS. In the presence of Ube2w, which transfers the first ubiquitin, Ubc13/Mms2 and Ube2k generate high molecular weight poly-ubiquitination products attached to BABS (Fig. 3.3B and C). Though Ubc13 and Ube2k interact with BABS (Fig. 3.2) poly-ubiquitination requires direct interaction between the E3 and E2 as mutation of the E2 (Ubc13-A98D and Ubek2-A103D), to disrupt interaction with the BRCA1 RING (Christensen et al., 2007) but not with BABS (Fig. 3.5A), eliminates transfer. The poly-ubiquitination products synthesized on BABS by Ubc13/Mms2 are Lys63-linked chains and those synthesized by Ube2k are Lys48-linked chains as Lys to Arg mutation of these residues abrogates chain elongation (Fig. 3.3B and C). In summary, these results demonstrate that Ubc13/Mms2 and Ube2k transfer ubiquitin to BABS in collaboration with BRCA1/BARD1. Consistent with their function in poly-ubiquitin chain elongation Ubc13/Mms2 and Ube2k do not transfer ubiquitin directly to BABS but rather build a poly-ubiquitin chain onto a mono-ubiquitinated BABS (Fig. 3.3 B and C). Intriguingly, the E2s that interact with BABS are the same E2s that can efficiently ubiquitinate BABS in the presence of BRCA1/BARD1 as the E3. Hence, BRCA1/BARD1 can ubiquitinate BABS *in vitro* and different types of ubiquitination products are synthesized on BABS depending on the E2 used.

BABS, A BARD1-Associated BRCA1 Substrate

The two-hybrid screen results implicate BARD1 residues proximal to the RING domain as a primary BABS substrate binding site (Fig. 3.1B). Our standard *in vitro* assays include additional BRCA1 residues not contained in the two-hybrid screens (BRCA1 residues 99-304). To define the region of BRCA1/BARD1 required for BABS ubiquitination more clearly we tested the ability of BRCA1/BARD1 heterodimers of different lengths to ubiquitinate BABS. BC112/BD327 ubiquitinates BABS at a level similar to BC304/BD327 with the E2s UbcH5 and Ube2w (Fig. 3.4, lanes marked 1 and 2) indicating that for BRCA1 only the RING domain is required. However, BRCA1/BARD1 heterodimers lacking the BABS binding-site (BARD1 residues 140 to 327) are unable to efficiently transfer ubiquitin to BABS (Fig. 3.4, lanes marked 3 and 4). During a substrate ubiquitination assay with BABS, the E3 subunits BC304 and BD327 are also auto-ubiquitinated (data not shown). Removal of the substrate binding-site on BARD1 (BC304/BD140) only eliminates BABS and BARD1 ubiquitination, BC304 auto-ubiquitination is unaffected (data not shown). Thus, removal of the substrate binding-site only affects ubiquitination of BABS and not the intrinsic E3 activity of BRCA1. Disruption of the E2 binding-site on BRCA1 by the Ile26Ala mutation or omission of BRCA1/BARD1 from the assay also eliminates BABS ubiquitination (Fig. 3.4, lanes marked 5 and 6). These results demonstrate that although UbcH5c and Ube2w interact with BABS, these E2s are unable to transfer ubiquitin to BABS on their own. Taken together, the results confirm that BABS is a ubiquitination substrate of BRCA1/BARD1 that is recognized primarily by the BARD1 subunit.

Substrate-E2 Interaction as a Mechanism for E3-Dependent BABS Ubiquitination

A single ubiquitin attached to BABS is the main product of BRCA1/BARD1-dependent ubiquitination when UbcH5c is the E2. This is in stark contrast to the ability of UbcH5c to poly-ubiquitinate BRCA1/BARD1 in an auto-ubiquitination assay (Christensen et al., 2007; Brzovic et al., 2003). In the presence of BABS the E3, BC304/BD327, is still poly-ubiquitinated by UbcH5 (data not shown). Two other reported *in vitro* substrates of BRCA1/BARD1 are also mono-ubiquitinated by UbcH5c, γ-tubulin (Starita et al., 2004) and ERα {Eakin, 2007 #65}. The mechanism underlying UbcH5c-mediated mono-ubiquitination of these substrates is unknown. However, UbcH5c-mediated poly-ubiquitination of BRCA1 requires a non-covalent interaction between ubiquitin and the β-sheet surface of UbcH5c (Brzovic et al., 2006). Only a single ubiquitin is transferred to BRCA1 using UbcH5c with a mutation in the β-sheet surface (Ser22Arg) that disrupts the ability of ubiquitin to bind non-covalently.

A possible mechanism for the transfer of a single ubiquitin to BABS could involve disruption of the non-covalent interaction between ubiquitin and UbcH5c. For example, BABS could disrupt the non-covalent ubiquitin-UbcH5 interaction by binding to an overlapping surface on UbcH5. We found that UbcH5c-S22R does not interact with BABS in the yeast two-hybrid assay (Fig. 3.5A), consistent with an overlap in the binding-sites of ubiquitin and BABS. BRCA1/BARD1-dependent ubiquitination of BABS is also substantially reduced using UbcH5c-S22R as the E2 (Fig. 3.5B). Together the results suggest that BABS binds to the β-sheet surface of UbcH5c and

provides further evidence for the requirement of a substrate-E2 interaction for BRCA1/BARD1-dependent ubiquitination of BABS.

Discussion

We report the discovery of a new BARD1-binding protein that can serve as a ubiquitination substrate for the BRCA1/BARD1 heterodimeric RING E3 ligase, *in vitro*. We have named the novel substrate (KIAA0701) BABS, for BARD1 Associated BRCA1 Substrate. Unexpectedly, characterization of BABS revealed evidence for substrate-E2 interactions within the ubiquitination pathway. We have recently reported that eight E2s have BRCA1-binding properties and can transfer activated ubiquitin to BRCA1 in an auto-ubiquitination assay (Christensen et al., 2007). In the present study, we find that a subset of the BRCA1-interacting E2s can interact with BABS in the yeast two-hybrid system. Furthermore, we find a strong correlation between BRCA1-interacting E2s that bind to BABS, as determined by yeast two-hybrid, and those that function with BRCA1/BARD1 to ubiquitinate BABS *in vitro*. The ubiquitination of BABS requires three pairwise interactions: substrate-E3, E3-E2, and substrate-E2. Hence, the BRCA1/BARD1-mediated ubiquitination of BABS differs from the current model of ubiquitination where the E3 is assigned the primary role in substrate recognition and the E2 is only passively involved. Our data provide strong evidence in support of a model in which the E2 interacts directly with a substrate and this interaction is important for E3-dependent ubiquitin transfer (Fig. 3.5).

Eight different E2s interact with the same surface of BRCA1 and seven of these (UbcH5, UbcH6, Ube2e2, UbcM2, Ube2k, Ubc13/Mms2, and Ube2w) transfer

ubiquitin to BRCA1 (Christensen et al., 2007). Our results imply an additional requirement for ubiquitin transfer to BABS. Neither substrate-E3 nor E3-E2 interactions alone are sufficient for the BRCA1/BARD1 mediated ubiquitination of BABS, as not all BRCA1-interacting E2s transfer ubiquitin to BABS. The requirement of a substrate-E2 interaction is further demonstrated by a mutation of UbcH5c (Ser22Arg) that disrupts both binding and ubiquitin transfer to BABS. Thus, there is a perfect correlation between E2s that interact with BABS and E2s that support ubiquitin transfer to BABS. The small level of ubiquitination of BABS observed with UbcH5c-S22R, UbcH6, Ube2e2, and UbcM2 is likely do to the close proximity of the E2 with the substrate generated by the ability of both to interact with BRCA1/BARD1. However, robust ubiquitination occurs only with E2s that can interact with BABS.

BRCA1/BARD1-dependent ubiquitination of BABS also requires an interaction between the E3 and E2. This interaction can be disrupted by mutation of the BRCA1 RING (Ile26Ala) or by mutation of the loop L2 of the E2 (Ubc13-A98D and Ube2k-A103D) (Christensen et al., 2007). Though UbcH5c, Ubc13, Ube2k, and Ube2w all interact with BABS, disruption of the E2-E3 interaction by mutation of the BRCA1 RING or the E2 eliminates ubiquitin transfer. Thus, the substrate-E2 interaction alone is not sufficient for ubiquitin transfer.

Consistent with current models of ubiquitination, the substrate-E3 interaction between BABS and BRCA1/BARD1 is absolutely required for ubiquitin transfer. The substrate-E3 interaction is mediated by residues that reside outside of the BARD1 RING (Fig. 3.1B). These BARD1 residues (140 to ~327) define the substrate binding site and are absolutely required for ubiquitin transfer to BABS (Fig. 3.4). Removal of

the substrate binding site does not affect the ubiquitin ligase activity of BRCA1. Hence, loss of ubiquitination of BABS by removal of the substrate binding site is due solely to the inability of the active E3 to recognize and bind the substrate.

BABS is an uncharacterized protein with unknown cellular function. Therefore it is also not known how ubiquitination of BABS by BRCA1/BARD1 relates to any of the reported cellular functions of BRCA1/BARD1. BABS is over 40% identical to UHRF1BP1, a protein recently identified based on its ability to interact with UHRF1 (also referred to as ICBP90) (Unoki et al., 2004). UHRF1BP1 is localized to the nucleus and appears to suppress cellular proliferation (Unoki et al., 2004), although its cellular function is unknown. Given the high degree of identity, UHRF1BP1 and BABS may have similar functions. We hypothesize that BABS is also likely to be localized to the nucleus were BRCA1/BARD1 is found and where they function to help regulate cellular proliferation (Kubista et al., 2002b). BRCA1/BARD1-dependent ubiquitination of BABS may serve a role in BRCA1's inhibitory effect on cellular proliferation. Another intriguing possibility involves a role in DNA damage repair. BRCA1/BARD1(Westermark et al., 2003), Ubc13 (Zhao et al., 2007), and Lys-63 linked poly-ubiquitin chains (Hofmann and Pickart, 1999) are all linked to the process of DNA-damage repair. The ability of BRCA1/BARD1 and Ubc13/Mms2 to build Lys63-linked poly-ubiquitin chains onto BABS *in vitro* may signify that BABS is a target of Lys63-linked poly-ubiquitination by BRCA1/BARD1-Ubc13/Mms2 at sites of DNA damage.

In summary, our results contribute compelling evidence in support of a new model for protein ubiquitination that incorporates substrate-E2 interactions as a critical

determinate of ubiquitin transfer. Whether all substrates bind to the same surface of an E2 or if a substrate-E2 interaction is required for all substrates remain open questions. Within the confines of our own assay system BRCA1 auto-ubiquitination and ubiquitination of BABS appear to occur *via* different mechanisms, as mutation of the β-sheet surface of UbcH5c has different consequence on the ubiquitination products observed on the two substrates. Finally, we note that a requirement of a set of three interactions among E3, E2, and substrate affords the possibility of regulation of BRCA1/BARD1-dependent ubiquitination of BABS, and presumably other substrates, at multiple levels.

Experimental Procedures

Yeast Two-Hybrid Screens

The bait construct BC98FBD327 was created by sequential ligation of PCR products encoding BRCA1 residues 1-98 and BARD1 residues 4-327 into pGBKT7 (Clontech) using Nde1, BamH1, and Sal1 restriction sites. The six amino acid linker was also incorporated between BRCA1 and BARD1 subunits. The bait pGBKT7-BC98FBD327 was transformed into the yeast strain AH109 (Clonetech). Positive transformants were selected on SD -Trp media (Clontech) and retransformed with the Matchmaker Human Ovary cDNA library (Clonetech) and yeast cells harboring positive interactions between bait and prey constructs were selected on SD –His/-Leu/-Trp media with 5 mM 3AT (3-amino-1,2,4-triazole, Sigma). Respective prey plasmids were isolated from yeast cells capable of growth on selective media and sequenced (UW, Biochemistry Sequencing Facility) to determine the identity of the encoded protein.

For directed screens, respective "bait" (pGBKT7) and "prey" (pACT2) plasmids were co-transformed into the yeast strain AH109 (Clontech). Positive transformants were selected on minimal SD -Leu/-Trp media (Clontech). A single colony for each bait/prey combination was suspended in 100 μL of sterile water in a 96 well plate. Using a replica plater (Sigma) yeast cells from the single colony were spotted onto selective media (SD -His/-Leu/-Trp with 0, 1, 2.5, 5, and 10 mM 3AT (Sigma) and non-selective control media (SD -Leu/-Trp). Yeast were incubated at 30°C for 7 days and photographed. All mutations and truncations of the bait and prey constructs were introduced by Quikchange (Stratagene) and confirmed by DNA sequencing (UW, Biochemistry Sequencing Facility).

Bacterial Expression Plasmids, Protein Expression, and Purification

DNA encoding BABS residues 1145 to 1402 was amplified by PCR and cloned into the vector pET151D-TOPO (Invitrogen) according to the manufacturer's instructions, this vector incorporates an N-terminal His_6 and V5 tag. For protein expression, pET151-BABS was transformed into BL21 Star (DE3) (Invitrogen) and positive transformants were selected on LB medium containing ampicillin (RPI). Bacteria were grown in rich LB media and expression was induced at an O. D. 600nm of 0.6 by addition of 1 mM IPTG. Four hours after induction of protein expression cells were harvested and frozen.

For purification of BABS, bacterial cells were lysed by French Press and BABS was purified by Ni^{2+}-affinity chromatography, according to the manufacturers instructions (Sigma) followed by size exclusion chromatography in 25 mM Sodium

Phosphate pH 7.0, 150 mM NaCl. Plasmid constructs, expression, and purification of BRCA1/BARD1 constructs, E2s, E1, and ubiquitin were performed as described previously ((Brzovic et al., 2006; Christensen et al., 2007).

BABS Ubiquitination Assays

150 µL reaction mixtures for BRCA1-directed ubiquitination assays included 1.0 µM BRCA1/BARD1 heterodimer, 1.0 µM specified E2, 20 µM ubiquitin, and 0.5 µM wheat Uba1. All reactions with Ube2k were assayed with 0.2 µM human E1 (BioMol). Reactions were initiated by addition of 5 mM ATP and 10 mM $MgCl_2$. Samples were collected at 0, 30, 60, and 120 minutes after addition of ATP. Reaction products were resolved on a NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen) and transferred onto PVDF membranes (Biorad). The membranes were probed with mouse anti-V5 antibody (Invitrogen), followed by goat anti-mouse secondary antibody conjugated to Alexa Fluor 680 (Molecular Probes). Blotted proteins were detected using an Odyssey infrared imaging system (Licor).

A

I26A

126 170 225 327

B

Bait: BC98(I26A)FBD327, BC98(I26A)FBD225, BC98(I26A)FBD170, BC98(I26A)FBD126, BD126-327, BC98FBD327, BC98FBD126, DB-vector

Prey: 1 2 3 4 5 6 7 8

BABS 933-1464

AD-Vector

-His/-Leu/-Trp + 2.5 mM 3AT

BABS 933-1464

AD-Vector

-Leu/-Trp

Figure 3.1. A Fused BRCA1/BARD1 "Bait" Construct Identifies a New BARD1 Associated Protein.

A) Liner representation of the BRCA1/BARD1 fused bait. The bait is comprised of the BRCA1 RING domain in grey (residues 1-98) at the N-terminus and BARD1 residues at the C-terminus (blue), creating the bait BC98FBD327. Various truncations of BARD1 are noted with arrows, as well as the BRCA1 I26A RING mutation in yellow. B) Directed yeast two-hybrid screens between BABS and various BRCA1/BARD1 constructs. Plasmids encoding respective bait and prey constructs were co-transformed in the yeast strain AH109. Two colonies for each bait and prey pair were replica plated on selective and non-selective media. Yeast that can grow under selective conditions (upper panel) indicate an interaction between the respective bait (Gal4 DNA-binding domain (DB)) and prey (Gal4 activation domain (AD)) fusions. Lower panel shows that the yeast are viable under control conditions.

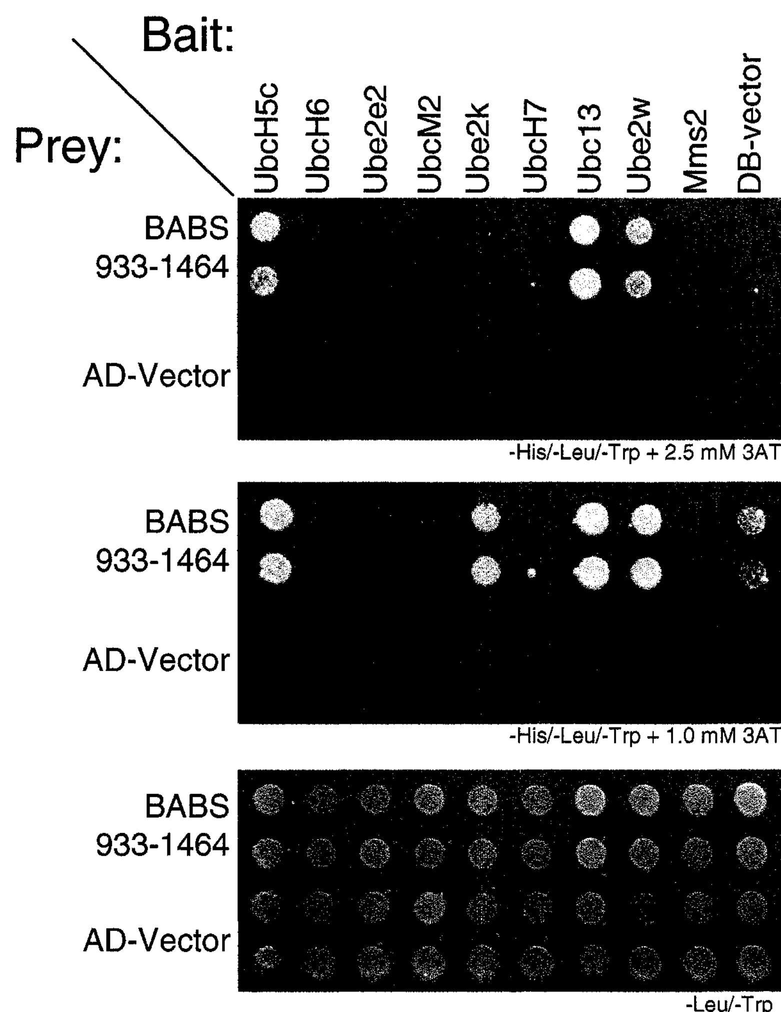
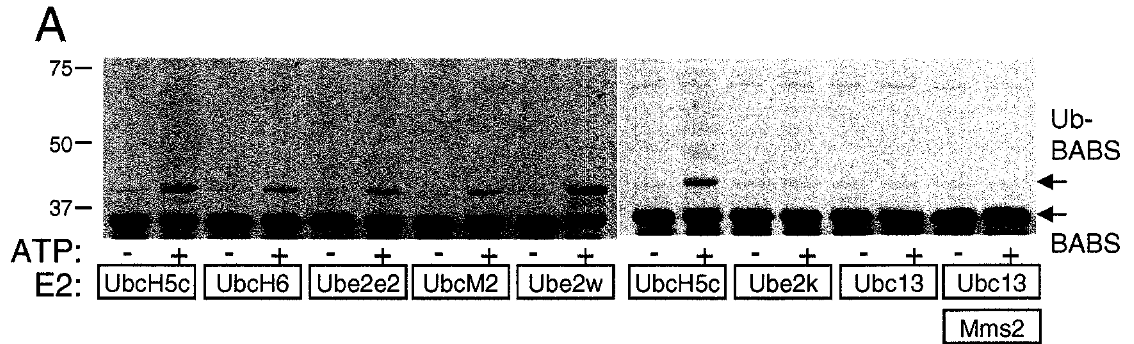
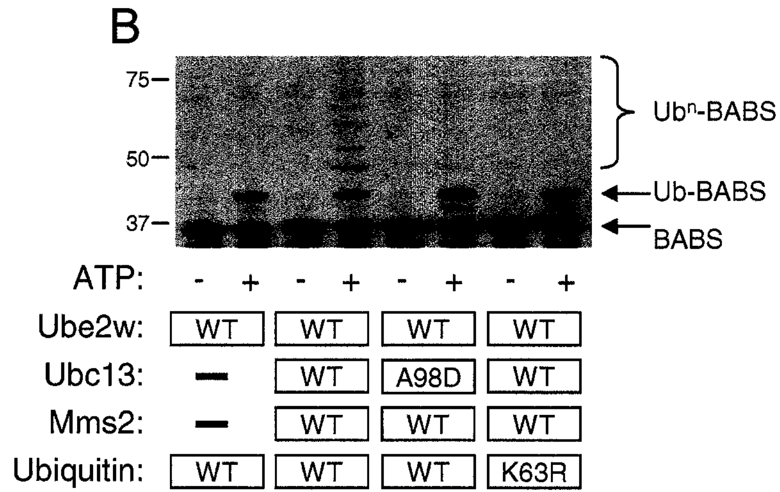
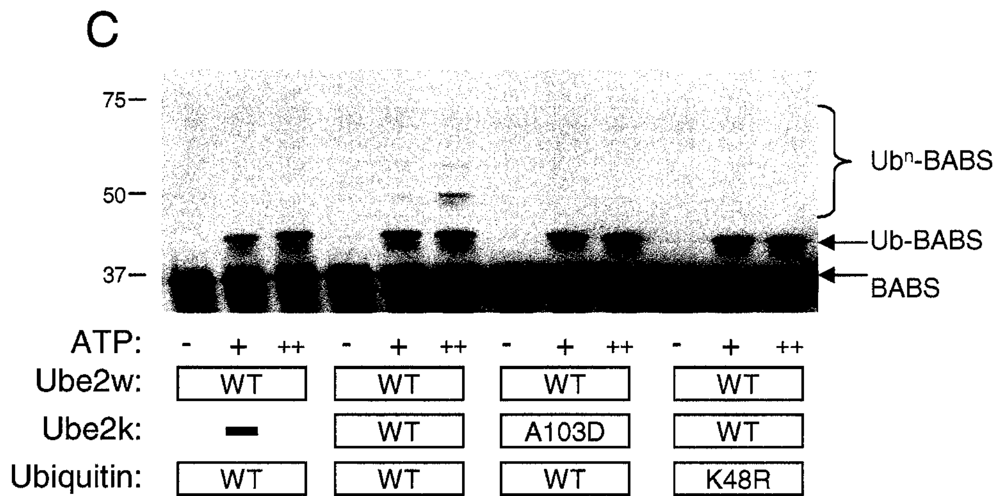


Figure 3.2. BABS Associates with a Subset of the BRCA1-Interacting E2s.

Directed yeast two-hybrid screens between BABS and E2s that interact with the RING of BRCA1. Two colonies for each bait and prey pair were replica plated on selective and non-selective media. The amount of 3AT controls the stringency of selection. The ability of a bait and prey pair to grow at any level above the background of BD- and AD-vector controls is indicative of a specific interaction.


A
75
50
37
Ub-
BABS
BABS
ATP: - + - + - + - + - + - + - + - + - +
E2: UbcH5c UbcH6 Ube2e2 UbcM2 Ube2w UbcH5c Ube2k Ubc13 Ubc13
Mms2


B
75
50
37
Ubn-BABS
Ub-BABS
BABS
ATP: - + - + - + - +
Ube2w: WT WT WT WT
Ubc13: WT A98D WT
Mms2: WT WT WT
Ubiquitin: WT WT WT K63R


C
75
50
37
Ubn-BABS
Ub-BABS
BABS
ATP: - + ++ - + ++ - + ++ - + ++
Ube2w: WT WT WT WT
Ube2k: WT A103D WT
Ubiquitin: WT WT WT K48R

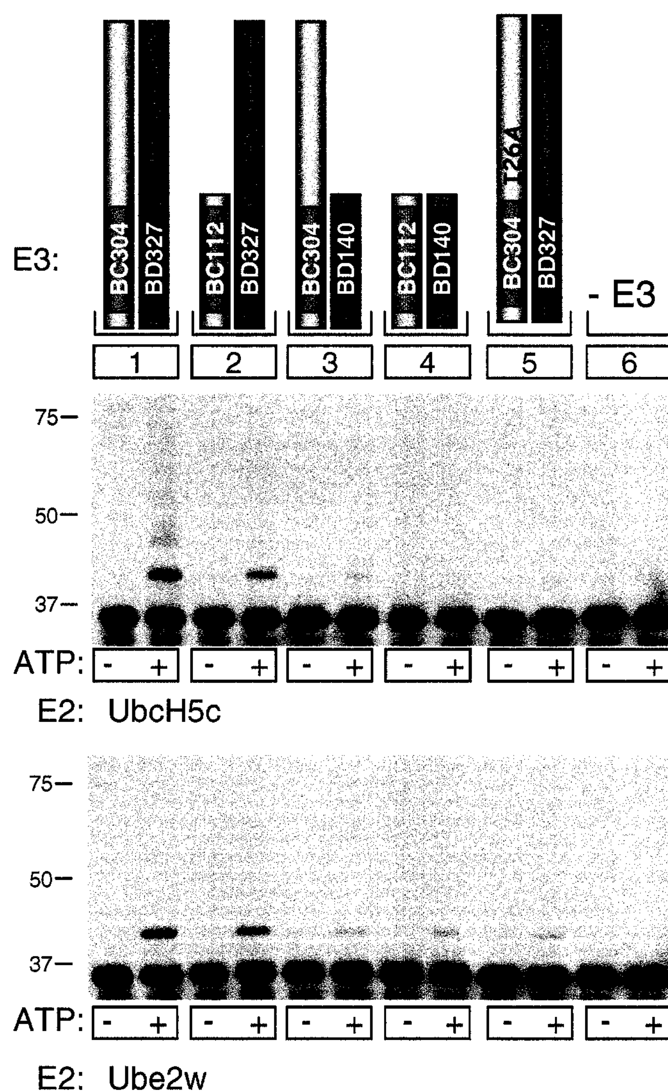


Figure 3.4. A BARD1-Associated BRCA1 Substrate.

Ubiquitination assays of BABS using different lengths of BRCA1 and BARD1. α-V5 western blot showing unmodified and ubiquitinated BABS at time 0 (-) and 1 hr (+) after addition of ATP for each indicated BRCA1/BARD1 E3. Upper panel are ubiquitination assays using UbcH5c as the E2. Lower panel are ubiquitination assays using Ube2w as the E2.

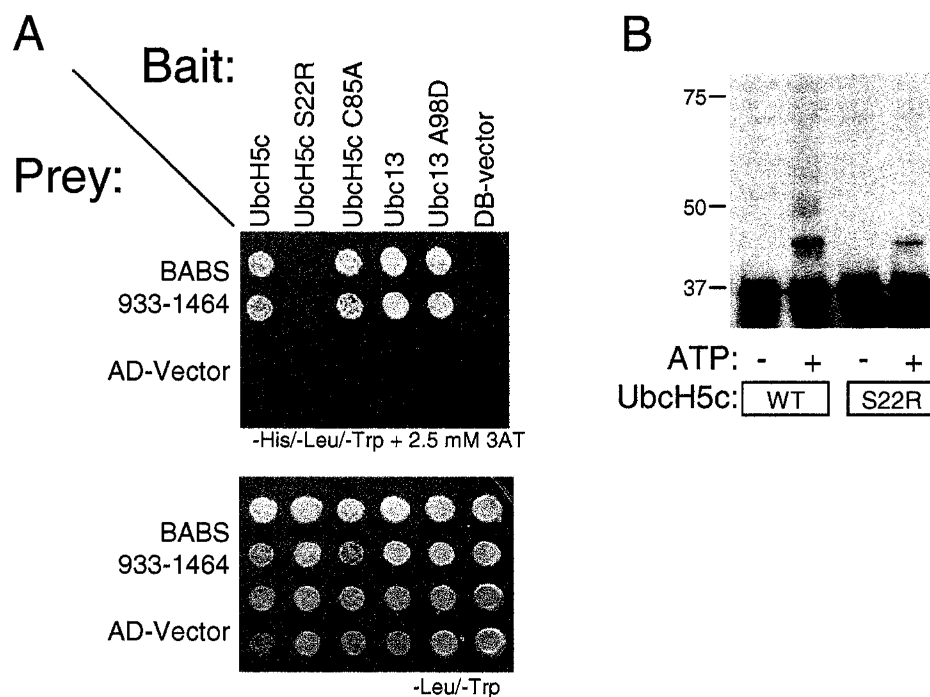


Figure 3.5. Mutation of the β-Sheet Surface of UbcH5c Disrupts Both BABS Binding and Ubiquitination.

A) Directed yeast two-hybrid screens between BABS and E2s with mutations on different surfaces. B) BRCA1/BARD1 ubiquitination of BABS using wild-type and mutant UbcH5c. α-V5 western blot showing unmodified and ubiquitinated V5-BABS at time 0 (-) and 1 hr (+) after addition of ATP.

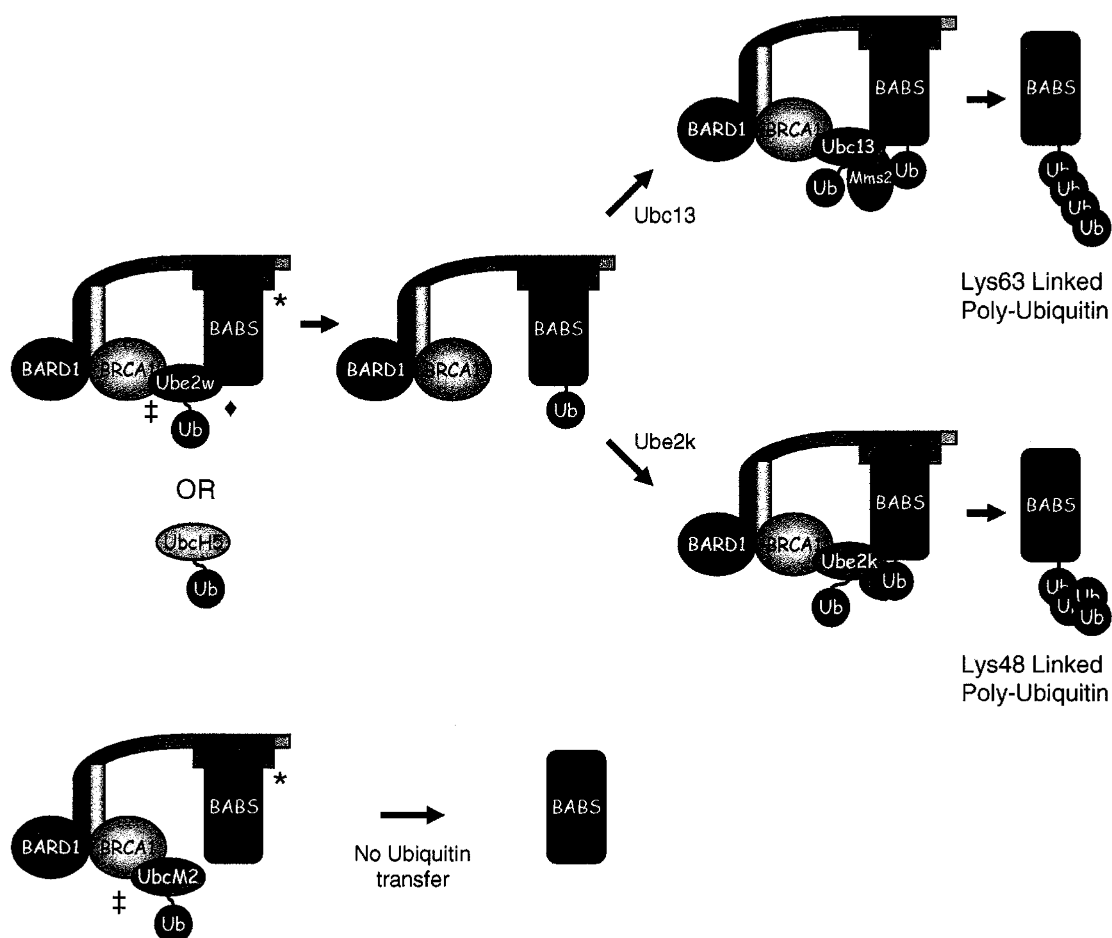


Figure 3.6. Model of BRCA1/BARD1 Dependent Ubiquitination of BABS.

A set of three independent interactions are required for ubiquitination of BABS. First, BABS is recognized by BARD1 residues outside of the RING domain which define the substrate binding-site and mediates the substrate-E3 interaction (*). Second, the E2-E3 (‡) interaction is mediated by the RING of BRCA1 directly interacting with an E2. Third, the newly defined substrate-E2 (♦) interaction between BABS and one of four different E2s. The E2s UbcH5c, Ube2w, Ubc13, and Ube2k can all function to bridge the interaction between the RING of BRCA1 and the substrate BABS. Depending on the E2 used during a BRCA1/BARD1-dependent ubiquitination assay different types of ubiquitination product are synthesized on BABS. Though an E2 can interact with the BRCA1 RING, if it does not interact with BABS no ubiquitin transfer occurs.

Chapter 4. The Tight Junction Protein ZO-2 Interacts with a Substrate Binding Site of the BRCA1/BARD1 Ubiquitin Ligase

Introduction

The breast and ovarian cancer susceptibility gene, *BRCA1*, is expressed in most tissues of the body. However, cancer predisposing mutations of BRCA1 lead primarily to breast and ovarian tumorigenesis. The mechanisms underlying the tissue specific nature of cancer associated mutations of BRCA1 are not known. Subsequently, BRCA1 has been highly investigated to determine its normal cellular function so as to understand why mutations lead to breast and ovarian cancers. Many cellular functions have been attributed to BRCA1 such as, DNA damage repair, centrosome duplication, chromatin remodeling, cellular proliferation, and transcription. Currently, it is unclear how the loss of BRCA1 function, by cancer associated mutations, during any of these processes results in tissue specific cancer predisposition.

A possible explanation for the tissue specific nature of cancer predisposing BRCA1 mutations might be through modulation of estrogen receptor alpha (ERα) activity. The steroid hormone estrogen induces the proliferation of normal breast (Clarke, 2006) and ovarian (Syed et al., 2001) epithelial cells. Prolonged exposure to estrogen is considered a risk factor for the development of tumors in estrogen responsive tissues such as breast, ovary, and uterus (Ho, 2003; Ikeda and Inoue, 2004). Over expression of BRCA1 has been found to inhibit cellular proliferation in breast and ovarian cancer cell lines (Holt et al., 1996), while inhibition of BRCA1 expression is associated with accelerated proliferation rates (Thompson et al., 1995). The ability of BRCA1 to inhibit cellular proliferation may counteract the proliferative effects of

estrogen on breast and ovarian epithelial cells. In fact, transient over expression of BRCA1 inhibits estrogen signaling through ERα (Fan et al., 1999). A link between BRCA1 and estrogen signaling is further demonstrated in BRCA1-deficient mice. ERα-positive breast epithelial cells normally do not proliferate (Clarke et al., 1997), but are believed to promote the proliferation of neighboring ERα-negative epithelial cells in response to estrogen by secreting growth factors *via* a paracrine pathway. However, in the absence of functional BRCA1 a significant number of ERα-positive mouse mammary epithelial cells were found to be proliferating (Li et al., 2007). Furthermore, ectopically supplied estrogen in mice deficient for BRCA1 in the mammary glad, promotes tumor formation (Li et al., 2007). These results provide further evidence in support of a link between BRCA1, ERα signaling, and breast cancer.

Although there appears to be a strong relationship between BRCA1 and ERα activity this may not explain the tissue specific nature of breast and ovarian cancer associated mutations. As BRCA1 is expressed in most tissues (Durocher et al., 1997), loss of the inhibitory effect of BRCA1 on ERα by cancer associated mutations does not explain why BRCA1 has not been linked to other estrogen-dependent tumor types such as endometrial and cervical cancers. Hence, further investigation into the tissue specific nature of BRCA1 cancer associated mutations is needed and may require a closer look into the biochemical properties of this molecule.

BRCA1 encodes a large protein of 1863 amino acid residues, with a RING domain at its N-terminus and two tandem BRCT repeats at the extreme C-terminus.

BARD1, another RING containing protein, forms an obligate heterodimer with BRCA1 (Brzovic et al., 2001) and the two proteins appear to function as a single complex within the cell (McCarthy et al., 2003; Yu and Baer, 2000). The only biochemical activity known for the BRCA1/BARD1 heterodimer is the ability to function as a ubiquitin ligase *in vitro* (Hashizume et al., 2001).

Ubiquitination is the covalent attachment of the C-terminal Gly76 of ubiquitin to substrate lysine residues. The process is mediated by a cascade of three enzymes, ubiquitin activating (E1), ubiquitin conjugating (E2), and a ubiquitin ligase (E3). In the final step of the cascade the E3 functions by directly interacting with both an E2 and a substrate and somehow mediates the transfer of ubiquitin from the E2 active-site to a substrate lysine residue. This process can occur once resulting in a single ubiquitin attached to the substrate or through multiple rounds of the cascade a poly-ubiquitin chain can be synthesized on the substrate were ubiquitin attached during one round of the cascade becomes the substrate for the next ubiquitin transfer. There are seven lysine residues in ubiquitin and all have the potential to function as linkage sites for poly-ubiquitin chains. Subsequently, ubiquitination is a diverse signaling mechanism, as the type of ubiquitin attached targets the substrate for different fates. The most characterized ubiquitin signal is mediated by Lys48-linked poly-ubiquitin chains of at least four subunits, which targets the attached substrate to the proteasome for degradation.

Cancer associated mutations of BRCA1 are located throughout the protein with a considerable number residing within the RING domain and these eliminate ubiquitin ligase activity (Brzovic et al., 2003). Hence, there is a direct link between loss of

ubiquitin ligase activity and breast and ovarian cancer susceptibility, suggesting that loss of the BRCA1/BARD1-dependent ubiquitin transfer to at least one cellular target specific to these tissues leads to breast and ovarian tumorigenesis. Many *in vitro* substrates have been reported for BRCA1/BARD1 including; γ-tubulin (Starita et al., 2004), NPM (Sato et al., 2004), RNA polymerase II (Kleiman et al., 2005), CtIP (Yu et al., 2006), and ERα (Eakin et al., 2007). However, it is unclear how loss of the BRCA1/BARD1-dependent ubiquitination of any of these substrates leads to the tissue specific nature of cancer predisposing BRCA1 mutations. Therefore, we went searching for additional cellular targets of the BRCA1/BARD1 ubiquitin ligase.

Each of the reported substrates of the BRCA1/BARD1 ubiquitin ligase appear to bind to the BRCA1 subunit of the complex. BARD1 is an integral member of this complex and its association is required to maintain BRCA1 in the appropriate structural conformation necessary for ubiquitin ligase activity. Furthermore, BARD1 represents another polypeptide to which substrates can bind and be recognized for BRCA1 RING mediated ubiquitin transfer. Using a yeast two-hybrid "bait" with a bias toward identifying BARD1-associated proteins in the context of the BRCA1/BARD1 RING:RING heterodimer, we have previously identified a BARD1 Associated BRCA1 Substrate, BABS. The *in vitro* ubiquitination of BABS by BRCA1/BARD1 requires a substrate-E3 interaction mediated by BARD1 residues outside of its RING domain. Our yeast two-hybrid screen also identified another protein that interacts with BARD1 at the same substrate binding-site used for recognition of BABS. We report the interaction between BARD1 and the tight junction protein ZO-2 (zonula occludens-2).

ZO-2 is a suspected tumor suppressor protein found to be down regulated in breast and pancreatic carcinomas (Chlenski et al., 2000). ZO-2 has also been linked to the unique oncogenic property of human adenovirus type 9 (Ad9) in rodents, which elicits exclusively estrogen-dependent mammary tumors (Glaunsinger et al., 2001). Another link between ZO-2 and estrogen signaling is provided by the direct interaction between ZO-2 and the heterogeneous nuclear ribonucleoprotein scaffold attachment factor-B (SAFB1) (Traweger et al., 2003), an estrogen receptor co-repressor (Oesterreich et al., 2000) with expression found to be deregulated in clinical breast cancer samples (Townson et al., 2000). Hence, the interaction between BARD1 and ZO-2 may be a critical determinate in the suppression of breast tumors and may provide another link between BRCA1/BARD1, suppression of ERα signaling, and breast and ovarian tumorigenesis.

Results

ZO-2 Interacts with BARD1 and a Subset of the BRCA1-Interacting E2s in the Yeast Two-Hybrid Assay

As reported previously, we conducted a yeast two-hybrid screen with a novel fusion protein comprised of the RING domain of BRCA1 (residues 1-98) fused to BARD1 residues 29-327 creating BC98FBD302. This bait construct maintains the appropriate structural conformation of the RING:RING heterodimer necessary for ubiquitin ligase activity and a bias for identifying BARD1-associated proteins by including additional residues (126-327) beyond the BARD1 RING. The ligase inactive mutation of BRCA1 Ile26Ala (Brzovic et al., 2003) was also incorporated into the bait

(BC98(I26A)FBD327) to avoid the potential interference of yeast ubiquitin pathway enzymes during activation of the yeast two-hybrid reporter cassette when the BRCA1/BARD1 E3 bait interacts with a potential substrate. In an attempt to identify tissue specific substrates of the BRCA1/BARD1 ubiquitin ligase, BC98(I26A)FBD327 was screened against a human ovarian cDNA library. In addition to identifying BABS, as reported previously, this screen also identified the tight junction protein ZO-2 as a potential BRCA1/BARD1-interacting protein.

ZO-2 (accession number AAM28524) is 993 amino acid residues in length. Two different ZO-2 constructs were identified which encode residues 133-993 and 886-993. Directed two-hybrid screens with various truncations and mutations of BRCA1/BARD1 were used to determine the region of BC98(I26A)FBD327 recognized by ZO-2. Both ZO-2 constructs promote growth of yeast in the presence of BC98(I26A)FBD170 and BD126-327 (BARD1 residues 126-327) (Fig. 4.1, columns 3 and 5) but not with BC98(I26A)FBD126 or with the DNA binding (DB) vector control (Fig. 4.1, columns 4 and 8). These results indicate that ZO-2 interacts with BARD1 residues outside of the RING domain (residues 126-170). This is the same binding-site used to interact with BABS for BRCA1/BARD1-dependent ubiquitination *in vitro*. However, there are likely to be some differences in the manner in which BABS and ZO-2 are recognized by BARD1 as mutation of a conserved tryptophan to alanine (Trp146Ala) disrupts the interaction with ZO-2 by negatively affecting yeast growth at high 3AT concentrations (Fig. 4.1, column 10, 10 mM 3AT panel) but does not affect the interaction with BABS under the same conditions (data not shown).

The smaller ZO-2 prey construct encoded by residues 886-993 also supports growth of yeast with the bait BC98FBD126 (Fig. 4.1, column 7), but not with the ligase inactive version of the same construct (Fig. 4.1, column 4). As the Ile26Ala RING mutation specifically disrupts the ability of BRCA1 to interact with E2s (Christensen et al., 2007; Brzovic et al., 2003), these results suggest a possible bridging interaction, mediated by yeast E2s, between the BRCA1 RING and ZO-2. This potential bridging interaction suggests the possibility that ZO-2 interacts with a member of the ubiquitin-conjugating enzyme family. We have previously reported that BRCA1 can interact with eight different human E2s and depending on the E2 used in an *in vitro* auto-ubiquitination assay different ubiquitination products are synthesized on BRCA1/BARD1 (Christensen et al., 2007). To test for possible E2s that could bridge an interaction between ZO-2 and BRCA1, we used directed two-hybrid screens between ZO-2 and the subset of E2s that interact with BRCA1: UbcH5c, UbcH6, Ube2e2, UbcM2, UbcH7, Ube2k, Ubc13, Ube2w, and Mms2. As shown in Figure 4.2, Ubc13 and UbcH5c support growth of yeast with ZO-2 886-993 indicating an interaction between these E2s and ZO-2. UbcH6, Ube2e2, UbcM2, and Ube2w also support growth of yeast with ZO-2 133-993 at a stringency level slightly better than empty vector controls indicting that these E2s may also interact with ZO-2 (Fig. 4.2). These results are similar to those obtained for BABS in that a primary interaction is mediated by BARD1 residues proximal to the RING domain and the ability to associate with a subset of the BRCA1-interacting E2s.

Domain Architecture and Interaction of ZO-2 with BARD1

ZO-2 is a multidomain protein comprised of three PDZ domains, an SH3 domain, and an inactive guanylate kinase-like domain (Fig. 4.3A). There are at least three isoforms reported for ZO-2, A, C, and 3 (Chlenski et al., 2000; Huang et al., 2002). The ZO-2 protein identified in our yeast two-hybrid screen is isoform 3 which is alternatively spliced resulting in a protein with a different C-terminus than reported for ZO-2 isoforms A and C. Residues 1-960 are identical with ZO-2 isoforms A and C, however, residues 961 to 993 are unique to isoform 3 (Fig. 4.3B). The interaction of ZO-2 886-993 with BARD1 in the yeast two-hybrid system suggests that this interaction might be specific to isoform 3. To test whether potentially all isoforms of ZO-2 could interact with BARD1 or if this interaction is specific to isoform 3, we conducted directed two-hybrid screens between various truncations of the two ZO-2 prey constructs identified. Removal of residues encoded by the shorter ZO-2 construct (886-993) from the larger construct (ZO-2 133-886) eliminates the two-hybrid interaction with BC98(I26A)FBD327 (Fig. 4.3C). This result suggests that the interaction between ZO-2 and BARD1 is mediated solely by ZO-2 residues within amino acids 886-993. Truncation of the last 23 residues in ZO-2 (ZO-2 886-970) also disrupts the interaction with BC98(I26A)FBD327 (Fig. 4.3C). From this we conclude that the interaction between BARD1 and ZO-2 is unique to isoform 3. Furthermore, the simultaneous mutation of two cysteines, Cys988 and Cys989, to serines within the unique C-terminus of ZO-2 isoform 3 negatively effects the ability to interact with BARD1 (Fig. 4.3C).

Confirmation of Interaction Between ZO-2 and BARD1

To validate the interaction between ZO-2 and BARD1 inferred for our yeast two-hybrid data, we used transient transfections of HBL-100 cells followed by immunoprecipitation. A plasmid encoding full length ZO-2 isoform 3 with an N-terminal HA epitope tag as well as an empty vector control plasmid were transfected into HBL-100 cells. 30 hours after transfection the cells were lysed and the soluble fraction was immunoprecipitated using anti-HA antibodies. As shown in Figure 4.4, BARD1 is co-immunoprecipitated with ZO-2 but not in cells transfected with the empty pcDNA3HA vector. These results demonstrate that ZO-2 over expressed in HBL-100 cells interacts with endogenous BARD1, confirming the interaction between these two proteins.

Discussion

The interaction between ZO-2 and BARD1 has several implications with regard to the tissue specific nature of BRCA1-cancer associated mutations. First, ZO-2 expression is down regulated in breast cancer samples and breast epithelial cell lines (Chlenski et al., 2000). Second, ZO-2 is directly linked to the unique oncogenic property of human Ad9 in rodents, which displays exclusively estrogen-dependent mammary tumors (Glaunsinger et al., 2001; Javier et al., 1991). Third, ZO-2 interacts with the estrogen receptor co-repressor, SAFB1 (Traweger et al., 2003), also found to be deregulated in breast cancer samples (Townson et al., 2000). From this it is apparent that ZO-2 has already been linked to estrogen signaling and breast cancer. Our, discovery that ZO-2 interacts with BARD1, which forms an obligate heterodimer with

BRCA1, may serve as a functional link between BRCA1 and cancers of the breast and ovaries.

The most commonly observed cancer-associated missense mutation in BRCA1 (Cys61Gly) eliminates ubiquitin ligase activity. Hence, there is a direct link between loss of ubiquitin ligase activity and breast and ovarian cancer susceptibility, suggesting that loss of ubiquitination of at least one critical substrate leads to breast or ovarian tumorigenesis. Although not tested directly, ZO-2 is a likely ubiquitinated substrate of BRCA1/BARD1. Evidence in support of ZO-2 as a substrate is found with its ability to interact with BARD1 using the same substrate binding site as BABS, another *in vitro* substrate of BRCA1/BARD1. Also, as was found with BABS, ZO-2 binds to a subset of the BRCA1-interacting E2s presenting the possibility of a substrate-E2 interaction. Therefore, by analogy with BABS, ZO-2 is also likely to be a substrate recognized by the BARD1 subunit for BRCA1-mediated ubiquitin transfer. Interaction with Ubc13 suggests the possibility that ZO-2 may be poly-ubiquitinated with Lys63-linked chains. This would require a prior mono-ubiquitination event that may be mediated by UbcH5c, which also interacts with ZO-2 in the yeast two-hybrid assay, as this E2 leads to the mono-ubiquitination of other reported substrates such as γ-tubulin, ERα, and BABS. UbcH6, Ube2e2, UbcM2, or Ube2w may also function to mono-ubiquitinate ZO-2 with BRCA1/BARD1 as they also appear to interact with ZO-2 in the yeast two-hybrid assay.

Cellular localization studies have found ZO-2 concentrated at the cell membrane, as part of tight junctions which form a physiological boundary sealing epithelial cells together to create a selective permeability barrier (Gumbiner, 1996), and

as clusters in the nucleus (Islas et al., 2002). The confluency of tissue culture cells is directly associated with the cellular localization of ZO-2. In sparse cultures ZO-2 accumulates within the nucleus. As tissue culture cells become more confluent and cells come in contact with other cells, ZO-2 redistributes to the cell membrane at tight junctions. The rate of cellular proliferation is a major difference between confluent and sub-confluent tissue culture cells. It has been postulated that ZO-2 may act as a sensor in modulating cellular proliferation (Islas et al., 2002) based on its distinct localization pattern between actively growing (sub-confluent) and non-proliferating cells (confluent).

Human adenoviruses are primarily associated with respiratory, gastrointestinal, and ocular infections. However, when injected subcutaneously in rodents, human Ad9 leads to estrogen dependent mammary tumors (Javier et al., 1991) and is the only adenovirus with this property suggesting that Ad9 possesses an oncogenic determinate not found in other adenoviruses. The oncogenic property of Ad9 has been isolated to the viral protein encoded by the E4 region open reading frame 1 (E4-ORF1) (Javier, 1994). The E4-ORF1 proteins from representative adenoviral subgroups display between 45% to 51% identity (Glaunsinger et al., 2001) and a common ability to bind to cellular PDZ domain containing proteins (Lee et al., 1997). Unique to Ad9 E4-ORF1 is the ability to bind to ZO-2, whereas E4-ORF1 proteins from other adenovirus subgroups do not (Glaunsinger et al., 2001). Interestingly, both E4-ORF1 and SAFB1 bind to the first PDZ domain of ZO-2. Whether binding of E4-ORF1 prevents SAFB1 binding to ZO-2 is not known. The interaction between SAFB1 and ZO-2 appears to occur in the nucleus (Traweger et al., 2003), were as E4-ORF1 sequesters ZO-2 in the

cytoplasm (Glaunsinger et al., 2001). From these data it appears that E4-ORF1 could disrupt the functional aspects of the SAFB1-ZO-2 interaction.

There are at least three isoforms of ZO-2, isoform A has an additional 23 amino acids at its N-terminus as compared to isoform C (Chlenski et al., 1999) and isoform 3 has a unique C-terminus (Fig. 4.3A). The interaction of ZO-2 with BARD1 is specific to isoform 3 as binding is mediated by residues that are present only in this isoform. ZO-2 isoforms A and C are differentially expressed in a variety of tissues. Loss of isoform A is specifically associated with pancreatic carcinomas though isoform C is still present (Chlenski et al., 2000). ZO-2 isoform 3 may also display a unique tissue distribution. If ZO-2 isoform 3 links the observed estrogen-dependent mammary tumors induced by Ad9 in rodents to the tissue specific cancer susceptibility of BRCA1 mutations we would expect that within the subset of estrogen responsive tissues isoform 3 is highly expressed or limited to epithelial cells of the breast and ovary.

The interaction between ZO-2 and BARD1 presents an intriguing possibility of a mechanism whereby cancer associated BRCA1 mutations lead primarily to breast and ovarian tumorigenesis. Given the reported pairwise interactions between BRCA1-BARD1, ZO-2-SAFB1, SAFB1-ERα, and BRCA1-ERα I propose that these proteins form a single complex with in the cell, as illustrated in Figure 4.5. Estrogen induces proliferation of breast and ovarian epithelial cells. Disruption of the interaction between any two proteins within the complex may allow overactive ERα signaling leading to uncontrolled proliferation and cellular transformation. Inherited germ line mutations of BRCA1, loss of protein expression of ZO-2 or SAFB1, or sequestration of ZO-2 in the cytoplasm by E4-ORF1 in rodents are all reported events directly

associated with breast or ovarian tumors and repression of ERα signaling that would disrupt complex formation. Therefore, we predict that ZO-2 acts with BRCA1/BARD1 and SAFB1 to modulate ERα activity and suppress tumorigenesis in the breast and ovarian epithelial cells.

Experimental Procedures

Yeast Two-Hybrid Screens

The bait construct BC98FBD327 was created by sequential ligation of PCR products encoding BRCA1 residues 1-98 and BARD1 residues 4-327 into pGBKT7 (Clontech) using Nde1, BamH1, and Sal1 restriction sites. The six amino acid linker was also incorporated between BRCA1 and BARD1 subunits. The bait pGBKT7-BC98FBD327 was transformed into the yeast strain AH109 (Clonetech). Positive transformants were selected on SD -Trp media (Clontech) and retransformed with the Matchmaker Human Ovary cDNA library (Clonetech) and yeast cells harboring positive interactions between bait and prey constructs were selected on SD –His/-Leu/-Trp media with 5 mM 3AT (3-amino-1,2,4-triazole, Sigma). Respective prey plasmids were isolated from yeast cells capable of growth on selective media and sequenced (UW, Biochemistry Sequencing Facility) to determine the identity of the encoded protein.

For directed screens, respective "bait" (pGBKT7) and "prey" (pACT2) plasmids were co-transformed into the yeast strain AH109 (Clontech). Positive transformants were selected on minimal SD -Leu/-Trp media (Clontech). A single colony for each bait/prey combination was suspended in 100 μL of sterile water in a 96 well plate. Using a replica plater (Sigma) yeast cells from the single colony were spotted onto

selective media (SD -His/-Leu/-Trp with 0, 1, 2.5, 5, and 10 mM 3AT (Sigma) and non-selective control media (SD -Leu/-Trp). Yeast were incubated at 30°C for 7 days and photographed. All mutations and truncations of the bait and prey constructs were introduced by Quikchange (Stratagene) and confirmed by DNA sequencing (UW, Biochemistry Sequencing Facility).

Tissue Culture and Immunoprecipitation

Human breast epithial cells HBL-100 were maintained in McCoy's 5A media supplemented with 10% FBS (Hyclone), penicillin, and streptomycin. For immunoprecipitation assays one near confluent 10 cm plate of HBL-100 cells were transfected using 60 μL lipofectamine 2000 (Invitrogen) and 24 μg of indicated pcDNA3 (Invitrogen) vector. Media was replace four hours after transfection. 30 hours after transfection cells were lysed in buffer containing 50 mM Tris pH 8.0, 50 mM NaF, 100 mM NaCl, and 1% Triton X-100. Immunoprecipitation was conducted using μMacs protein A micro beads and MACS separation μ columns (Miltenyi Biotec) according to the manufactures instructions. Briefly, 2 μL of α-HA antibody (Covance) was added to the soluble cell lysate with 50 μL of protein A micro beads and incubated on ice for one hour. For separation a MACS μ column was placed in a μMACS magnet and lysates were loaded onto column by gravity flow. The columns were washed several times and immunoprecipitated proteins were eluted using reducing SDS-sample buffer. The eluted samples were resolved on a NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen) and transferred onto PVDF membranes (Biorad). The membranes were probed with mouse α-HA antibody (Covance), followed by goat anti-mouse secondary

antibody conjugated to Alexa Fluor 680 (Molecular Probes). Blotted proteins were detected using an Odyssey infrared imaging system (Licor).

A

I26A

126 170 225 327

B

Bait:

Prey:

1 BC98(I26A)FBD327
2 BC98(I26A)FBD225
3 BC98(I26A)FBD170
4 BC98(I26A)FBD126
5 BD126-327
6 BC98FBD327
7 BC98FBD126
8 DB-vector
1 BC98(I26A)FBD327
9 BC98(I26A)FBD327(W146A)
8 DB-vector

ZO-2 133-993

ZO-2 886-993

AD-vector

-His/-Lue/-Trp + 10.0 mM 3AT

ZO-2 133-993

ZO-2 886-993

AD-vector

-His/-Lue/-Trp + 2.5 mM 3AT

ZO-2 133-993

ZO-2 886-993

AD-vector

-Leu/-Trp

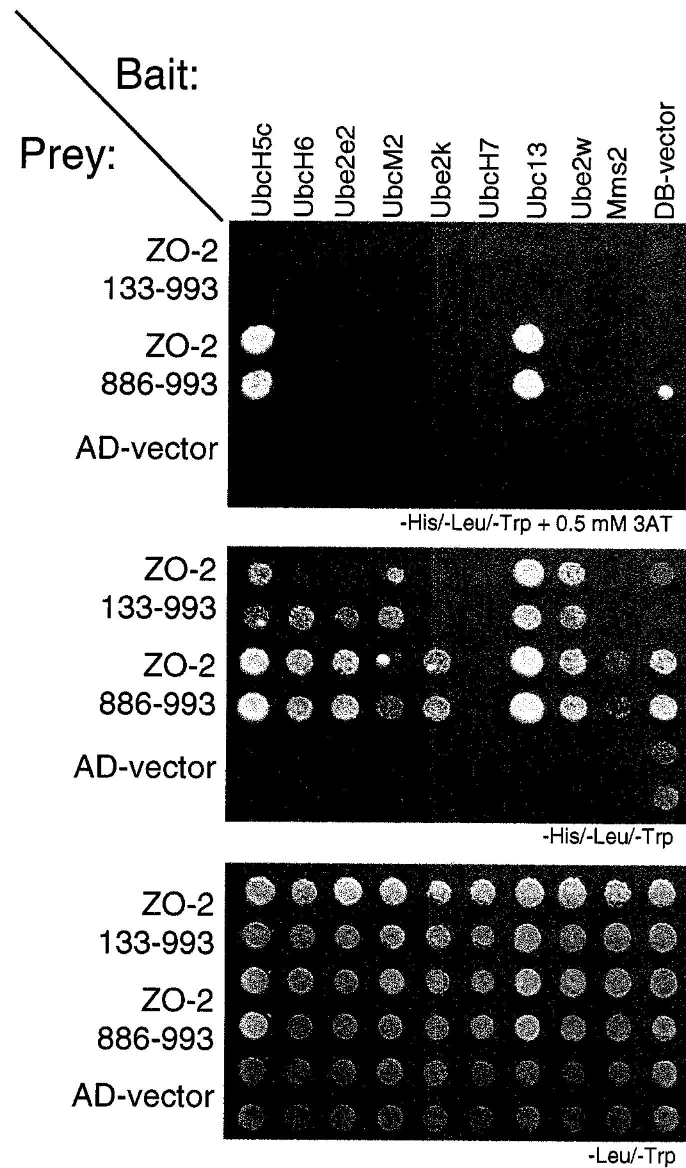
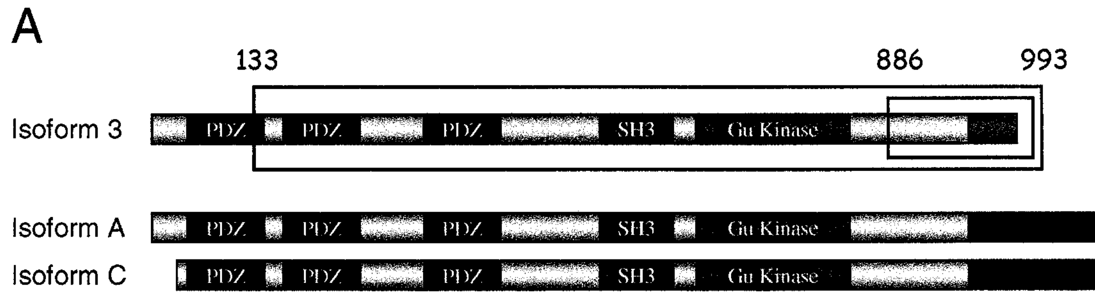
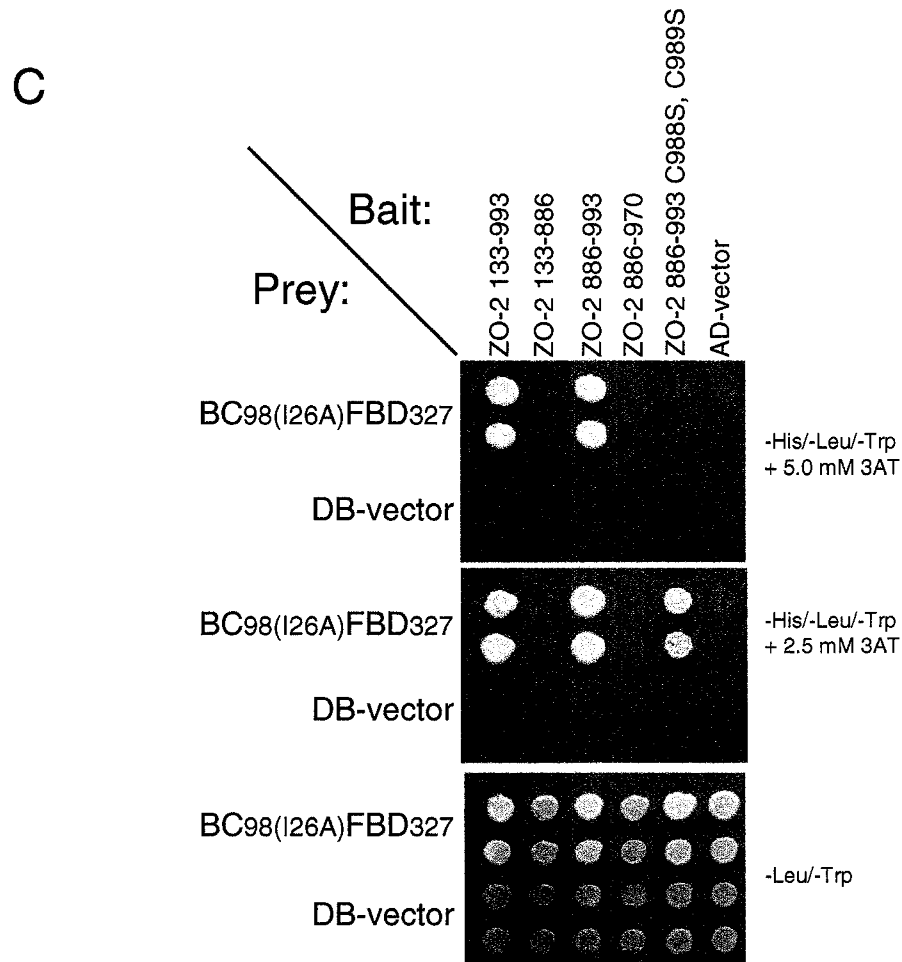


Figure 4.2. ZO-2 Associates with a Subset of the BRCA1-Interacting E2s.

Directed yeast two-hybrid screens between ZO-2 and E2s that interact with the RING of BRCA1. Two colonies for each bait and prey pair were replica plated on selective and non-selective media. The amount of 3AT controls the stringency of selection. The ability of a bait and prey pair to grow at any level above the background of BD- and AD-vector controls is indicative of a interaction.



B 961-VRRGRPRAGTGEPGVFLALSWTAVCSGCCGRHS



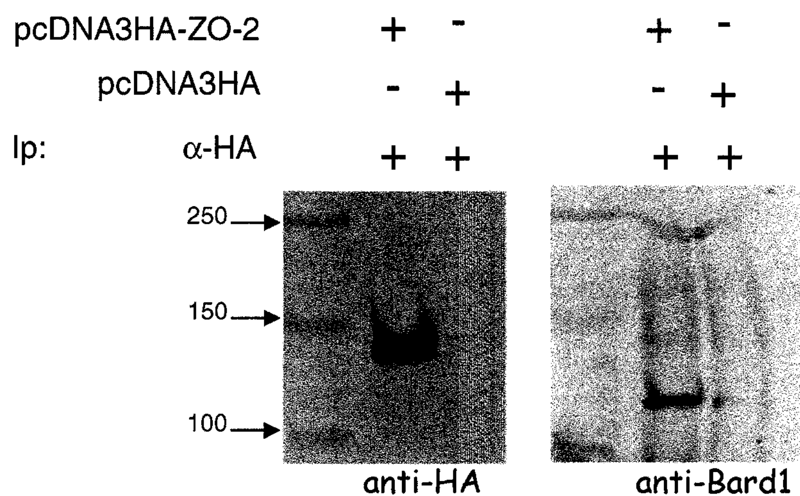


Figure 4.4. Confirmation of Interaction Between ZO-2 and BARD1.

HBL-100 cells were transfected with vector encoding HA-tagged ZO-2 (pcDNA3HA-ZO-2) or empty HA vector (pcDNA3HA) as a control. 30 hours after transfection cells were lysed and the soluble protein fraction was immunoprecipitated (IP) using anti-HA antibodies. α-BARD1 and α-HA western blots showing the presence of BARD1 only in the HA-ZO-2 IP indicating a specific interaction with ZO-2.

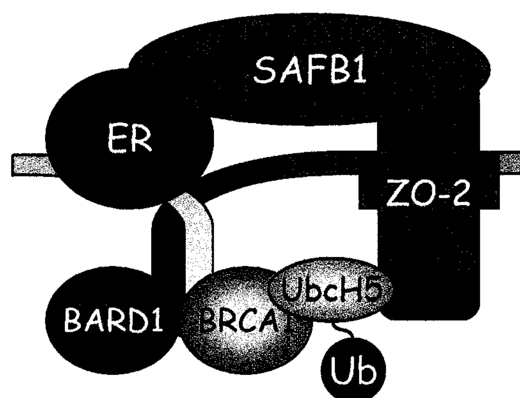


Figure 4.5. Model of a Potential Complex Formed by BRCA1/BARD1, ZO-2, SAFB1, and ERα.

We have demonstrated that ZO-2 can interact with BARD1 and ubiquitin conjugating enzymes, UbcH5 and Ubc13. The functional significance of this interaction may be associated with other reported interactions between ZO-2-SAFB1, ERα-SAFB1, BRCA1-ERα, and BRCA1-BARD1. Each of these proteins has been linked in someway to estrogen signaling and breast cancer, therefore, we propose that their functions may be linked together and these proteins may act as part of a single complex.

Chapter 5. Conclusions and a Potential BARD1 RING Domain Function

Summary and Conclusion

My thesis research directly addresses the need to understand the function of the BRCA1 RING domain. The most commonly observed cancer associated BRCA1 missense mutation (Cys61Gly) lies within the RING domain and eliminates ubiquitin ligase activity. This loss in activity suggests that loss of ubiquitin transfer to at least one critical substrate leads to breast and ovarian cancer susceptibility. The specific goals of my project were to:

1) Identify a novel substrate of the BRCA1/BARD1 ubiquitin ligase.
2) Evaluate the need for other protein factors (such as E2s) for BRCA1 mediated ubiquitination of putative substrates.

To accomplish these goals a structure-based yeast two-hybrid strategy was designed. The unique feature of our design was the construction of a novel "bait" based on the 3D structure of the BRCA1/BARD1 RING:RING heterodimer. Our bait design maintains the structural elements of the heterodimer necessary for ubiquitin ligase activity in a single polypeptide. Using structure-based BRCA1/BARD1 baits I have found:

1) that the BRCA1 RING domain interacts with ten different human E2s.
2) a BARD1-associated BRCA1 substrate, BABS.
3) a BARD1-interacting protein (ZO-2) which may provide a functional link between estrogen signaling and the tissue specific nature of cancer susceptibility of BRCA1 mutations.

These findings directly address my initial goals with respect to BRCA1 and provide new insight toward understanding the process of protein ubiquitination.

By searching for all possible E2s that could bind to BRCA1 we wanted to define the E2s that should be used for future assays with potential substrates. It was quite unexpected that as many as ten different E2s bind BRCA1. The demonstrated ability of BRCA1/BARD1 to synthesize different ubiquitination products depending on the E2 used in a ubiquitination assay opens the possibility for BRCA1 to target different substrates for different fates. All previous research reported for BRCA1 has assumed that UbcH5 is the only E2 with which BRCA1 can function. However, my finding that BRCA1 can also function with Ubc13/Mms2 and Ube2k for the synthesis of Lys63 and Lys48-linked ubiquitin chains, respectively, provides a biochemical explanation of the BRCA1-dependent ubiquitination products at sites of DNA damage(Polanowska et al., 2006; Zhao et al., 2007) and the BRCA1-dependent targeted degradation of RNA pol II(Kleiman et al., 2005). Hence, my discovery of six new BRCA1-E2 interactions opens new avenues for investigating the function of the ubiquitin ligase activity of BRCA1/BARD1.

All of the reported substrates of BRCA1 appear to bind to the BRCA1 subunit of the heterodimeric complex. I decided to search for substrates where others were not looking and therefore set out to address whether a substrate can bind to the BARD1 subunit. Two protein were identified that bind to BARD1 proximal to the RING domain, BABS and ZO-2. The BRCA1/BARD1-dependent ubiquitination of BABS requires a set of three interactions:

1) a substrate-E3 interaction between BABS and BARD1

2) an E3-E2 interaction between BRCA1 and UbcH5, Ube2w, Ubc13, or Ube2k
3) a substrate-E2 interaction between BABS and UbcH5, Ube2w, Ubc13, or Ube2k.

Interestingly, the substrate binding site on BARD1 is the only highly conserved region of the protein that has not previously been assigned to a specific domain (Fig. 5.1). ZO-2 also binds to the BARD1 substrate binding site and to a subset of the BRCA1-interacting E2s. Therefore, though not yet tested directly ZO-2 is a possible candidate substrate.

Unexpectedly, my results also have provided significant evidence towards a new model of the process of protein ubiquitination. Specifically, I have found that:

1) a single E3 can interact and function with multiple E2s (Fig. 2.11)
2) for BRCA1, the E2 dictates the type of ubiquitin modification to be synthesized on a substrate (Fig. 2.11)
3) a substrate-E2 interaction plays an important role in determining which E2 is used, among the set of E2s that interact with the E3, for ubiquitin transfer (Fig. 3.6).

These results are in contrast to current models of ubiquitination that imply a high level of specificity for E3-E2 interactions, where one or a few E2s are active with a single E3. Additionally, the mechanisms underlying substrate selectivity and selection of the type of ubiquitination product have not been well understood. Thus, my research has provided mechanistic details toward a better understanding of the process of ubiquitination.

The BARD1 RING Domain

Though BARD1 also possesses a RING domain, ubiquitin ligase activity is mediated solely by the RING of BRCA1. The function of the BARD1 RING is not known. As part of the structure-based yeast two-hybrid screen I had hoped to identify an E2 that interacts with the RING of BARD1. Although six new E2s were identified that interact with the BRCA1 RING, none were found to interact with BARD1. In the absence of any known function of the RING domain, BARD1 appears to be required to maintain the BRCA1 RING in an appropriate structural confirmation necessary for ubiquitin ligase activity and to recruit substrates (BABS) into close proximity to the BRCA1 RING for ubiquitin transfer. The RING domain of BARD1 does not appear to be involved during any of these processes, however, it is highly conserved from *homo sapiens* to *c. elegans* suggesting that this domain has an important cellular function.

The yeast two-hybrid system is a useful tool to identify protein-protein interactions, however, there are limitations. The BD126FBC109 bait used in the directed yeast two-hybrid screens appears to present the BRCA1 RING in a manner appropriate for activation of the reporter cassette for identifying BRCA1-interacting E2s. However, this orientation of the BRCA1 and BARD1 RING domains may not be optimal for identifying BARD1-interacting E2s. Therefore, I screened a fusion in which the order of the two protein sequences is switched, BC98FBD126, against the remaining non-BRCA1 interacting E2s using directed yeast two-hybrid screens. From this screen I identified another potential BRCA1/BARD1 interacting protein, Ft1 (Fig. 5.2A) a ubiquitin E2 variant (UEV). UEVs contain the same core Ubc domain as an E2 but lack

the active-cite cysteine residue necessary for ubiquitin transfer activity. The Ile26Ala mutation of BRCA1 does not eliminate the interaction of Ft1 suggesting the possibility that this protein interacts with the RING of BARD1 (Fig. 5.2B).

The potential interaction between BARD1 and Ft1 may relevant to the ubiquitin ligase activity of BRCA1. The two most well known UEVs, TSG101 and Mms2, both bind to ubiquitin non-covalently and Mms2 is necessary for the poly-ubiquitination activity of Ubc13. By analogy with Mms2, Ft1 may provide the necessary elements for BRCA1-dependent poly-ubiquitin chain synthesis by assisting UbcH7 or one of the mono-ubiquitination E2s, UbcH6, Ube2e2, UbcM2, or Ube2w. To gain further insight into how Ft1 may be functioning with the BRCA1/BARD1 heterodimer, I sought to determine if Ft1 interacts with an E2, similar to Mms2 with Ubc13. Directed yeast two-hybrid screens using Ft1 as "bait" with 23 different human E2s "prey" constructs identified Ube2u as a potential interacting partner (Fig. 5.3). As further support of this interaction, using Ube2u as bait with Ft1 as prey, yeast growth is also supported under selective conditions. These results suggest the possibility that Ft1 functions with Ube2u for transfer of ubiquitin or a ubiquitin-like protein (UBL) in a BRCA1/BARD1 dependent manner to substrates lysine residues.

Since BRCA1 can interact and function with multiple ubiquitin E2s it would seem unnecessary for the BARD1 RING domain, which is situated so closely to the BRCA1 RING, to also function with a ubiquitin E2. Therefore, I hypothesize that Ube2u is an E2 for a ubiquitin-like molecule rather than for ubiquitin. As further support of this hypothesis, helix-1 of Ube2u differs greatly compared to known ubiquitin E2s. This helix has been identified as an important E2 structural element that

interacts with an E1(Huang et al., 2005) and the differences in this helix suggests that Ube2u is not a ubiquitin E2.

If BARD1 can function with the potential Ft1/Ube2u heterodimer for the transfer of a UBL, what are its substrates? Given the close proximity of the BRCA1 and BARD1 RING domains it is possible that they could share common substrates. Since substrate-E2 interactions were discovered between BABS and the E2s UbcH5c, Ube2w, Ubc13, and Ube2k, I tested whether Ft1 or Ube2u also interact with BABS in the yeast two-hybrid assay. As shown in Figure 5.4, BABS and ZO-2 both interact with Ft1 suggesting that they may function as potential substrates for BARD1-dependent Ft1/Ube2u mediated transfer of a UBL.

These preliminary results suggest a unique mode of UBL transfer with the E2 Ube2u, although the specific modifier protein utilized is not known. The association of Ube2u with Ft1 would be the second reported E2-UEV heterodimer and the first for a UBL pathway. However, unlike Ubc13/Mms2 it appears as if the RING of the E3 interacts with the UEV rather than the E2. This RING-UEV interaction and begs the question of how UBL transfer would be promoted from the distant Ube2u active-site. These results also suggest that BRCA1/BARD1 may function as a double-headed ligase leading not only to the transfer of ubiquitin to substrate proteins, but the transfer of a UBL as well (Fig. 5.5). Through further research into these preliminary results much is to be gained into the various mechanisms utilized for transfer of ubiquitin or UBLs to protein substrates.

Many proteins have been suggested to function as a ubiquitin ligase based on the presence of one of three domains involved in ubiquitin transfer; RING, HECT, and U-

box. When investigating the function of one of these proteins, a major question is what are its substrates? Based on the results that I have presented in this thesis, prior to searching for substrates one should first determine the E2s that interact with the E3. Identifying the interacting-E2 is a critical step toward understanding the function of the E3 as this provides the researcher with knowledge of:

1) whether the E3 is a ubiquitin- or Ubl-ligase.
2) the subset of E2s that should be used in future Ubl transfer assays of putative substrates.
3) for ubiquitin E2s the type of ubiquitination event to expect on the substrate.
4) a molecule that may provide a bridging interaction between the E3 and substrate.

By identifying the E2s that interact with BRCA1 new avenues have opened toward understanding its function, in such processes as DNA damage repair. As additional substrates of BRCA1 are identified it will be interesting to learn which BRCA1-interacting E2s are used to transfer ubiquitin to each substrate. This will undoubtedly provide crucial information needed to determine the mechanisms used by different E2s for selection of their substrates for ubiquitin transfer. The potential interaction between the BARD1 RING and Ft1 also opens the door toward understanding the function of this domain, which may function as a ligase for transfer of a yet to be identified Ubl. As the ideas presented in this thesis become more and more accepted it will be interesting to learn of the E2s that interact and function with each E3 as this will lead to a better understanding of the mechanisms used to govern E2-E3 interactions. Also it will be interesting to determine the surface of the E2 used for interaction with a given

substrate and whether the substrate, such as BABS, recognizes the same surface on all of its interacting E2s. This thesis work has open the door into an underrepresented area in the field of protein ubiquitination. Further investigation into E2-E3 and E2-substrate interactions will lead to a better understanding of the mechanisms of ubiquitination.

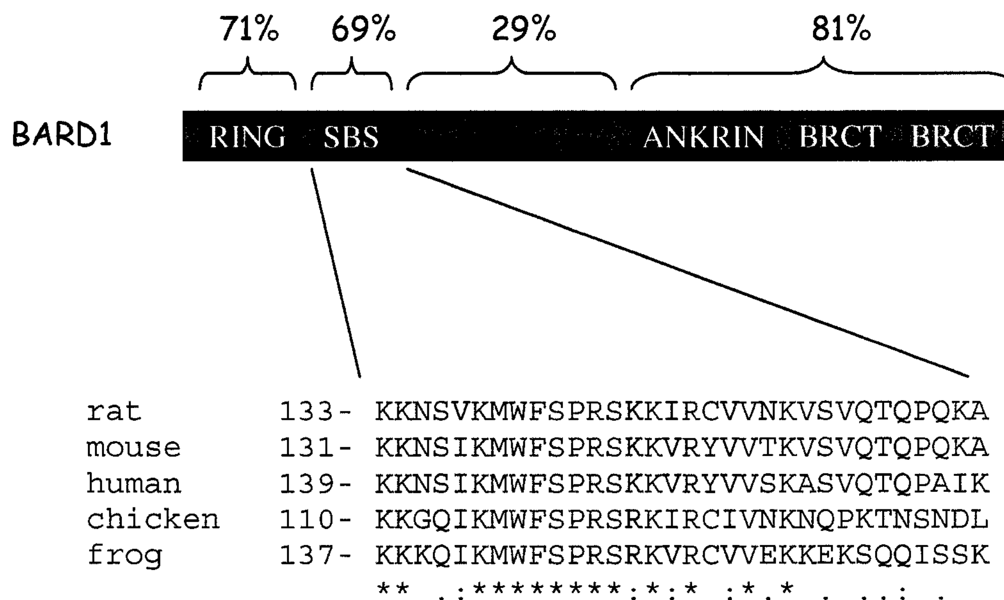


Figure 5.1. Conservation of BARD1 domains.

The BARD1 Substrate Binding-Site (SBS) is highly conserved compared to rat, mouse, chicken, and frog amino acid sequences. The numbers above each domain represent the percentage similarity between human and the organisms listed above. Residues that align with the following human sequences were used to determine similarity for each domain: 26-126 for the RING domain, 139-170 for the SBS, 171-422 for region lacking any recognizable domain, and 423-777 for the ANKRIN and BRCT domains.

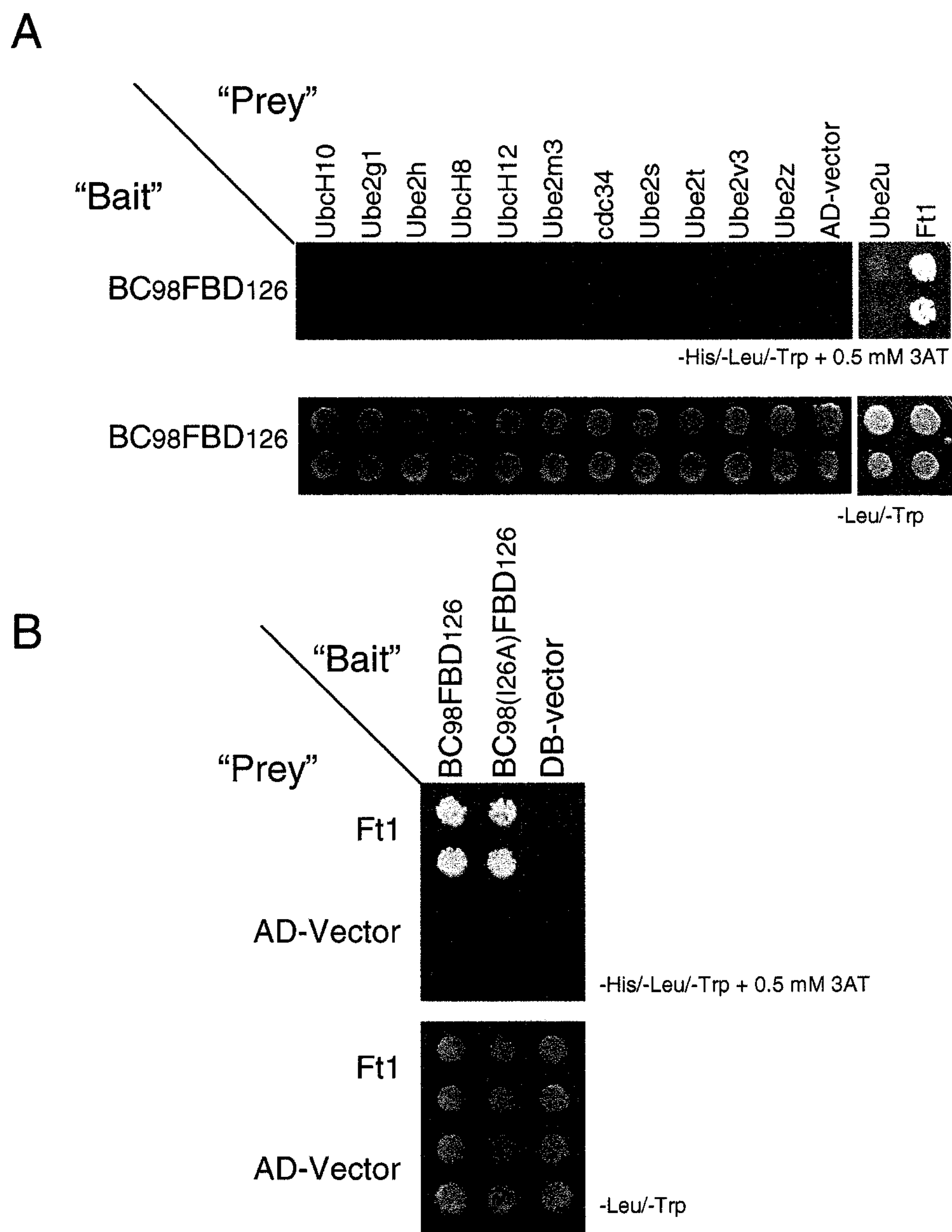


Figure 5.2. BRCA1/BARD1 RING:RING Heterodimer Interacts with Ft1.

Directed yeast two-hybrid screens between BC98FBD126 and the non-BRCA1 interacting E2s. Respective bait and prey constructs were co-transformed into the yeast strain AH109. Two colonies for each bait and prey pair were replica plated on selective and non-selective media. A) BC98FBD126 supports yeast growth with Ft1. B) the I26A mutation (BC98(I26A)FBD126) does not disrupt the interaction with Ft1.

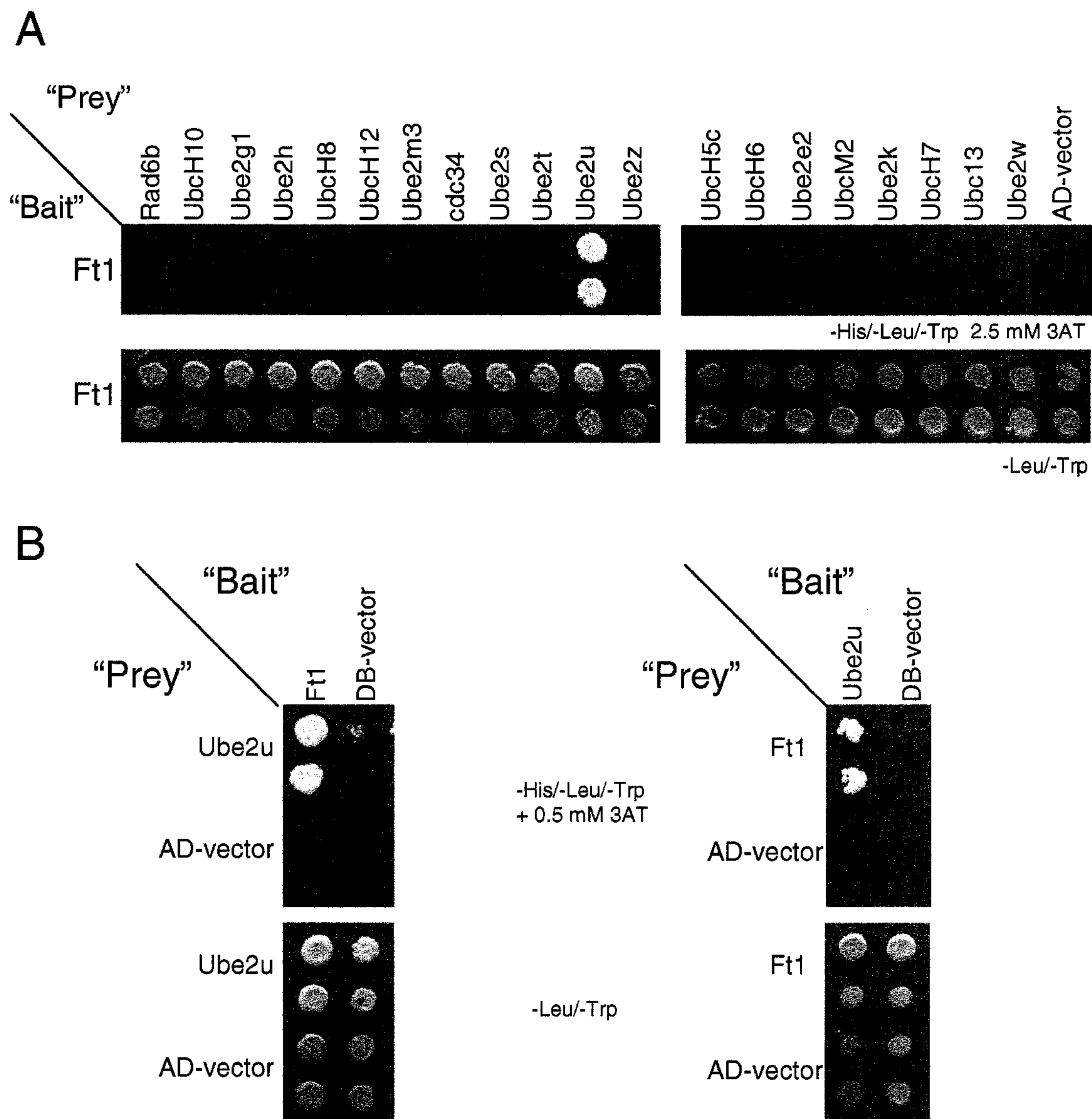


Figure 5.3. Ft1 Interacts with Ube2u.

Directed yeast two-hybrid screens between Ft1 and 20 different E2s. Respective bait and prey constructs were co-transformed into the yeast strain AH109. Two colonies for each bait and prey pair were replica plated on selective and non-selective media. A) Ft1 supports yeast growth with Ube2u on selective media. B) the interaction between Ft1 and Ube2u is also detected with Ube2u as bait and Ft1 as prey.

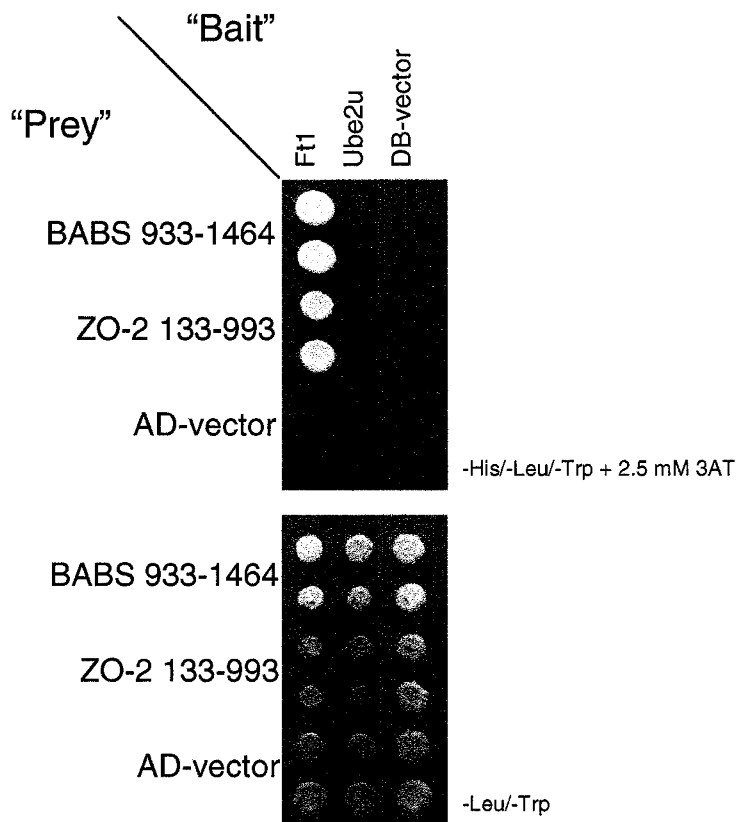


Figure 5.4. BABS and ZO-2 interact with Ft1.

Directed yeast two-hybrid screens to detect potential substrate-E2 interactions for proposed Ube2u mediated UBL pathway. Respective bait and prey constructs were co-transformed into the yeast strain AH109. Two colonies for each bait and prey pair were replica plated on selective and non-selective media. Yeast growth on selective media indicates Ft1 interacts with both BABS and ZO-2.

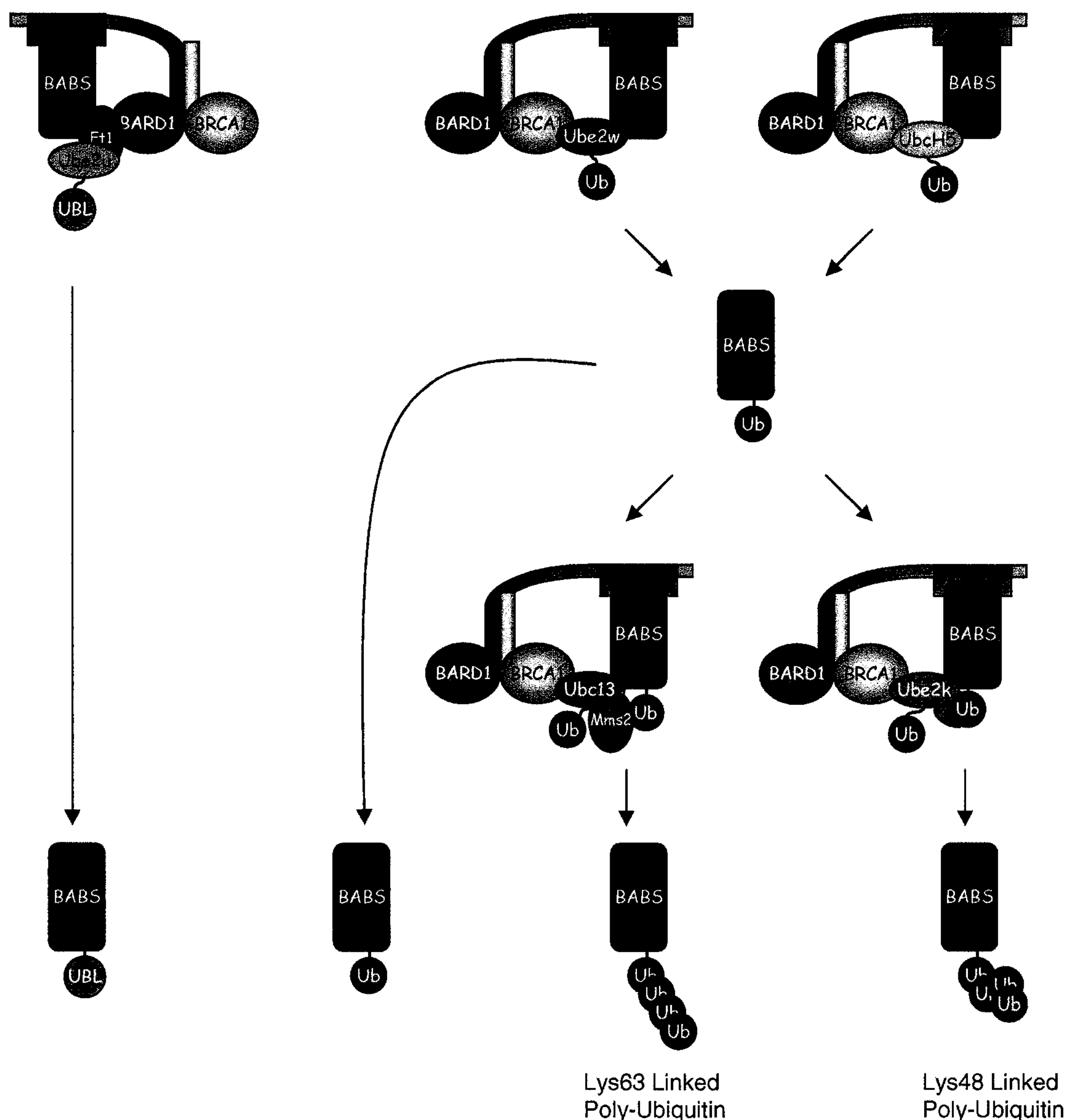


Figure 5.5. Proposed Model for Different Types of BRCA1/BARD1 Directed Ubiquitin or UBL Modification of BABS.

A set of three interactions are required for transfer of ubiquitin, or a UBL, to BABS. First, the substrate-E3 interaction between BABS and BARD1. Second, an E2-E3 interaction. For ubiquitin transfer the interaction is between the BRCA1 RING and the E2s UbcH5, Ube2w, Ubc13, or Ube2k. For UBL transfer Ft1 interacts with the BARD1 RING and the E2 Ube2u. Third, a substrate-E2 interaction between BABS and each of the indicated E2s. Depending on the E2 used different types of modifications are attached to BABS.

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Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., *et al.* (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature *416*, 703-709.

Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell *102*, 533-539.

Zuiderweg, E. R. (2002). Mapping protein-protein interactions in solution by NMR spectroscopy. Biochemistry *41*, 1-7.

Curriculum Vitae

Devin E. Christensen

EDUCATION

Doctor of Philosophy in Biochemistry
University of Washington 2007
Seattle, Washington

Bachelors of Science in Chemistry
Utah State University 2001
Logan, Utah
cum laude

AWARDS/HONORS/FELLOWSHIPS

Microbial Pathogenesis Post-Doctoral Training Grant 2007-2008
Competitive Award

Breast Cancer Research Program Predoctoral Traineeship Award 2004-2007
Department of Defense
Competitive Award

Benjamin Schultz Travel Fellowship, Department of Biochemistry 2004
University of Washington

Cellular and Molecular Biology Pre-Doctoral Training Grant 2003-2004
University of Washington
Competitive Award

Undergraduate Research and Creative Opportunities Grant 2000
Utah State University
Competitive Award

National Deans List 1995
Utah State University

PUBLICATIONS

Christensen, D. E., Brzovic, P. S., and Klevit, R. E. (2007). Novel E2:BRCA1 RING Interactions Dictate the Synthesis of Either Mono- or Specific Poly-Ubiquitin Chain Linkages. Nature Structure and Molecular Biology, Accepted 7/26/2007.

Brzovic, P. S., Lissounov, A., **Christensen, D. E.**, Hoyt, D. W., and Klevit, R. E. (2006). A UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. Mol Cell *21*, 873-880.

RESEARCH EXPERIENCE

Graduate Student Research Assistant 6/2001 - 9/2007
Department of Biochemistry, University of Washington
Supervisor: Dr. Rachel E. Klevit

Identified six new interactions between BRCA1 and the family of ubiquitin conjugating enzymes (E2s). Discovered that different ubiquitination products are synthesized by BRCA1 depending on the E2s used in an ubiquitination assay and poly-ubiquitin chain formation depends on a second, non-covalent, ubiquitin binding site on the E2. Identified an uncharacterized protein and named it BABS, and demonstrated that it is a BARD1-Associated BRCA1 Substrate. Found that BRCA1 mediated ubiquitination of BABS requires a set of three different interactions between the E3 ubiquitin ligase (BRCA1/BARD1), E2, and Substrate (BABS). This research has and will provide significant advancement in the field of protein ubiquitination.

Undergraduate Research Technician 3/1998 - 5/2001
Biotechnology Center, Utah State University
Supervisor: Dr. Steve Aust

Investigated two metalloproteins; ferritin and ceruloplasmin, and their mechanisms of iron storage and transport. Specifically, cloned, expressed, and purified a 19 kDa C-terminal fragment of ceruloplasmin and investigated it potential to facilitate iron incorporation into ferritin. Purified ferritin and ceruloplasmin from both recombinant and native tissues. Cloned full-length ceruloplasmin for expression in insect cells using the baculovirus expression system.

Undergraduate Research Technician 7/1998 – 1/1999
Nutrition and Food Science, Utah State University
Supervisor: Dr. Jeff Broadbent

Molecular cloning of a heat shock promoter from lactic acid bacteria

PRESENTATIONS/ABSTRACTS

Talks

"The BRCA1/BARD1 RING:RING Complex is an Ubiquitin Ligase,
and a SUMO ligase?"
The Ubiquitin Proteasome System in Health and Disease
Joint 59th Harden / EMBO Conference
Cirencester, UK 9/2004

Posters

"A Novel Structure-Based Yeast Two-Hybrid Screen to Identify Substrates
of the Ubiquitin Ligase BRCA1."
Ubiquitin and Cellular Regulation
FASEB Summer Research Conferences
Vermont Academy, Vermont 7/2006

"Identifying Substrates and Functional Roles of the BRCA1 Ubiquitin Ligase."
Era of Hope
Department of Defense Breast Cancer Research Program Meeting
Philadelphia, Pennsylvania 6/2005

"The BRCA1/BARD1 RING:RING Complex is an Ubiquitin Ligase,
and a SUMO ligase?"
Ubiquitin and Signaling
Keystone Symposia
Taos, New Mexico 2/2005

"The BRCA1/BARD1 RING:RING Complex is an Ubiquitin Ligase,
and a SUMO ligase?"
The Ubiquitin Proteasome System in Health and Disease
Joint 59th Harden / EMBO Conference
Cirencester, UK 9/2004

PROFICIENT LABORATORY TECHNIQUES

Molecular Cloning and Site-Directed Mutagenesis,
including traditional restriction digestions and ligation, site-directed exchange, recombinational cloning in yeast, and the gateway vector cloning system.

Protein Purification,
from native and recombinant sources.

Yeast Two-Hybrid System,
directed and library screens.

Ubiquitination Assays,
E2 thiol ester formation and E3-dependent substrate ubiquitination.

NMR,
^{1}H, ^{15}N HSQC-TROSY for protein interaction studies.

Tissue Culture
transient transfection, immunoprecipitation, and stable isotope labeling of cells for quantitative mass spectrometry.

Western Blots

DNA Sequencing

TEACHING EXPERIENCE

Biochemistry 440. Teaching Assistant, University of Washington, fall quarter 2002. Upper division course for biochemistry majors. Students learned protein structure/function relationships and glucose metabolism.

Biochemistry 426. Teaching Assistant, University of Washington, spring quarter 2003. Laboratory course for biochemistry majors where students learn about molecular cloning, protein purification, enzyme kinetics, and experimental assays.

PROFESSIONAL SERVICE

Department of Biochemistry Admissions Committee 2005
Graduate student representative and committee member for evaluating and accepting future graduate students.

BUSINESS

Center for Technology Entrepreneurship Summer Boot Camp 2004
University of Washington Business School