University of Alberta

Investigation of the BRCT repeats in human hereditary breast cancer and DNA damage response

by

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Abstract

The C-terminal region of breast cancer susceptibility gene 1 (BRCA1) contains a pair of tandem BRCT repeats that are critical for the tumour suppressor function of BRCA1. BRCT repeats are present in a large of number of proteins that are implicated in the cellular response to DNA damage. A subset of tandem BRCT domains, including those of BRCA1, functions as phosphorecognition modules. Aside from BRCA1, the precise molecular mechanisms of the BRCT repeats of other proteins remain largely unknown.

We determined the crystal structure of the tandem BRCT domain of human mediator of DNA checkpoint 1 (MDC1) at 1.45 Å resolution. Our structural and biochemical studies suggest that the tandem BRCT domain of MDC1 functions as the predominant histone variant, γH2AX phosphorecognition module and that the interaction is critically dependent on the free carboxylate group of the γH2AX C-terminal tail.

We also determined the crystal structure of the tandem BRCT domain of human BARD1, the *in vivo* binding partner of BRCA1. Our structure uncovers a degenerate phosphopeptide binding pocket that lacks the key arginine critical for phosphopeptide interactions in other BRCT proteins. Our biochemical studies reveal that a flexible tether links ankyrin and BRCT domains in BARD1. Furthermore, the linker is required for the interactions between the CstF-50 WD-40 domain and BARD1, allowing the

BARD1 C-terminus to convey DNA damage signals directly to RNA polymerase.

Finally, using protease-based and phosphopeptide pull-down assays, we directly assessed the structural and functional effects of 117 single amino acid substitutions in the BRCA1 BRCT domain derived from breast cancer screening programs. None of the variants showing enhanced sensitivity to proteolytic digestion were found to be active in peptide binding, indicating that these missense mutations contribute to BRCA1 loss of function through protein destabilizing effects. A subset of structurally stable variants was defective in peptide binding activity, suggesting that these variants may disrupt the phosphopeptide binding pocket. Taken together, the results reveal that 32% of the variants show structural stability and peptide binding activity that were indistinguishable from those of wild type.

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LIST OF ABBREVIATIONS

9-1-1	Rad9-Rad1-Hus1
53BP1	p53-binding protein
ANK	ankyrin
ATR	ataxia telangiectasia mutated and Rad3-related
BACH1	BRCA1-associated C-terminal helicase 1
BARD1	BRCA1-associated RING domain protein 1
BIC	breast cancer information core
BRCA1	breast cancer susceptibility gene 1
BRCT	BRCA1 C-terminal domain
CstF	cleavage stimulation factor
DDR	DNA damage response
DSB	double-strand break
FHA	fork-head associated
GD	Grantham deviation
GV	Grantham variation
HR	homologous recombination
IR	ionizing radiation
IRIF	ionizing radiation-induced nuclear foci
MDC1	mediator of DNA checkpoint 1
NHEJ	non-homologous end-joining
PTIP	Pax-transcription-activation domain-interacting protein
RAP80	receptor-associated protein 80
RNAP II	RNA polymerase II
SNP	single nucleotide polymorphism
TopBP1	topoisomerase (DNA) II binding protein 1

Chapter 1:

Introduction

DNA double strand break repair

Chromosomal double-strand breaks (DSBs) frequently develop from endogenous sources such as collapsed DNA replication forks, meiotic recombination within germ cells, reactive oxygen species generated by cellular metabolism and nucleases (Figure 1.1) (Thompson & Schild 2002, McKinnon & Caldecott 2007). These highly toxic DNA legions can also be induced exogenously by a variety of DNA damage agents, such as ionizing radiation (IR) and environmental chemicals. The IR and radio-mimetic drugs are often used in the treatment of cancer. In order to counter the threat to the genome from various DNA-damaging agents, cells use the DNA damage response (DDR) that senses DNA damage, catalyzes a multifaceted response and coordinates cell cycle arrest, apoptosis and DNA repair networks (Zhou & Elledge 2000, Rouse & Jackson 2002, Stucki & Jackson 2006, Bartek & Lukas 2007, Harper & Elledge 2007). The DDR must be tightly regulated to prevent the introduction of errors in critical genes that may lead to oncogenesis (Hahn & Weinberg 2002).

Upon DSB formation, certain DDR proteins relocalize to microscopically discernible sub-nuclear structures and appear as bright speckles or “foci” by fluorescence microscopy, which have been termed IR-induced nuclear foci (IRIF) (Fernandez-Capetillo, Celeste & Nussenzweig 2003). At the heart of each IRIF, there is one or a cluster of DSBs that are actively being repaired. A key member of IRIF formation in

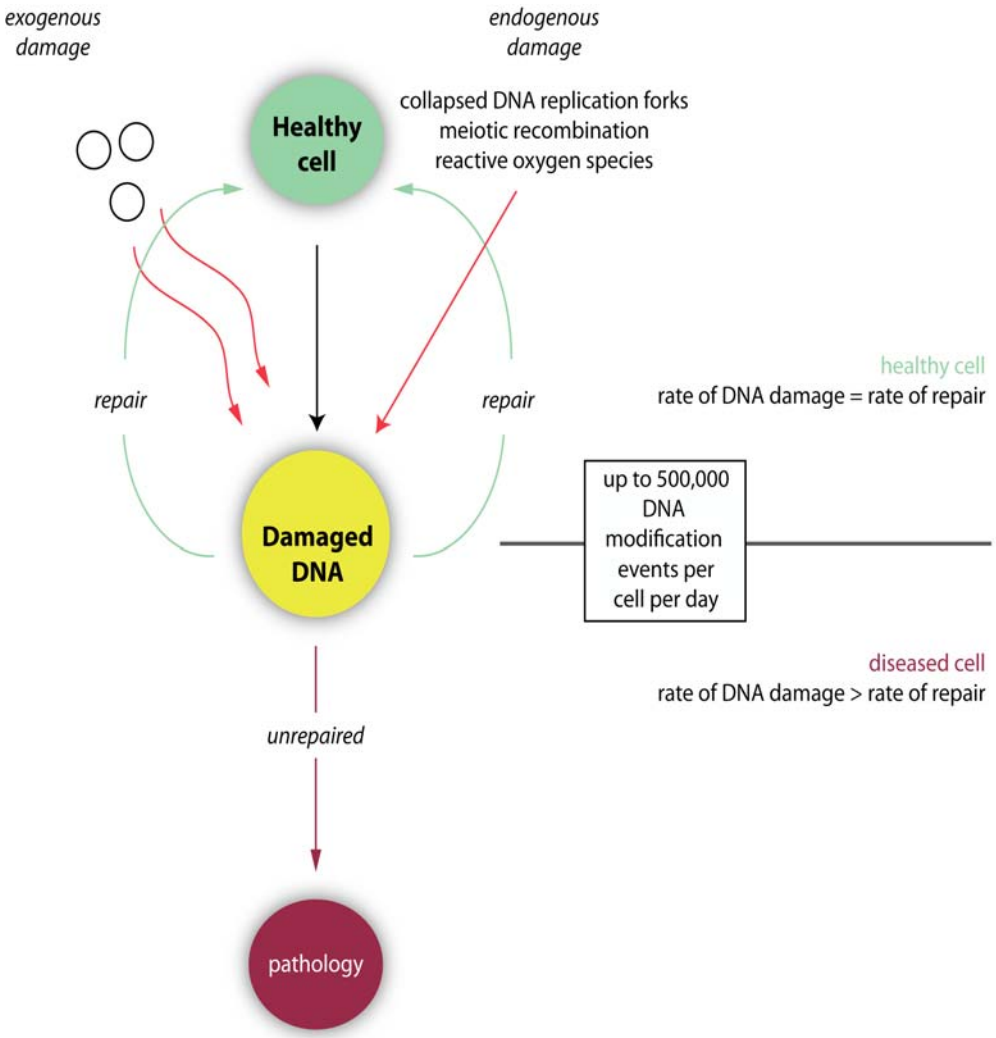

Figure 1.1. Chromosomal double-strand breaks frequently develop. The cells use the DNA damage response to prevent the introduction of errors that may lead to oncogenesis.

mammalian cells is the histone H2A variant, H2AX. H2AX is a component of the nucleosome core structure and comprises 10-15% of total cellular histone H2A in higher organisms (Celeste et al. 2002). In response to DSBs, H2AX is phosphorylated extensively on a conserved serine residue at its C-terminus in chromatin regions bearing and flanking the sites of the DNA DSBs (Rogakou et al. 1999). In normal cells, this phosphorylation is mediated primarily by ataxia telangiectasia mutated (ATM) kinase (Burma et al. 2001, Fernandez-Capetillo et al. 2002). When the gene for H2AX is disrupted to remove the H2AX C-terminal phosphorylation site, the IRIF formation by DDR factors is lost and organisms exhibit high levels of radiation-induced chromosomal aberrations (Celeste et al. 2003). This indicates that H2AX is involved in the signaling and/or repair of DNA DSBs (Celeste et al. 2002, Bassing et al. 2002). Notably, H2AX is required for the maximal efficiency and/or fidelity of the homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways, however it is not an essential component of either pathway (Celeste et al. 2002, Bassing et al. 2002, Petersen et al. 2001, Xie et al. 2004).

There are two predominant mechanisms for DNA repair – HR and NHEJ (Figure 1.2). The eukaryotic genome continuously cycles between diploid and tetraploid states and the ploidy has a dramatic influence on the processes available to repair the DNA damage. By definition, HR requires the presence of an intact sister chromatid and indeed, many DNA repair proteins that are directly involved in HR fail to concentrate at DSBs in G1

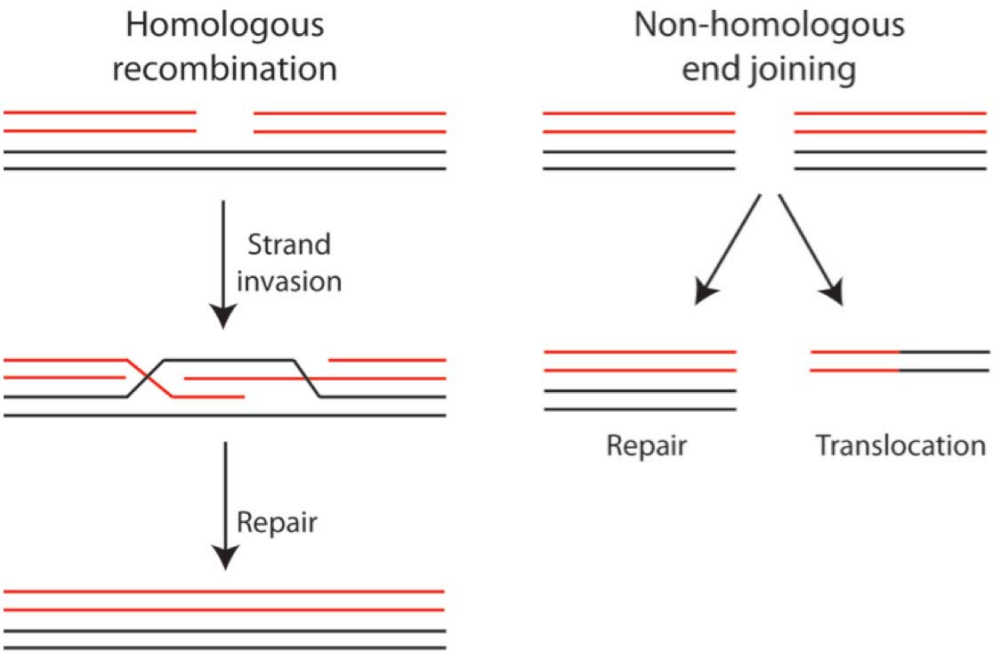

Figure 1.2. Models for DSB repair. DNA DSBs can be repaired by either homologous recombination or non-homologous end joining. Homologous recombination allows the repair of a DSB by copying the homologous region on the sister chromatid. Non-homologous end joining repairs DSBs by ligating contiguous (*red* or *black*) stretches of DNA or cause chromosomal translocations by ligating non-contiguous regions (*red* and *black*). Adapted from Greenberg 2008.

(Lisby et al. 2004). Therefore, G1 cells repair DSBs by NHEJ, while both NHEJ and HR can occur in S and G2 phases. Although accurate HR does not contribute to translocation events stemming from the presence of multiple DSBs induced by endonucleases, NHEJ is an error-prone process that enzymatically joins DNA ends (Weinstock et al. 2006).

Mutations in genes involved in any of the DDR steps can predispose an individual to cancer. For example, inactivating mutations in the breast cancer susceptibility genes, BRCA1 and BRCA2, account for the most common forms of hereditary breast and ovarian cancer. Statistical modeling hinted towards an existence of dominant breast cancer susceptibility genes (Williams & Anderson 1984) and the subsequent discovery of two major genes, BRCA1 and BRCA2, has shed some light on the genetic bases of the diseases (Hall et al. 1990, Miki et al. 1994, Wooster et al. 1994, Wooster et al. 1995). These gene products are required for efficient HR as well as NHEJ repair pathways of a DSB (Moynahan et al. 1999, Moynahan, Cui & Jasin 2001, Wang et al. 2001, Zhong et al. 2002a, Zhong et al. 2002b, Bau et al. 2004, Coupier et al. 2004).

BRCA1

Breast Cancer and its association with BRCA1

Breast cancer is the most common cancer among Canadian women and one in eight women in North America is expected to develop some form of breast cancer during her lifetime (Table 1.1). A phenotype in human breast cancer cells, HCC1937, harboring mutant BRCA1 is characterized by extensive chromosomal aberrations (Moynahan, Cui & Jasin 2001, Kachhap et al. 2001). Heterozygous BRCA1 mutations are capable of predisposing individuals to breast, ovarian and other secondary cancers and the second BRCA1 allele appears to be lost in a high percentage of familial breast carcinomas (Neuhausen, Marshall 1994). Although germline mutations in BRCA1 account for fewer than 5% of all breast cancers, it is estimated that the disruption of one of the alleles of BRCA1 is responsible for 50% of women with inherited breast cancer and up to 90% of women with combined breast and ovarian cancers (Miki et al. 1994, Couch & Weber 1996, Ford et al. 1998, Russell et al. 2000, Nathanson, Wooster & Weber 2001). Furthermore, male and female breast cancer patients with a BRCA1 defect are commonly diagnosed with secondary cancers such as pancreatic, prostate and melanoma at later stages (Liede, Karlan & Narod 2004, Hemminki et al. 2005). Many of the BRCA1 mutations are clearly pathological because they result in truncated proteins. However, a large portion of the documented mutations is single amino-acid changes within the RING or the BRCT domain of BRCA1 and they are more difficult to assess for cancer risk. Breast cancer screening programs where identification of individuals at risk prior to tumourigenesis

is now possible and the use of preventative method such as prophylactic mastectomy for women with germline BRCA1 mutations has been shown to decrease the breast cancer risk by as much as 60% (Rebbeck et al. 1999, Eisen & Weber 2001). Therefore, it is pivotal to understand how the single amino-acid mutations in the BRCA1 BRCT domain compromise the protein function in order to establish a correct individualized cancer risk assessment and to achieve eventual reduction of disease incidence.

The nuclear functions of BRCA1

BRCA1 plays critical roles in DNA damage repair, cell cycle control, transcription regulation and chromatin remodeling (Scully & Livingston 2000, Venkitaraman 2002, Powell & Kachnic 2003, Starita & Parvin 2003). This 1863 amino acid-long protein contains two well-known protein interacting modules: a RING domain at the N-terminus and a pair of BRCA1 C-terminal (BRCT) repeats at the C-terminus (Figure 1.3). A large central region rich in SQ and TQ dipeptide pairs separates the two modules. Intensive research has shown that the protein BRCA1 is involved in a multitude of critical cellular processes by interacting with a number of regulatory proteins (Table 1.2). Importantly, after genotoxic stress, BRCA1 localizes to distinct nuclear foci thought to represent sites of DNA damage. At the foci, BRCA1 is thought to function not only as a scaffold for the assembly of DNA repair complexes (Scully et al. 1997a, Wang et al. 2000), but also as a regulator of DNA repair, transcription and cell cycle.

Age of Breast Cancer onset	Proportion of women affected
By age 40	1 in 233
By age 50	1 in 69
By age 60	1 in 38
By age 70	1 in 27
Ever	1 in 8

Table 1.1. Lifetime probability estimates for Breast Cancer
Source: www.cancer.gov
Estimates are for the U.S. population, and based on incident rates in 2006

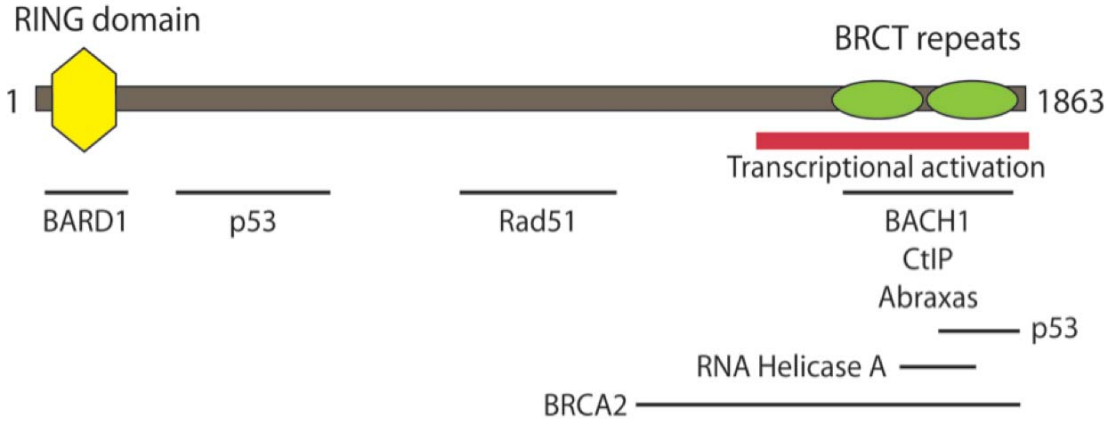

Figure 1.3. Features of the BRCA1 protein. BRCA1 contains the N-terminal RING domain and two C-terminal BRCT repeats. Interacting proteins are shown below the binding regions (black lines).

DNA repair	ATM, CHK2, ATR, BRCA2, RAD51, RAD50/MRE11/NBS1, BASC, PCNA, H2AX, c-Abl
Transcription	RNA polymerase II holoenzyme, HDAC1, HDAC2, E2F, CBP/p300, SWI/SNF complex, CtIP, p53, androgen receptor, ATF1, STAT1, estrogen receptor α, c-Myc, ZBRK1
Cell cycle	RB, CDK2, p21, p27, BARD1
Others	BAP1, BIP1, BRAP2, Importin α

Table 1.2. Proteins interacting with BRCA1. *Adapted from Yoshida and Miki 2004.*

The first evidence suggesting a role of BRCA1 in DNA repair was hyperphosphorylation of BRCA1 in response to DNA damage and relocation to the sites of replication forks marked by proliferating cell nuclear antigen (PCNA) (Scully et al. 1997a, Thomas et al. 1997). BRCA1 is phosphorylated at multiple sites by different kinases after DNA damage. For example, following IR exposure, BRCA1 is phosphorylated by ATM kinase and the G2/M control kinase, CHK2 on different serine residues (Bell et al. 1999, Chaturvedi et al. 1999, Cortez et al. 1999, Gatei et al. 2000). Unfortunately, how each type of phosphorylation influences the functions of BRCA1 is unknown. Nonetheless, studies implicate the involvement of BRCA1 in the repair of DSBs and HR. Rad51 is required for HR as well as the recombination during mitosis and meiosis (Shinohara, Ogawa & Ogawa 1992) and BRCA1 co-localizes with Rad51 to form complexes (Scully et al. 1997b, Chen et al. 1998). This co-localization strongly suggests that BRCA1 has a role in the repair of DSBs. Other studies indicate that BRCA1 co-localizes with Rad50, together with its partners Mre11 and NBS1 (Wang et al. 2000, Zhong et al. 1999) and regulates the Mre11-Rad50-NBS1 (MRN) complex (Wu et al. 2000). The MRN complex has well established roles in the repair of DSBs. BRCA1 binds DNA directly and inhibits the Mre11 nuclease activity, thereby regulating the length and the persistence of single-stranded DNA generation at the sites of DSBs (Paull et al. 2001). Therefore, BRCA1 indirectly influences HR-mediated repair of DSBs (Moynahan et al. 1999).

BRCA1 also fails to form DNA damage-induced foci in absence of histone H2AX, suggesting that a part of the BRCA1 response to DSBs may take place on chromatin (Celeste et al. 2002).

BRCA1 is required for effective S-phase and G2/M-phase checkpoints. BRCA1 variants defective for ATM-mediated phosphorylation are associated with a defect in G2/M arrest (Cortez et al. 1999). Moreover, BRCA1 induces 14-3-3σ, a major G2/M checkpoint control gene, in a p53-dependent manner (Aprelikova et al. 2001). Interestingly, another p53 target gene, G1 cyclin-dependent kinase inhibitor p21, is transactivated by BRCA1 to prevent S-phase entry in a p53-independent manner (Somasundaram et al. 1997). Notably, the cancer-associated BRCA1 variants fail to activate p21. Together, these works indicate that BRCA1 regulates S and G2/M-phase DNA-damage induced checkpoints.

BRCA1 as a transcription regulator

Given the high content of negatively charged amino acids in the C-terminus of the protein, BRCA1 was predicted to play a role in transcriptional regulation (Miki et al. 1994). The C-terminus of BRCA1 fused to a DNA-binding domain was shown to activate transcription in both yeast and mammalian cells (Figure 1.4) (Chapman & Verma 1996, Monteiro, August & Hanafusa 1996). This analysis was possible as a consequence of modular nature of transcription factors in which the DNA-binding and transactivation domains could function independently

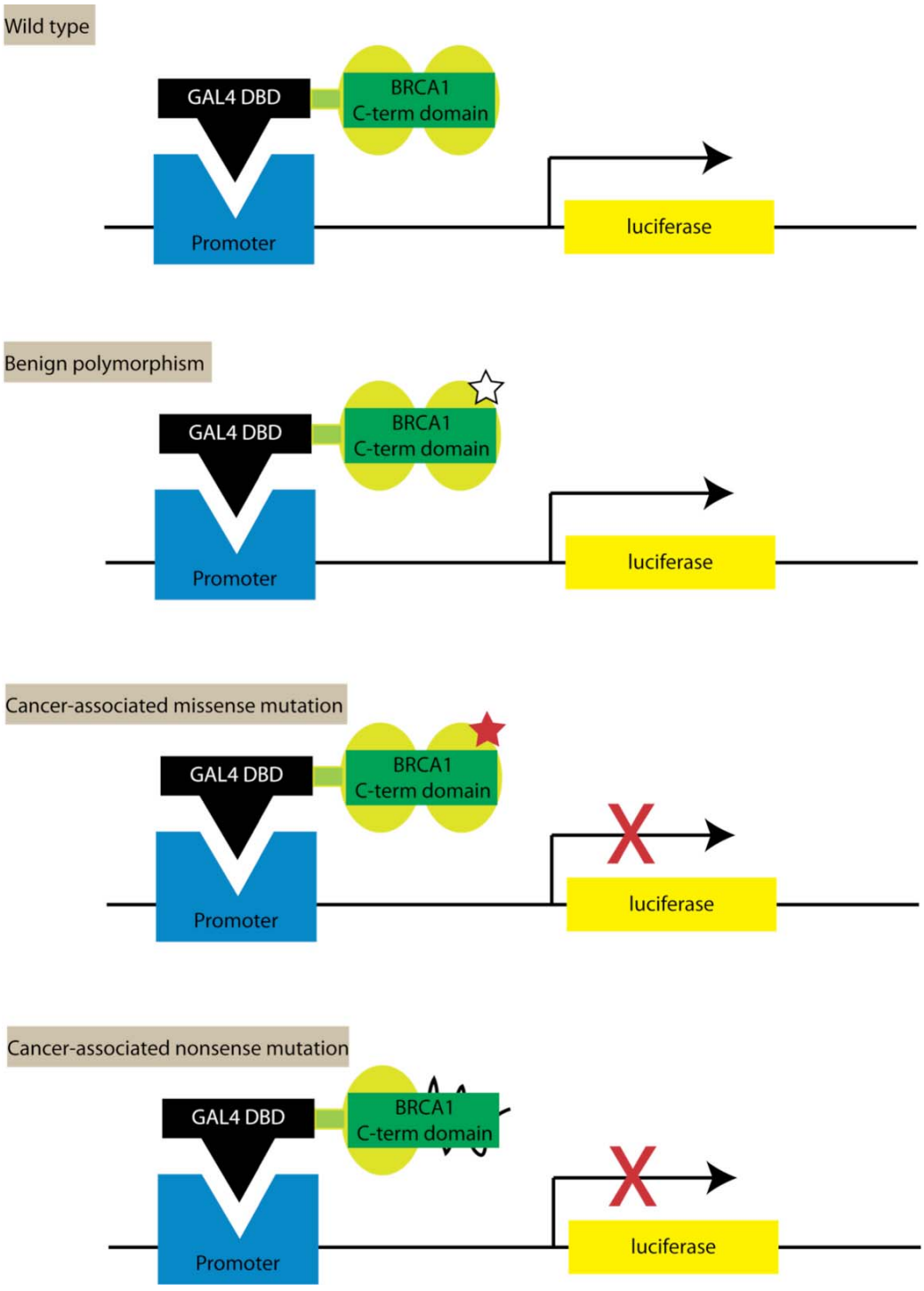

Figure 1.4. Transcription activation by the BRCA1 C-terminal region fused to a heterologous DNA-binding domain. Yellow-green circles represent the BRCA1 C-terminal repeats and black boxes represent the heterologous DNA-binding domain. In mammalian cells, fusion to a GAL4-DNA binding domain enables the fusion BRCA1 protein to recognize specific sequences in the promoter of a reporter gene. Introducing cancer-associated mutations (but not benign polymorphisms) obliterates reporter activation. Adapted from Monteiro 2000.

(Ptashne & Gann 1997). This activation was lost in early onset breast or ovarian cancer patients with BRCA1 C-terminal germline missense mutations, suggesting that the transcriptional activation of BRCA1 was indispensable to its tumour suppressor activity (Monteiro, August & Hanafusa 1996). The BRCT region of BRCA1 that is involved in phosphopeptide binding colocalizes with a region that is essential for transcription activation in the heterologous system of the transcription assay (Mirkovic et al. 2004). This colocalization may highlight a structural basis for the correlation among the transcription assay, the phosphopeptide binding assay and the integrity of the BRCA1 BRCT domain (Carvalho et al. 2007).

BRCA1 has been connected to the transcriptional regulation of several genes triggered in response to DNA damage. For example, the BRCA1/BARD1 heterodimer binds to RNA polymerase II (RNAP II) holoenzyme. Furthermore, BRCA1 overexpression stimulates p53-responsive cell cycle progression inhibitors and stress-response factors such as p21 and GADD45 (MacLachlan, Takimoto & El-Deiry 2002). Studies have revealed a role of BRCA1 as a co-activator for p53 through a direct physical interaction (Zhang et al. 1998a).

Characteristics of BRCT domains

Structure

At the C-terminal end of BRCA1, there are two tandem BRCT repeats that exhibit weak amino acid sequence similiary to the C-terminal regions of proteins involved in DNA repair, such as the p53-binding protein, 53BP1, and the yeast protein RAD9 (Koonin, Altschul & Bork 1996, Bork et al. 1997, Callebaut & Mornon 1997). The BRCT repeat has a conserved structure, composed of a four-stranded parallel β-sheet bordered by a pair of α-helices on one face and a single α-helix on the opposite face (Zhang et al. 1998b, Krishnan et al. 2001, Williams, Green & Glover 2001, Derbyshire et al. 2002, Joo et al. 2002, Lee et al. 2005, Stucki et al. 2005, Birrane et al. 2007, Edwards et al. 2008). The repeat is ~90-100 amino acids long and can occur singly or as multiple repeats.

In BRCA1, two BRCT repeats pack in a head-in-tail manner involving α2 of the N-terminal repeat and α1 and α3 of the C-terminal repeat, in addition to an inter-repeat linker (Figure 1.5) (Williams, Green & Glover 2001). This packing arrangement is conserved in many other dual BRCT repeat proteins that are involved in the cellular response to DNA damage (Figure 1.6) (Derbyshire et al. 2002, Joo et al. 2002, Lee et al. 2005, Edwards et al. 2008). Many breast and ovarian cancer-associated BRCA1 missense mutations are localized at the interface between the two BRCT repeats, indicating that the correct packing arrangement of the two repeat is critical to BRCA1 function (Williams, Green & Glover 2001, Williams & Glover 2003, Williams et al. 2003).

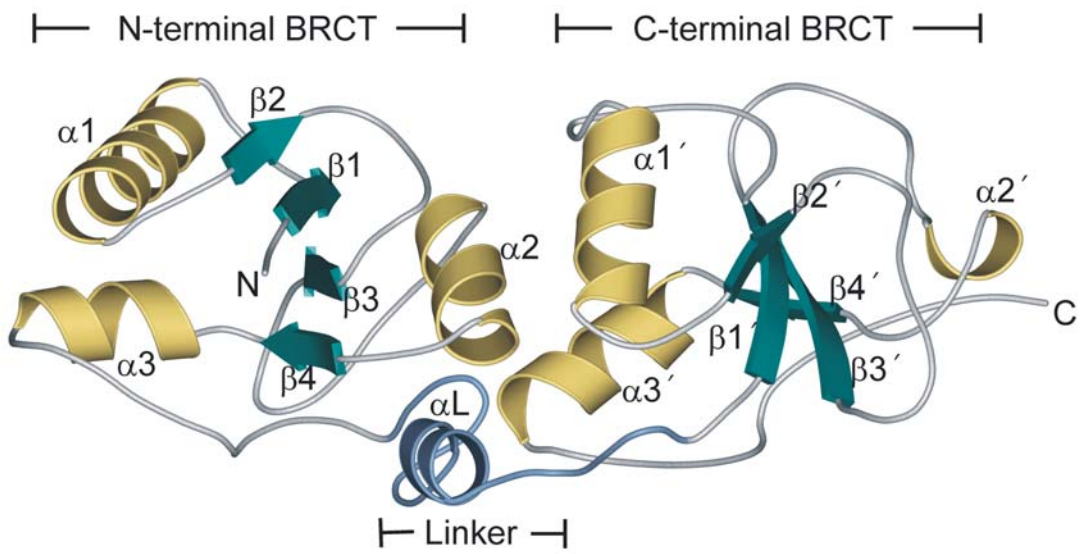

Figure 1.5. Structure of the tandem BRCT repeats of BRCA1. The secondary structure elements in the C-terminal BRCT repeat are labelled 'prime.'

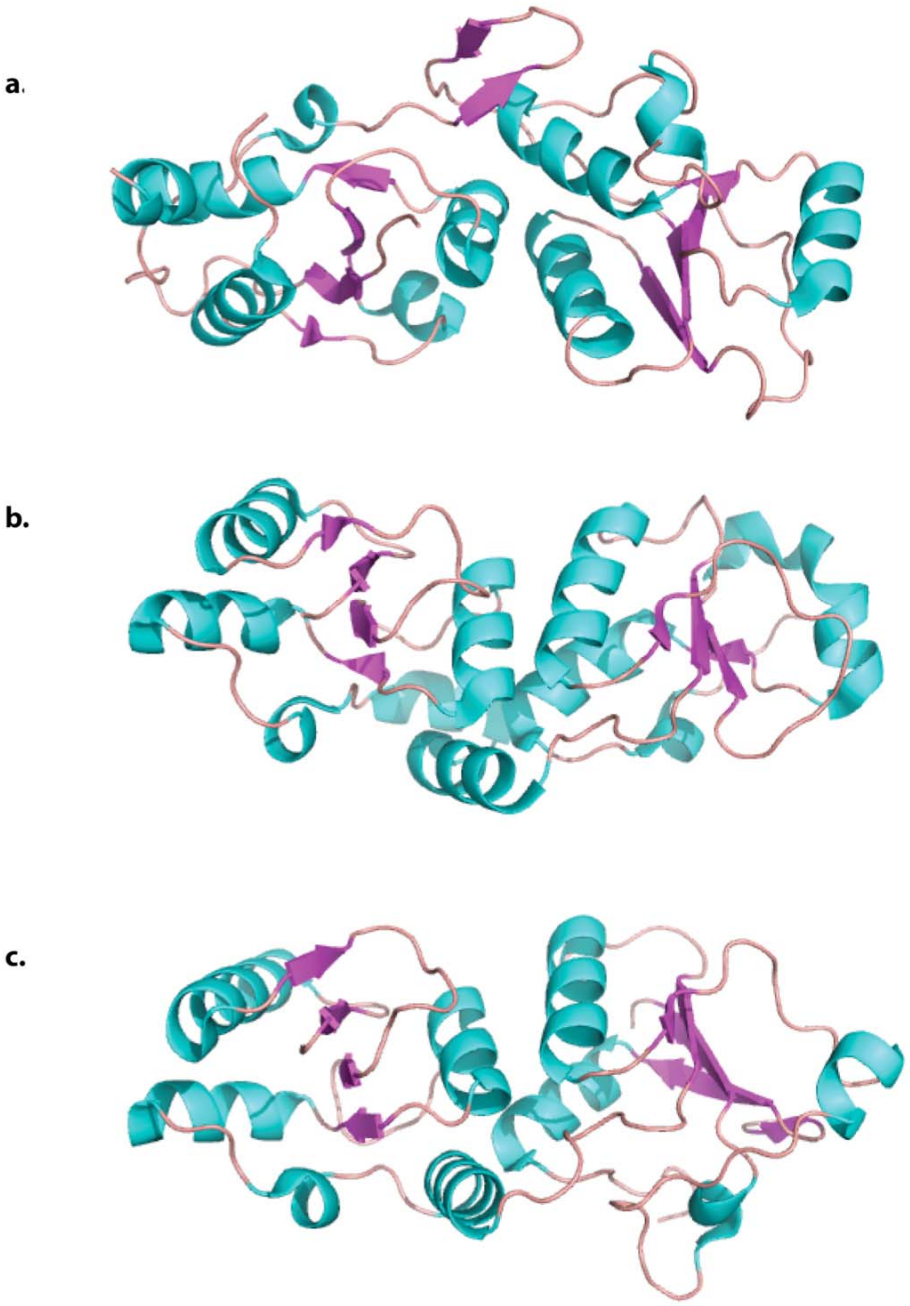

Figure 1.6. Structures of the tandem BRCT repeats. a. 53BP1-BRCT (pdbID-1KZY, Joo et al. 2002) **b.** MDC1-BRCT (pdbID-2ADO, Lee et al. 2005) **c.** BARD1-BRCT (pdbID-2R1Z, Edwards et al. 2008)

BRCT as a peptide binding domain

The most common mode of BRCT-mediated interactions occurs between the BRCT domain and a non-BRCT partner (Yu et al. 1998, Cantor et al. 2001), yet some BRCT domains can interact with each other, as in the case of XRCC1 and DNA ligase III (Taylor et al. 1998). The base excision repair protein XRCC1 (X-ray Repair Cross Complementation 1 protein) contains two BRCT domains separated by a 125-residue linker region. XRCC1 interacts with the BRCT domain of Poly (ADP-ribose)polymerase (PARP) via its N-terminal BRCT and with the BRCT domain of DNA ligase III via its C-terminal BRCT (Kubota et al. 1996, Nash et al. 1997, Taylor et al. 1998). BRCT domains are also capable of mediating protein-DNA interactions. For example, several of the TopBP1 (topoisomerase II binding protein 1) BRCT domains show binding to DNA fragments, independent of DNA sequences and forms of DNA termini (Yamane, Tsuruo 1999, Choi et al. 2009). Interestingly, TopBP1 shows preferential binding to longer DNA fragments (Choi et al. 2009).

A subset of tandem BRCT domains, including those of BRCA1, functions as phosphoserine/phosphothreonine-recognition modules (Manke et al. 2003, Rodriguez et al. 2003, Yu et al. 2003). This suggests that some BRCT-mediated interactions in DNA damage response and cell cycle control are controlled by protein phosphorylation (Manke et al. 2003). The oriented peptide library screening of the tandem BRCA1 BRCT domains showed that not only the interaction depended on

phosphorylation of Ser, but also the binding specificity extended from the pSer(+1) to the pSer(+5) position (Rodriguez et al. 2003). The binding specificity of BRCA1 was particularly high in the pSer(+3) position, for Phe (Manke et al. 2003, Rodriguez et al. 2003).

Phospho-dependent BRCA1 BRCT interacting proteins

Multiple proteins interact with the BRCA1 BRCT domain in a phosphorylation-dependent manner and collaborate functionally with BRCA1 to cooperate in multiple cellular processes. The biochemical understanding of these protein complexes containing BRCA1 helps to illustrate signaling pathways that recruit BRCA1 in response to DNA damage.

BRCA1-associated C-terminal helicase 1 (*BACH1)*

A screen for proteins that directly bind the BRCA1 BRCT domain identified BACH1 (Cantor et al. 2001). Indeed, the BRCA1 BRCT binds with high affinity to pSer-Pro-Thr-Phe motif in BACH1 (Manke et al. 2003, Yu et al. 2003) and this interaction is essential for the activation of the G2/M cell cycle checkpoint in DDR (Yu et al. 2003). BRCA1 variants that either lack a BRCT domain or contain cancer-associated mutations (P1749R and M1775R) fail to interact with BACH1, highlighting the importance of an intact BRCT domain structure for its function (Cantor et al. 2001).

CtIP (CtBP-interacting protein)

A two-hybrid screen to find BRCA1 BRCT domain interacting partners identified the transcriptional suppressor CtBP binding partner, CtIP (Yu et al. 1998). The BRCA1-binding region of CtIP, a Ser-Pro-Val-Phe motif (aa 327-330), resembles the phosphorylation motif on BACH1 and the residue Ser327 becomes phosphorylated during G2 (Yu & Chen 2004). The BRCA1/CtIP complex exists only during G2 and is essential for the G2/M transition checkpoint and DNA damage-induced Chk1 kinase activation (Yu & Chen 2004). Moreover, the cancer-associated BRCA1 variants (A1708E and P1749R) and the nonsense mutation that eliminates the C-terminal 11 amino acids of BRCA1 (Y1853delta) abrogate the *in vivo* interaction between BRCA1 and CtIP (Yu et al. 1998). It should be noted that prolonged G2 accumulation after DNA damage requires the BRCA1/BACH1 complex, but not the BRCA1/CtIP complex (Yu & Chen 2004).

CtIP also promotes ataxia telangiectasia mutated and Rad3-related (ATR) kinase activation and HR (Sartori et al. 2007). CtIP promotes endo- but not exo-nucleolytic activities along with the MRN complex to create ssDNA and to mediate DSB resection. Strikingly, CtIP shows sequence homology with *S. cerevisiae* Sae2 (Sartori et al. 2007). Sae2 generally cooperates with the yeast MRN complex to promote DSB resection (McKee & Kleckner 1997, Prinz et al. 1997, Rattray et al. 2001, Lobachev et al. 2002, Clerici et al. 2005, Deng et al. 2005, Lisby et al. 2004). In human cells, the DSB resection is regulated by cyclin-dependent kinase

(CDK)-mediated phosphorylation of CtIP on Thr-847 in a manner that is similar to CDK phosphorylation of a related consensus site on Sae2 (Huertas et al. 2009). These findings establish evolutionarily conserved roles for CtIP-like proteins in HR.

Abraxas/CCDC98 and receptor-associated protein 80 (RAP80)

Abraxas/CCDC98 directly binds to the BRCA1 BRCT domain through a pSer-Pro-Thr-Phe motif and this binding is mutually exclusive with BACH1 or CtIP interaction with BRCA1 (Kim, Huang & Chen 2007, Liu, Wu & Yu 2007, Wang et al. 2007). Subsequent studies found another ubiquitin-binding protein, RAP80, to associate with the Abraxas/CCDC98-BRCA1 complex, with Abraxas/CCDC98 bridging the interaction between RAP80 and BRCA1 (Kim, Chen & Yu 2007, Kim, Huang & Chen 2007, Liu, Wu & Yu 2007, Sobhian et al. 2007, Wang et al. 2007). Abraxas/CCDC98 mediates the BRCA1-dependent G2/M checkpoint activation and the formation of BRCA1 foci in response to DNA damage. Both Abraxas/CCDC98 and RAP80 are required for DNA repair and G2/M checkpoint control.

Structure of the complex formed between the BRCA1-BRCT domain and its phosphopeptide ligand

The structure of the BACH1 phosphopeptide-bound BRCA1 BRCT domain is essentially identical to that of unliganded domain (r.m.s. deviation ~0.4Å for all Cα atoms) (Figure 1.7) (Clapperton et al. 2004,

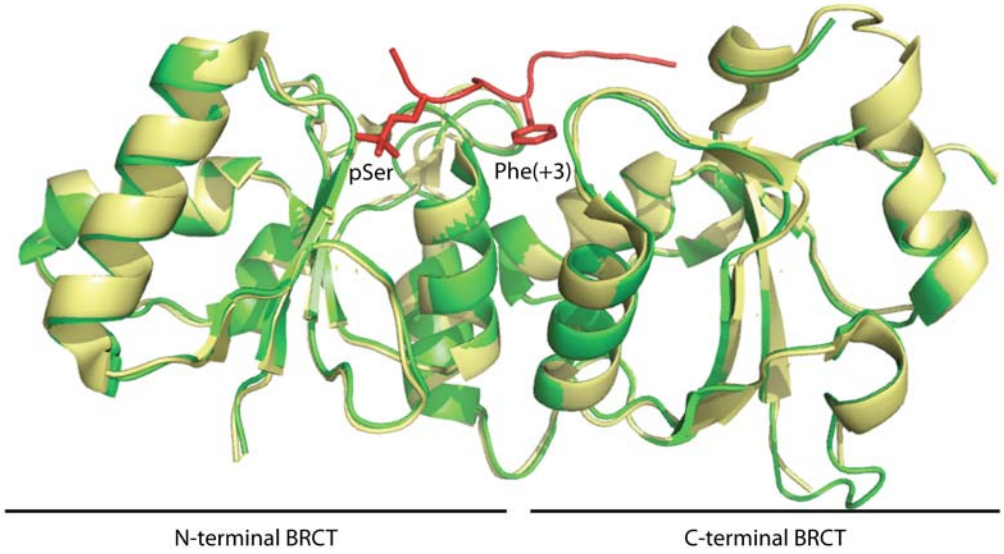

Figure 1.7. The phosphopeptide bound BRCA1 BRCT domain aligned with the non-liganded BRCA1 BRCT domain. The X-ray crystal structure of the BRCA1 BRCT repeat is in green (pdbID-1JNX, Williams et al. 2001). The structure of the peptide(in red)-bound BRCA1 BRCT repeat in light yellow (pdbID-1T2V, Williams et al. 2004).

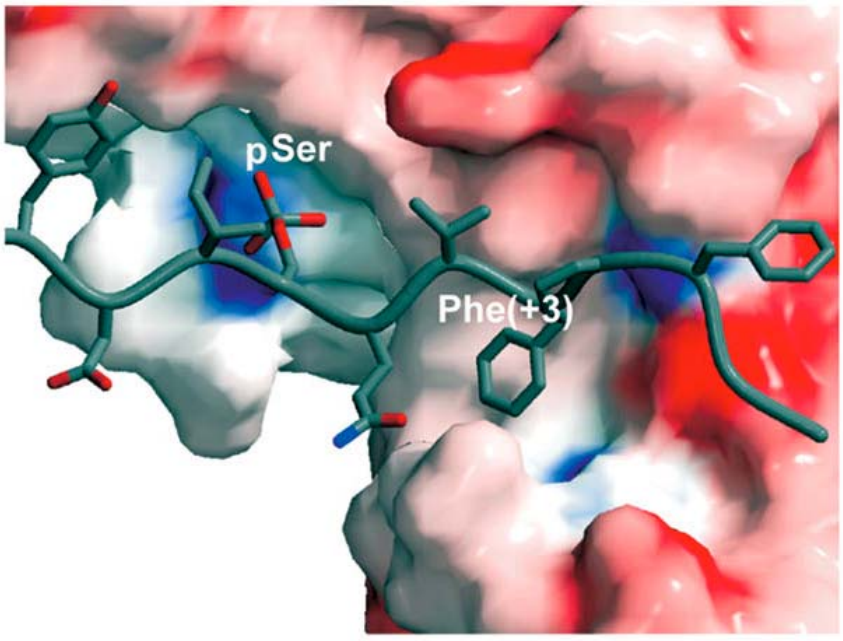

Figure 1.8. Details of the BRCA1-BRCT phosphopeptide interaction. Electrostatic surface representation of the BRCT domain with the pSer- and Phe-binding pockets is shown. The phosphopeptide is shown in gray-green. The pSer moiety fits into a shallow, basic pocket whereas the Phe(+3) fits into a deeper, hydrophobic pocket located at the BRCT interface. (Williams et al. 2004).

Shiozaki et al. 2004, Williams et al. 2004). The peptide binds in an extended conformation to a groove that sits across the two BRCT repeats. The groove is on the opposite side of the BRCT-BRCT interface that was involved in the interaction between p53 and 53BP1, which mainly occurs through the link region between the BRCT repeats (Clapperton et al. 2004). Furthermore, the phosphoserine fits into a shallow, basic pocket through interactions involving residues from the N-terminal BRCT repeat, whereas the Phe(+3) side chain is recognized by a deeper, hydrophobic pocket at the BRCT interface between the two BRCT repeats (Figure 1.8) (Clapperton et al. 2004, Shiozaki et al. 2004, Williams et al. 2004). Together, the structure explains why both BRCT repeats are required for efficient phosphopeptide binding (Manke et al. 2003, Rodriguez et al. 2003). The BRCA1 BRCT domain binds to the BACH1 pSer phosphate moiety by making three direct hydrogen bonds involving the hydroxyl group of Ser1655, the main chain NH of Gly1656 and the positive side chain N of Lys1702 (Figure 1.9) (Clapperton et al. 2004, Shiozaki et al. 2004, Williams et al. 2004). The structural comparison between the liganded and unliganded BRCT domains shows that the hydrogen bond donors lining the pSer-binding pocket are prealigned for recognition of the phosphate (Figure 1.9) (Williams et al. 2004). Moreover, this mode of binding where the phosphate oxygens are arranged by main chain NH, serine OH and positively charged side chain is comparable the way other protein modules, such as the fork-head associated (FHA) and 14-3-3

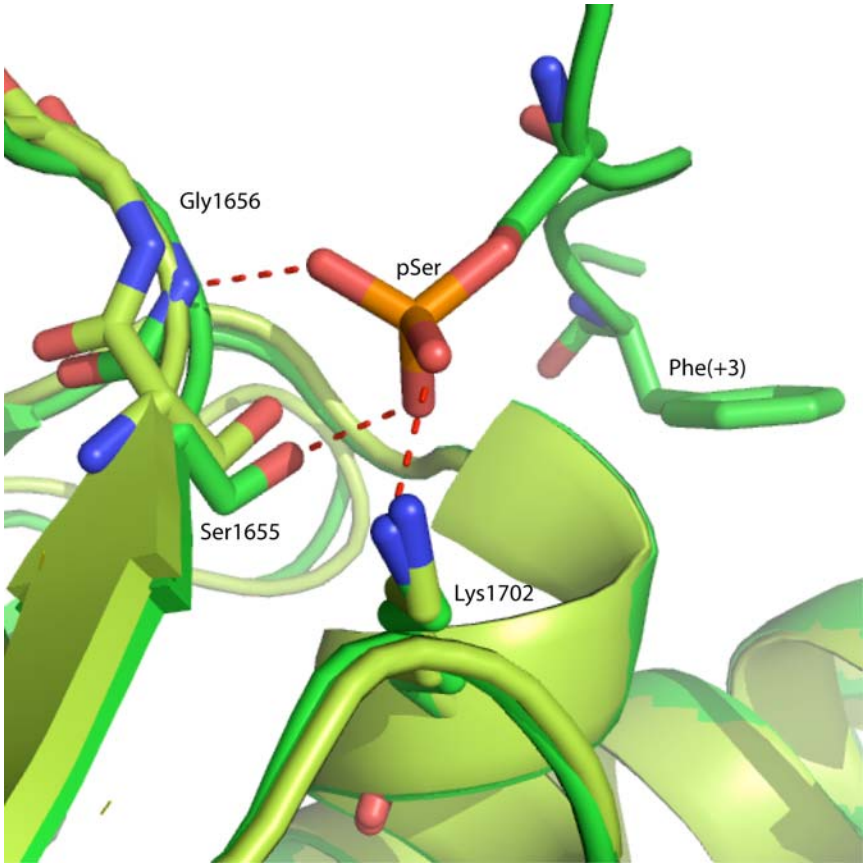

Figure 1.9. Contacts between the BRCA1 BRCT domain and its phosphopeptide ligand. The BRCA1 BRCT domain binds to the BACH1 pSer phosphate moiety by making three direct hydrogen bonds involving Ser1655, Gly1656 and Lys1702 (pdbID-1T15, Clapperton et al. 2004). The hydrogen bond donors lining the pSer-binding pocket are prealigned for recognition of the phosphate (The unliganded structure is in light green, pdbID-1JNX, Williams et al. 2001).

families, bind their phosphorylated residues (Yaffe, Smerdon 2001). All of the three residues, Ser1655, Gly1656 and Lys1702, are absolutely conserved in BRCA1 homologs (Clapperton et al. 2004), emphasizing the importance of these residues in regards to the protein function. The high resolution structure at 1.85 Å also revealed that the phosphate and some peptide main chain atoms are tethered through networks of water molecules (Figure 1.10) (Clapperton et al. 2004). This indirect protein-solvent-peptide interaction is an uncommon phosphorylation dependent protein-protein interaction, although it does occur in structures of phosphopeptide bound human Plk1 Polo-box domain (Cheng et al. 2003, Elia et al. 2003).

The hydrophobic Phe(+3) binding pocket is lined with residues from the N- and C-terminal BRCT repeats (Figure 1.11). The side chains of Phe1704, Met1775 and Leu1839 from both repeats comprise the bottom of the pocket (Clapperton et al. 2004, Williams et al. 2004), supporting the strong selection for aromatic amino acids in the +3 position from the pSer (Manke et al. 2003, Rodriguez et al. 2003). The main chain NH of the Phe(+3) forms a hydrogen bond with the main chain carbonyl of Arg1699 and the main chain carbonyl of the Phe(+3) interacts with the guanidinium group of Arg1699 (Williams et al. 2004). This explains the observation that the BRCA1 R1699W mutation eliminates BRCA1-phospholigand binding (Williams et al. 2004) and explains the mutant's association to cancer.

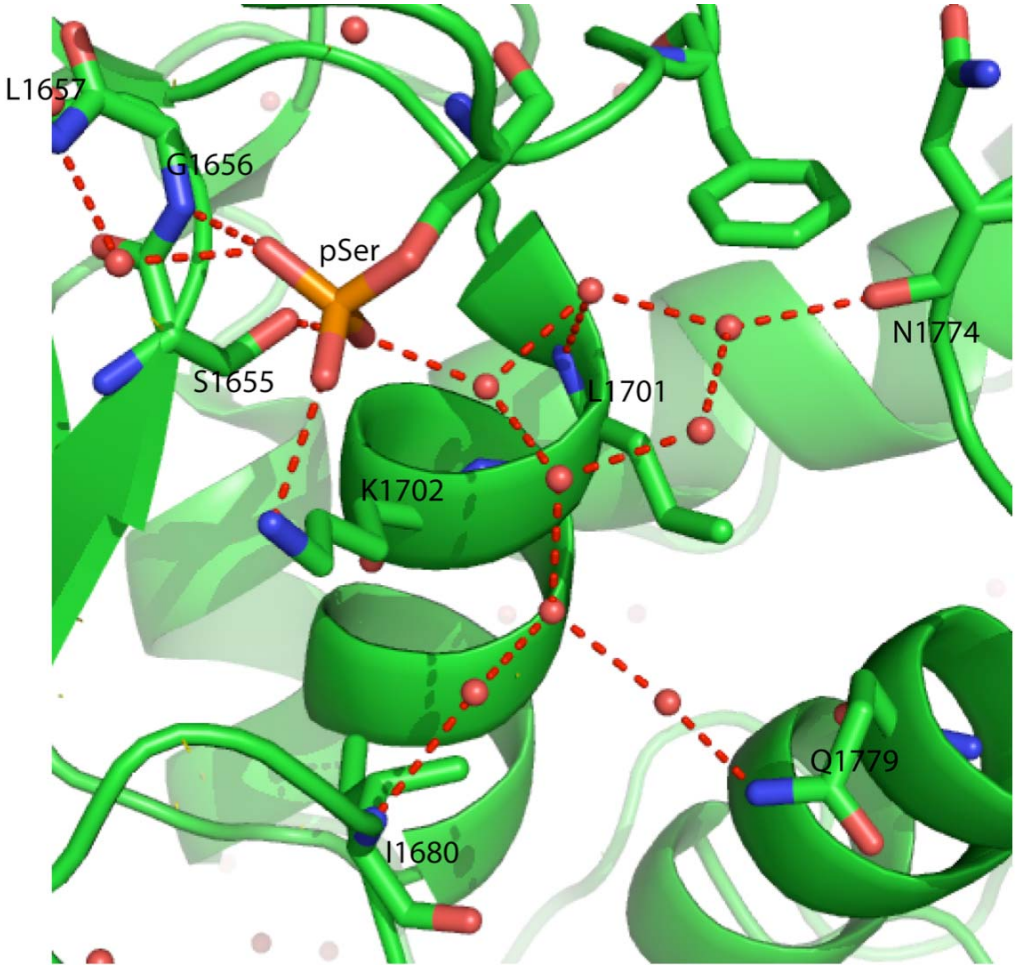

Figure 1.10. The phosphate moiety of the pSer and some peptide main chain atoms are connected via networks of water molecules. Key residues that recognize the pSer of the phosphopeptide are shown. *Red dashed lines* - hydrogen bonds; *red circles* – water molecules. (pdbID-1T15, Clapperton et al. 2004).

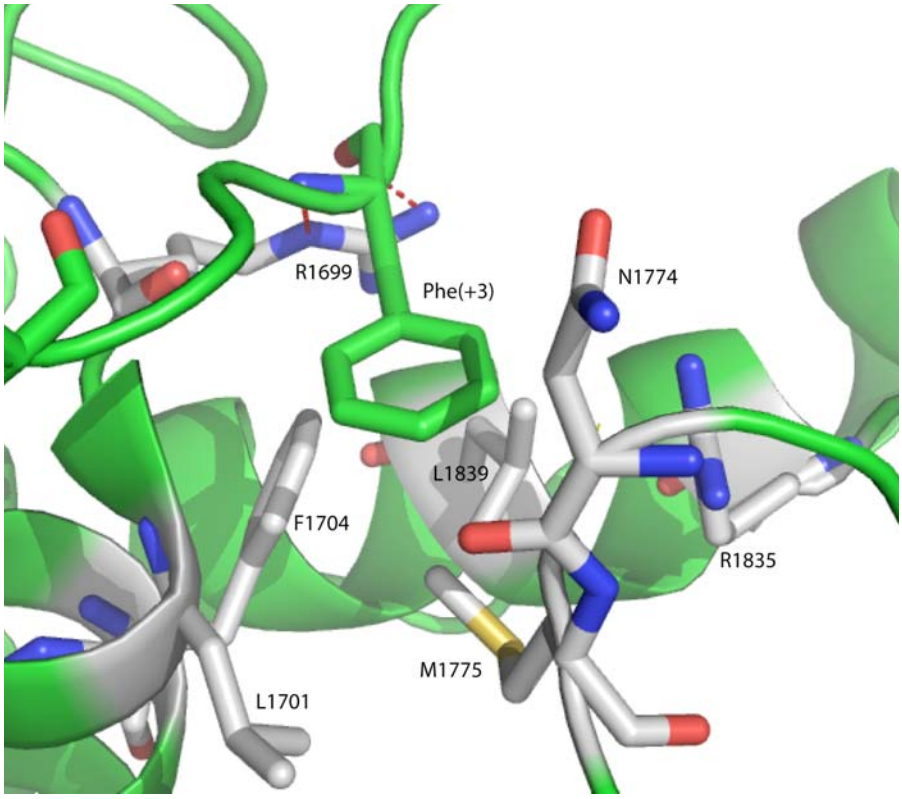

Figure 1.11. Structure of the Phe(+3) binding pocket. Key residues that recognize the Phe(+3) residue of the phosphopeptide are shown. The residues Leu1701, Phe1704, Met1775 and Leu1839 line the bottom of the pocket. The sides of the pocket are composed of Arg1699, Asn1774 and Arg1835. (pdbID-1T15, Clapperton et al. 2004).

Examples of BRCT proteins and their roles in the DDR

The tandem BRCT domains were originally identified as having weak sequence similarity to the C-termini of the human p53 binding protein, 53BP1 and *S. cerevisiae* RAD9 (Koonin, Altschul & Bork 1996, Bork et al. 1997, Callebaut & Mornon 1997). Subsequent studies refined a conserved hydrophobic clustering signature for these domains and more than 50 unique proteins, many of which had been connected to cell cycle control and DNA repair pathways, were recognized to contain the BRCT domain (Bork et al. 1997, Callebaut & Mornon 1997). Examples include DNA damage response or repair and cell cycle checkpoint proteins (BRCA1, MDC1, XRCC1, 53BP1), the Pax-transcription-activation domain-interacting protein PTIP and BARD1 (Bork et al. 1997, Callebaut & Mornon 1997, Glover, Williams & Lee 2004).

Mediator of DNA checkpoint 1 (MDC1)

Many focused on finding the protein that would recognize an epigenetic signal created by phosphorylation of the H2AX C-terminal tail. It has been established that phospho-specific interactions are indispensible in the DDR and several DDR proteins contain domains that interact with other proteins in a phosphorylation-dependent manner. Two notable domains are FHA domains and BRCT domains. Whereas tandem BRCT domains have the capacity to interact with proteins containing phosphorylated serine residues in a sequence specific context, FHA

domains can interact with proteins containing phosphorylated threonine (Durocher et al. 2000). Many DDR proteins that harbor FHA and/or BRCT domains accumulate in IRIF in an H2AX-dependent manner, such as BRCA1, 53BP1, MDC1, NBS1 and TopBP1 (Scully et al. 1997a, Carney et al. 1998, Schultz et al. 2000, Yamane, Wu & Chen 2002, Goldberg et al. 2003, Lou et al. 2003, Stewart et al. 2003).

Studies indicated that phosphorylation of the H2AX C-terminal tail is predominantly recognized by MDC1. MDC1 contains a FHA and two BRCT domains and forms foci that colocalize along with the phosphorylated H2AX (γH2AX) within minutes after exposure to IR (Goldberg et al. 2003, Stewart et al. 2003). Furthermore, cells lacking MDC1 were unable to activate the intra-S phase and G2/M phase cell cycle checkpoint properly following exposure to IR (Goldberg et al. 2003, Stewart et al. 2003). The oriented phosphopeptide library screen with the purified the MDC1 tandem BRCT repeats showed that MDC1 selectively recognized phosphorylated peptides (Rodriguez et al. 2003). In addition, the MDC1 BRCT domain preferred glutamic acid at the +2 position and tyrosine and phenylalanine at the +3 position, after the phosphoserine. The fact that the amino acid selection closely matched the sequence of the phospho-epitope within the H2AX C-terminal tail led Glover et al. to propose a MDC1 BRCT-γH2AX model (Figure 1.12) (Glover, Williams & Lee 2004). The model suggested that, in contrast to the BRCA1-BACH1 complexes where the conserved Arg1699 interacts with the main chain

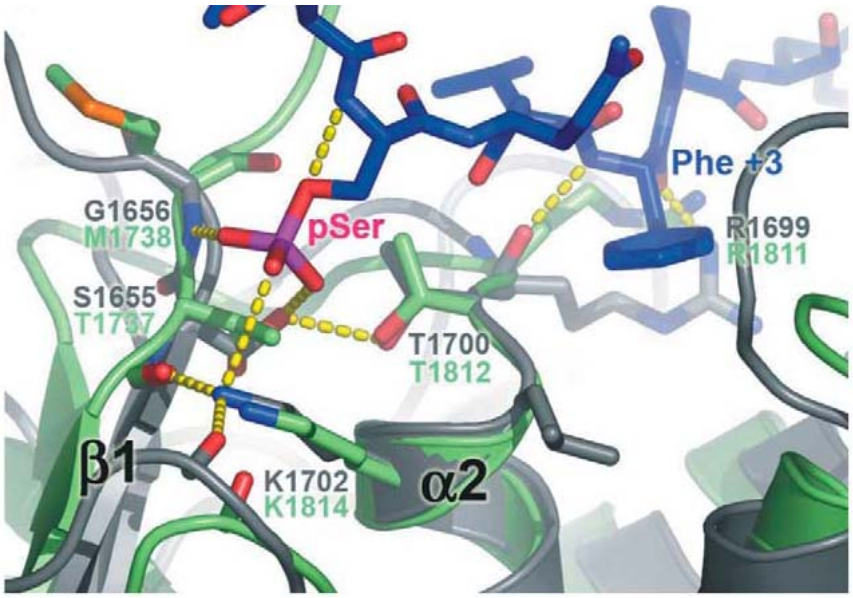

Figure 1.12. Phospho-peptide recognition by the BRCT domains. The phosphopeptide is shown in blue, the BRCA1-BRCT is in grey and the 53BP1-BRCT in green. Hydrogen bonding and salt bridges are indicated by yellow dashes. The tyrosine carboxylate of γH2AX could form a salt bridge with the guanidinium group of an arginine residue (Arg1699 in BRCA1 and Arg1933 in MDC1) (Glover et al. 2004).

carbonyl of Phe(+3) of the BACH1 peptide via an uncharged hydrogen bond, the guanidinium group of Arg1933 in MDC1 could form a strong, salt bridge with the tyrosine carboxylate of the γH2AX peptide.

PTIP

The transcriptional regulatory protein PTIP is required for the survival of cells exposed to IR, most likely due to its role in regulating genome stability and mitosis (Cho, Prindle & Dressler 2003, Jowsey, Doherty & Rouse 2004, Munoz et al. 2007). A pair of tandem BRCT repeats of PTIP appears to have a specific and high-affinity binding activity for peptides containing a pS/T-Q-V-F sequence (Manke et al. 2003). After DNA damage, the BRCT repeats of PTIP bind ATM-phosphorylated Ser25 on 53BP1 and this interaction is responsible for PTIP localization to nuclear foci that contain 53BP1 and γH2AX (Munoz et al. 2007). Interestingly, the tandem BRCT repeats on PTIP are also capable of interacting with ATM-phosphorylated epitopes other than the pS/T-Q-V-F, suggesting the versatility of the BRCT domains in binding different phosphorylated targets.

Topoisomerase (DNA) II binding protein 1 (TopBP1)

TopBP1 contains eight BRCT repeats and shares sequence similiarity with the budding yeast DPB11 protein and the fission yeast Rad4/Cut5 protein, both of which are essential for DNA damage and/or replication checkpoint controls (Yamane, Wu & Chen 2002). Previous

work in *Xenopus* has indicated that TopBP1 is required for the initiation of DNA replication as well as the activation of the ATR kinase at stalled replication forks (Van Hatten et al. 2002, Hashimoto & Takisawa 2003, Kumagai et al. 2006). Located between the BRCT 6 and 7 motifs, the ATR activation domain of TopBP1 interacts with DNA-bound ATR and ATR-interacting protein (ATRIP) to stimulate ATR kinase activity (Kumagai et al. 2006, Mordes et al. 2008). Recently, TopBP1 has been shown to act as a critical sensor of replication stress by directly recruiting DNA polymerase α and thereby the Rad9-Rad1-Hus1 (9-1-1) complex to stalled DNA replication forks (Yan Michael 2009). The 9-1-1 complex is also required for ATR activation. Furthermore, through its sixth BRCT domain, TopBP1 interacts with and represses a transcription factor, E2F1, not only after DNA damage, but also during G1/S transition to inhibit E2F1-dependent apoptosis (Liu et al. 2003, Liu et al. 2004). Phosphorylation by Akt induces oligomerization of TopBP1 through its seventh and eighth BRCT domains and this oligomerization is cruicial for TopBP1 to interact with and repress E2F1 (Liu et al. 2006).

BRCA1-associated RING domain protein 1 (BARD1)

BARD1 was discovered as a protein interacting with BRCA1 through their RING domains and adjacent helical domains to form a heterodimeric E3 ubiquitin ligase (Wu et al. 1996, Brzovic et al. 2001, Brzovic et al. 2003). Its expression is correlated to that of BRCA1, and together they control the cell cycle in response to DNA damage (Irminger-

Finger & Leung 2002, Scully, Xie & Nagaraju 2004). Accumulating evidence, such as tumour-derived BARD1 mutations, suggests that BARD1 may be a tumour suppressor protein. However, its role in carcinogenesis and cancer progression remains unclear. BARD1 has been detected mostly in the nucleus, but is also found in the cytoplasm due to a nuclear export signal (Rodriguez et al. 2004). The distribution of BRCA1 is similar to that of BARD1 (Henderson 2005). When BARD1 and BRCA1 form a heterodimer, they most likely mask each other's nuclear export signals thus leading to the nuclear entrapment of the BRCA1/BARD complex. This entrapment is important for cell survival, as well as its presumed role in DNA repair (Baer & Ludwig 2002). BARD1 is structurally homologous to BRCA1 as it contains the conserved RING finger and BRCT domains (Wu et al. 1996). In addition, BARD1 harbors multiple tandem ankyrin (ANK) repeats of unknown function in the central region of the protein.

Functions of the BARD1 BRCT repeats

The surface cleft in the BRCA1 BRCT repeats is highly conserved in BARD1 (Wu et al. 1996). Research shows that the tandem BARD1 BRCT repeats preferentially bind phosphoserine over its nonphosphorylated counterpart (Rodriguez et al. 2003, Yu, Chen 2004). In support of this finding, the residues that coordinate binding to phosphoserine in the phosphopeptide binding pocket are highly conserved in the BARD1 BRCT repeats (Figure 1.13). In contrast, the BRCA1 BRCT

residues that recognize the Phe(+3) are quite different in the BARD1 BRCT repeats (Figure 1.13). This evidence implicates the BARD1 BRCT domain in an interaction with phosphopeptides, while preferring a different residue at the +3 position from the phosphoserine than BRCA1 (Shiozaki et al. 2004). The oriented peptide library analyses demonstrate that the tandem BARD1 BRCT repeats prefer Asp/Glu residues at the +1 and +2 positions from the pSer residue (Rodriguez et al. 2003). Moreover, the protein specifically prefers a Glu residue at the +3 position (Rodriguez et al. 2003). Thus, the binding motif for the BARD1 BRCT domain is predicted to be pS-[D/E]-[D/E]-E.

Transcription and DNA repair

Upon DNA damage, the DNA damage-induced inhibition of transcription is activated and mRNA levels decrease in cells (Hanawalt 1994). The poly(A) tail is found on almost all eukaryotic mRNAs and the tail plays critical roles in regulation of mRNA stability, translation and RNA transport from the nucleus (Neugebauer 2002, Mangus, Evans & Jacobson 2003, Anderson 2005). Following the polyadenylation reaction, a large number of protein factors, including the cleavage stimulation factor (CstF), are required for 3' processing. One of the subunits of CstF, CstF-50, interacts with the RNAP II C-terminal domain, most likely to assist the RNAP II-mediated activation of 3' processing (McCracken et al. 1997, Hirose & Manley 1998). In nuclei, the BRCA1/BARD1 heterodimer is a component of the core transcriptional machinery, as it copurifies with

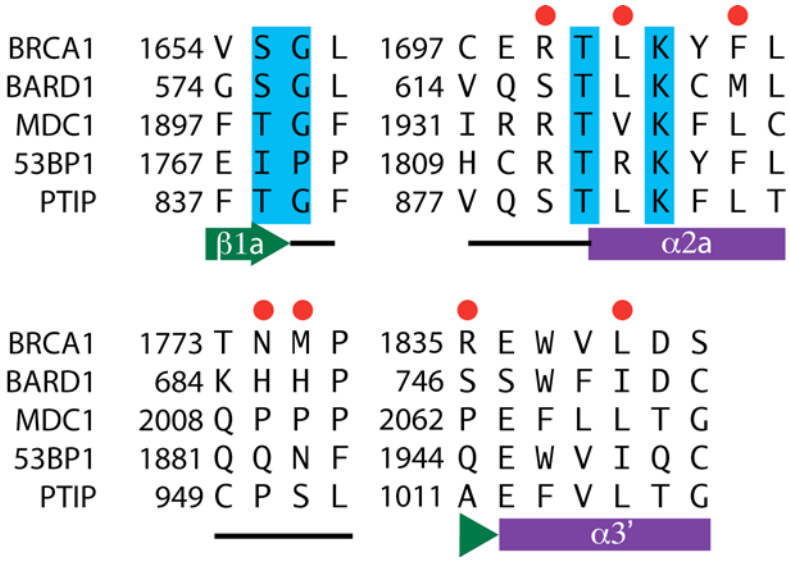

Figure 1.13. The sequence alignment of the BRCT domains of BRCA1, BARD1, MDC1, 53BP1 and PTIP. The secondary structural elements of the BRCA1-BRCT are indicated below the sequence alignment. BRCA1 residues that coordinate pSer990 of BACH1 (shaded in blue) are conserved in BARD1, MDC1, 53BP1 and PTIP. In contrast, the BRCA1 residues that interact with Phe993 of BACH1 (marked by red circles) are not well conserved.

the RNAP II holoenzyme complex (Anderson et al. 1998, Chiba & Parvin 2002). Studies show that the BARD1 C-terminus is involved in mRNA 3' processing in response to DNA damage, through a direct interaction with CstF-50 (Kleiman & Manley 1999, Kleiman & Manley 2001, Kleiman et al. 2005, Mirkin et al. 2008). Further studies show that the BARD1-CstF interaction as well as proteosome-mediated degradation of RNAP II results in inhibition of 3' processing upon DNA damage (Figure 1.14) (Kleiman et al. 2005). In this case, CstF-50 may play a role as a cofactor for ubiquitination of RNAP II by the BRCA1-BARD1 heterodimer (Kleiman et al. 2005, Starita et al. 2005). This ubiquitination process would also facilitate DDR by allowing access to the repair machinery, while concurrently preventing polyadenylation of aborted nascent mRNAs (Mirkin et al. 2008). In this scenario, a loss of CstF-50 would enhance cell death. These findings highlight a functional interplay between BARD1, CstF and RNAP II following DNA damage.

Research overview:

Great progress has been made in understanding cellular response to DSBs. Research has revealed the mechanism by which the BRCA1 BRCT repeats mediate their function, however, the precise molecular mechanisms of the BRCT repeats of other DDR proteins remain largely unknown. Several key questions are unanswered: 1. How do missense

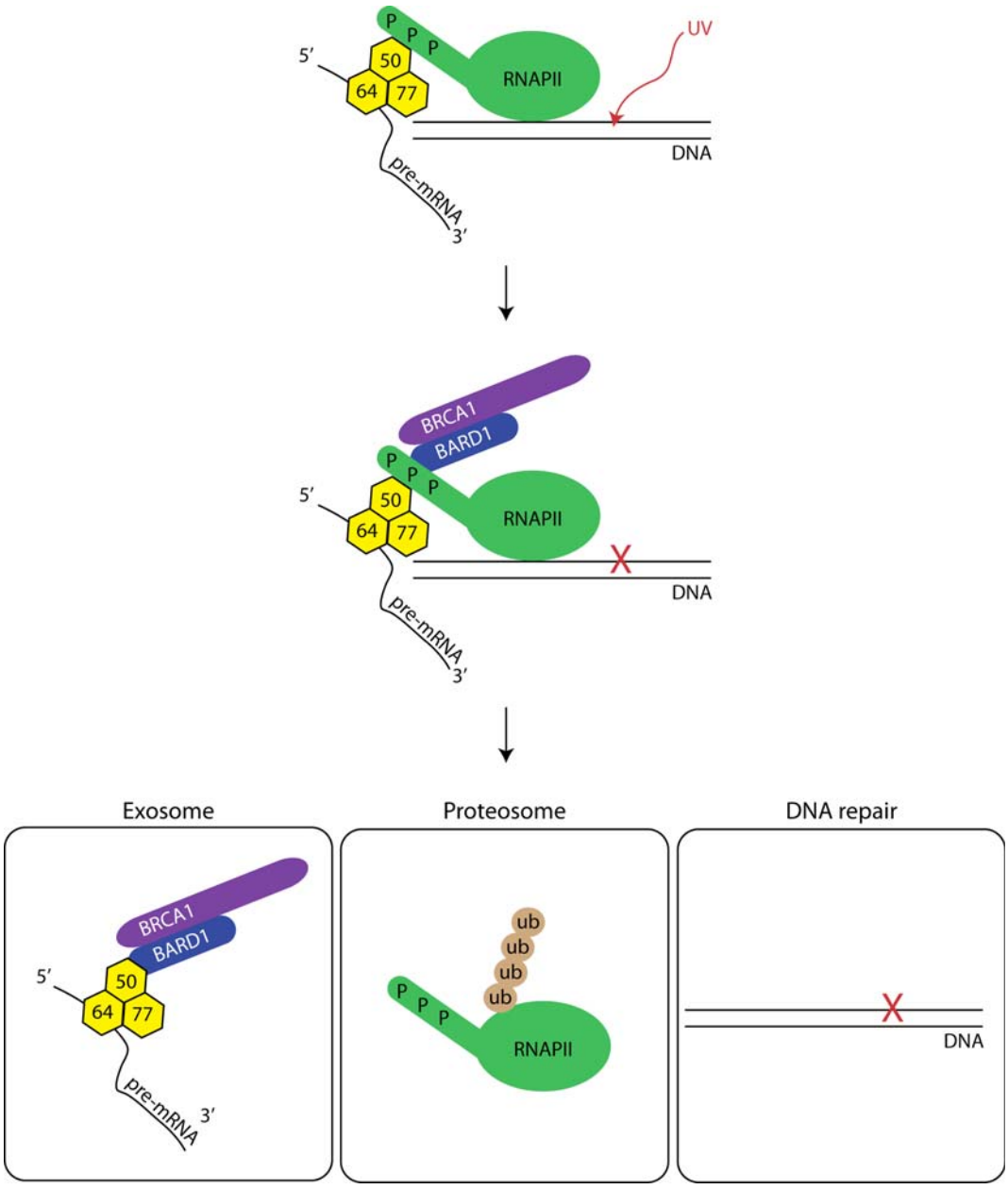

Figure 1.14. Model of BARD1 and CstF in DNA damage response. Upon DNA damage, the elongating RNAPII-CstF holoenzyme stalls at the damage site (*yellow hexagons* - CstF subunits). The BRCA1/BARD1 heterodimer is then recruited to the sites of damage where it inhibits RNAPII and the associated polyadenylation machinery (possibly by ubiquitination of the RNAP IIO). The RNAP IIO is degraded thereby allowing access to the repair machinery. Aborted nascent mRNAs are eliminated by exosome-mediated degradation. Adapted from Mirkin et al. 2008.

mutations affect the BRCA1 BRCT repeats? 2. How does the BRCA1/BARD1 heterodimer play a role in a complex response to DNA damage in mammalian cells? 3. How does the H2AX phosphorylation create a major signal for the recruitment of DDR proteins to DSBs?

In ***Chapter 2***, I demonstrate that mammalian MDC1 directly binds to γH2AX by specifically interacting with the phosphoepitope at the γH2AX carboxyl terminus. Further, through X-ray crystallographic approaches, I propose the molecular details of the MDC1-γH2AX complex. The data constitutes compelling evidence that the MDC1 BRCT domain is the major amplifier of γH2AX signal following DNA damage.

Chapter 3 establishes a structural platform to understand the mechanism by which the BRCA1/BARD1 heterodimer aids in DDR. Through a combination of biochemical and X-ray crystallographic approaches, the work reveals that BARD1 is responsible for CstF-50 binding despite a degenerate phosphopeptide binding pocket in the BARD1 BRCT domain. Protein pull-down experiments indicate that BARD1 interacts with the CstF-50 WD-40 domain via the ANK-BRCT linker.

Chapter 4 outlines the structural and functional dissection of missense mutations in the BRCA1 BRCT domain. This work characterizes 117 patient-derived missense mutations and we envision that the results

will assist clinicians in assessing the cancer-risk associated with the BRCT missense mutations.

In ***Chapter 5***, I discuss the results of my work in the context of recent studies describing a hierarchical model of the DDR proteins after IR exposure.

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Chapter 2:

Structure of the BRCT repeat domain of MDC1 and its specificity for the free C-terminal end of the γH2AX histone tail

Summary

MDC1 (mediator of DNA damage checkpoint protein 1) regulates the recognition and repair of DNA double strand breaks in mammalian cells through its interactions with nuclear foci containing the C-terminally phosphorylated form of the histone variant, H2AX. Here we demonstrate that the tandem BRCT repeats of MDC1 directly bind to the phosphorylated tail of H2AX – pSer-Gln-Glu-Tyr - in a manner that is critically dependent on the free carboxylate group of the C-terminal Tyr residue. We have determined the X-ray crystal structure of the MDC1 BRCT repeats at 1.45 Å resolution. By a comparison with the structure of the BRCA1 BRCT bound to a phosphopeptide, we suggest that two arginine residues in MDC1, Arg^{1932} and Arg^{1933} may recognize the C-terminus of the peptide as well as the penultimate Glu of H2AX, while Gln^{2013} may provide additional specificity for the C-terminal Tyr.

Introduction

Tandem BRCT repeats, initially discovered at the C-terminus of the breast cancer-associated protein, BRCA1, are phospho-protein recognition modules that play key signaling roles in the cellular response to DNA damage (Glover, Williams & Lee 2004). Individual repeats are approximately 90-100 amino acids in size. While they can fold independently, they often exist in tandem pairs where they pack in a head-tail manner (Glover, Williams & Lee 2004, Joo et al. 2002, Derbyshire et al. 2002). The BRCT repeats of BRCA1 have been shown to specifically bind to pSer-x-x-Phe peptide targets, such as the BACH1 helicase (Yu et al. 2003, Manke et al. 2003), and the transcriptional co-repressor CtIP (Yu & Chen 2004). Structural studies reveal that the N-terminal BRCT repeat is responsible for phospho-serine recognition, while the phenylalanine side chain is recognized by a pocket at the interface between the N- and C-terminal repeats (Williams et al. 2004, Clapperton et al. 2004, Shiozaki et al. 2004). Mutations that perturb the phenylalanine recognition pocket disrupt phosphopeptide binding and explain the enhanced cancer risks associated with some of these mutations.

MDC1 (mediator of DNA damage checkpoint protein 1) is another BRCT repeat protein that plays a critical role in the DNA damage response. MDC1 has been implicated in the recognition and repair of DNA double strand breaks through its rapid co-localization with γH2AX, the C-terminally phosphorylated form of the histone variant H2AX, at the sites of

double strand breaks in mammalian nuclei (Stewart et al. 2003, Goldberg et al. 2003). MDC1 also facilitates the recruitment of other repair proteins to these foci, including the Mre11 complex, and the BRCT proteins 53BP1 and BRCA1, and is required for the efficient repair of ionizing radiation-induced DNA damage. The C-terminal BRCT repeats of MDC1 can specifically bind to phosphopeptides with specificity for a tyrosine residue at the +3 position relative to the phosphoserine, and some specificity for a glutamic acid at the +2, matching the sequence of the γH2AX tail: pSer-Gln-Glu-Tyr (Rodriguez et al. 2003). Here we demonstrate that the MDC1 BRCT repeats bind to the γH2AX tail in a manner that is critically dependent on the free carboxylate group of the C-terminal tyrosine residue. The crystal structure of the MDC1 BRCT repeats reveals a phospho-serine binding pocket and adjacent structural features that may explain the novel selectivity of this domain for the free C-terminus of its phospho-peptide target. The majority of this chapter was originally published in the *Journal of Biological Chemistry* (Lee et al. 2005).

Experimental procedures

BRCT expression and purification

Four different constructs of human MDC1 BRCT domain, MDC1(1,888-2,086), MDC1(1,888-2,089), MDC1(1,891-2,086) and MDC1(1,891-2,089), were used in the initial expression experiments. The constructs were expressed and purified as GST-fusion protein by glutathione-affinity chromatography. The MDC1 polypeptides were then cleaved from GST using PreScission protease (Amersham-Pharmacia).

Native MDC1 BRCT domains, MDC1(1,888-2,086) and MDC1(1,891-2,086), were expressed in *E. coli* strain BL21 Gold99 and purified as GST-fusion proteins by glutathione-affinity chromatography. MDC1 was then cleaved from GST using PreScission protease (Amersham-Pharmacia), and the C-terminal MDC1 polypeptide was purified from GST by ion exchange chromatography.

Crystallization and structure determination of MDC1 BRCT

MDC1 BRCT crystals were grown by vapor diffusion in hanging drops at room temperature. Native MDC1 (10 mg ml^{-1}) in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM DTT was mixed with an equal volume of the reservoir solution (22% PEG 8000, 0.1 M HEPES, pH 8.0). Crystals of Se-methionine-substituted MDC1 were grown by mixing 2 µl of 10 mg ml^{-1} BRCT domain with a 1.5-fold molar excess of a γH2AX peptide in protein solution (150 mM NaCl, 1 mM DTT and 10 mM Tris-HCl, pH 7.5) with 2 µl

of well solution (1.4 M NaH_2PO_4, 0.4M K_2HPO_4, 0.1 M citrate, pH 4.2). No γH2AX peptide was found to be bound to MDC1 in these crystals.

For cryopreservation, single crystals were soaked in the appropriate well solution supplemented with 26% (v/v) glycerol and then flash-frozen in liquid nitrogen. All data were collected at beamline 8.3.1 of the Advanced Light Source, Lawerence Berkeley National Laboratory. Data reduction and scaling were done using the HKL package (Otwinowski, Minor 1997).

Crystallographic phases for MDC1 were determined from a multiple wavelength anomalous dispersion (MAD) experiment on the Se-methionine-substituted protein derivative to 2.7 Å resolution. Multiple MDC1 molecules were assumed to be present in the asymmetric unit, and eight Se sites were located with SOLVE (Terwilliger 2004) using data collected at two wavelengths. Electron density maps calculated from this solution were improved by density modification and NCS averaging in RESOLVE (FOM 0.67) and revealed three molecules in the asymmetric unit. Automatic (RESOLVE) and manual model building (using XFIT (McRee 1999)) produced a complete MDC1 model, which was partially refined using CNS (Brunger et al. 1998) and REFMAC (Murshudov, Vagin & Dodson 1997). This model was then used as the search model for molecular replacement using MOLREP (Navaza 2001) against the native 1.45 Å data. Two molecules were placed in the native asymmetric unit and these were re-built using ARP/warp (Morris, Perrakis & Lamzin 2003). Cycles of minimization and individual isotropic B-factor refinement and

manual model building produced the completed model having 390 residues and 256 waters with an R_{free} of 0.195 and an R_{factor} of 0.212 (Table 2.1). Atomic coordinates and structure factors have been submitted to Protein Database (RCSB accession code 2ADO).

Isothermal titration calorimetry

Peptides used for microcalorimetry contained the C-terminal sequence of H2AX, with an N-terminal "KKK" sequence to improve peptide solubility (acetyl-KKK**TQApSQEY***, γH2AX sequence in bold*) and were prepared with or without an amide group blocking the C-terminus (Alberta Peptide Institute). MDC1 and the phosphopeptides were prepared in 10 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl and 2 mM TCEP. The Micro Calorimetry System (Microcal, Amherst, MA) was used to perform the ITC measurements for the interaction between the BRCT repeats and the peptides. The titration data, collected at 22 ^{o}C, were analyzed using the ORIGIN data analysis software (Microcal Software, Northampton, MA). Thermodynamic parameters reported are the average of three independent experiments.

Results and Discussion

MDC1 recognition of the γH2AX C-terminus

Intriguingly, not only the sequence of the H2AX tail, but also its position at the C-terminus of the chain, is absolutely conserved in mammals (Pilch et al. 2003). To test if the free carboxyl terminus of the tail is an important determinant of recognition, we used isothermal titration calorimetry to determine the thermodynamics of binding between recombinant MDC1 tandem BRCT repeats, and synthetic phosphopeptides corresponding to the γH2AX tail, with or without an amide group blocking the carboxyl terminus (Figure 2.1). The results show that MDC1 binds to the γH2AX tail with a free C-terminus with high affinity (K_d = 2.2 +/- 0.2 μM), similar to binding affinity of the BRCA1 BRCT for pSer-x-x-Phe targets (Yu et al. 2003, Shiozaki et al. 2004). The binding reaction is driven by favorable enthalpic (ΔH = -6.3 kcal/mol) and entropic (ΔS = 4.8 cal/mol/deg) contributions. In contrast, the same peptide with an amidated, and therefore uncharged, C-terminus showed approximately 100-fold weaker binding for MDC1 (K_d ~ 150 μM). This reduction in binding affinity could be due to the loss of charge on the C-terminal residue, and/or steric repulsion between MDC1 and the terminal amide. This result demonstrates that, unlike BRCA1, the MDC1 BRCT domain recognizes the C-terminal carboxylate of its phospho-peptide target with a high degree of specificity.

Engineering the BRCT for crystallization

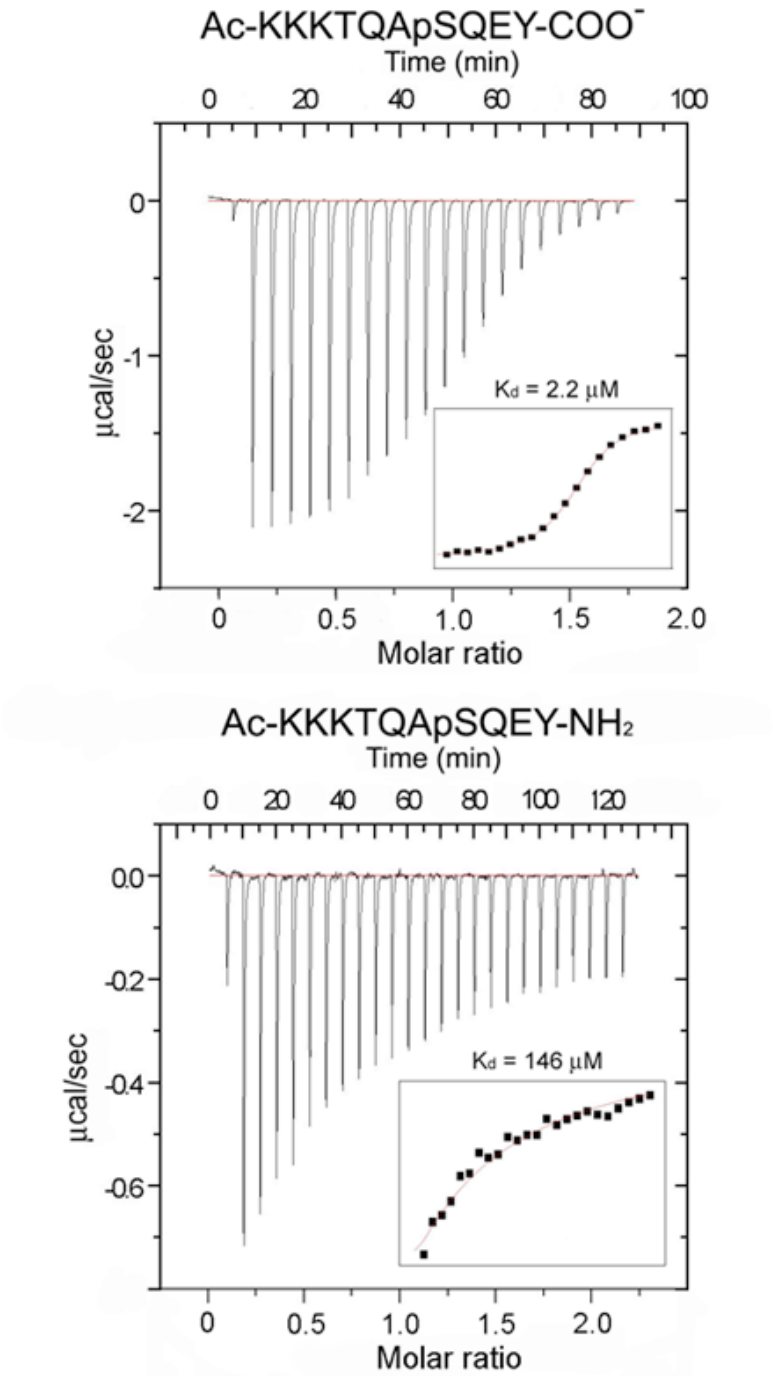


Figure 2.1. The MDC1 BRCT domain specifically recognizes the free carboxyl terminus of the γH2AX tail. Shown are sample ITC experiments showing titration of (*top panel)* the MDC1 BRCT domain with the γH2AX peptide bearing a free carboxylate at the COOH terminus or (*bottom panel*) an amidated COOH terminus. The titration curves (*insets*) were used to determine the dissociation constants. The *Kd* values shown are the average of three independent experiments.

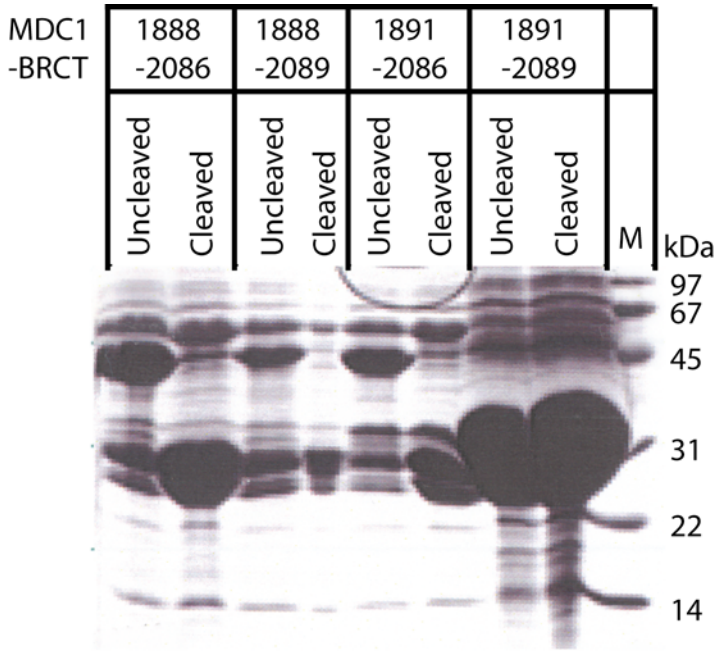


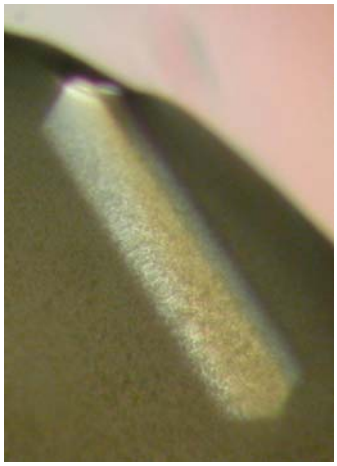
Figure 2.2. Defining the BRCT domain boundary by expression and cleavage analysis. *E. coli* lysates harboring the indicated MDC1-BRCT residues were analyzed by SDS-PAGE. Uncleaved and cleaved fractions are the protein preparations before and after cleavage with PreScission protease.

Based on sequence alignment with the BRCA1 BRCT domain, we designed four different MDC1-BRCT constructs. As shown in Figure 2.2, the removal of 3 residues from the C-terminus improved protein expression. Protein preparations of MDC1(1,891-2,089) with or without PreScission protease cleavage resulted in a large amount of proteins approximately 25 kD in size. This construct was not studied further. Both MDC1(1,888-2,086) and MDC1(1,891-2,086) produced crystals in many different crystallization conditions (Figure 2.3 and 2.4). However, the best of these crystals was produced with the MDC1(1,891-2,086) construct and diffracted to 1.45 Å. The phase information was obtained from the seleno-methionine substituted protein crystals grown with a 1.5-fold molar excess of a γH2AX peptide. No γH2AX peptide was found to be bound to MDC1 in these crystals.

Overall structure

To begin to understand the structural basis for MDC1 phospho-peptide recognition, we determined the X-ray crystal structure of the tandem BRCT repeats of MDC1 using multi-wavelength anomalous diffraction and a selenomethionine-substituted protein (Table 2.1). The structure reveals that MDC1 bears a structure that is strikingly similar to that of the tandem BRCT repeats of BRCA1, in spite of the fact that these two protein domains are only 15% identical at the sequence level (Figure 2.5). Both proteins share many of the same major secondary structure elements and the N- and C-terminal BRCT repeats pack in the same

a.



Crystallization condition

28% PEG8000
0.1M NaCl
0.1M Imidazole, pH 8.0

b.

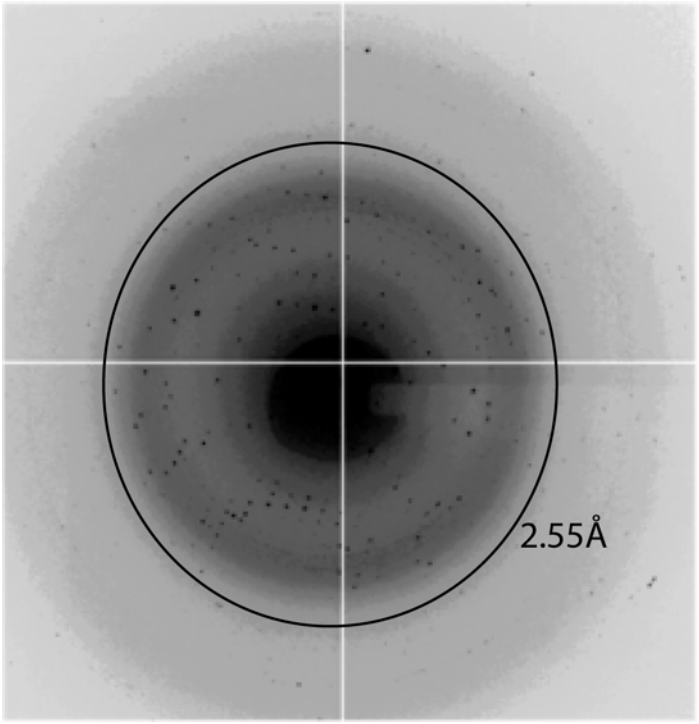
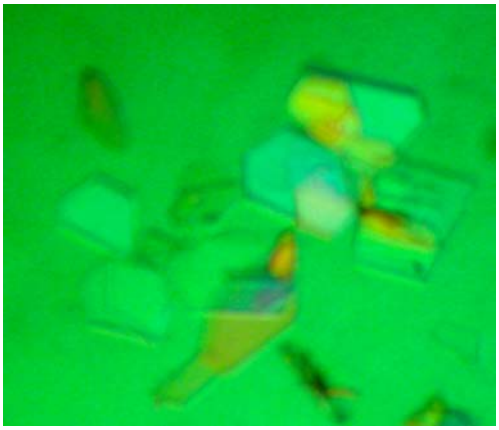


Figure 2.3. MDC1(1,888-2,086) crystal form. a. A crystal grown with PEG8000 as the precipitant. b. Sample diffraction pattern from the crystal (Space group P1) at the home source.

a.



Crystallization condition

20% PEG8000
0.1M HEPES, pH 7.5

b.

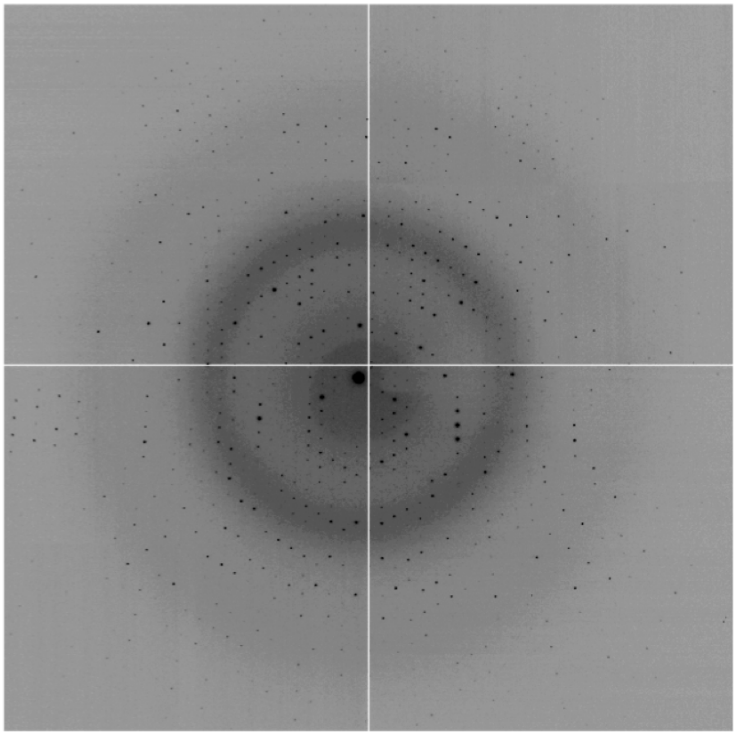


Figure 2.4. MDC1(1,891-2,086) crystal form. a. Crystals grown with PEG8000 as the precipitant. b. Sample diffraction pattern (1.6 Å) from a single crystal at the home source.

Table 2.1. Crystallographic data collection, phasing and refinement statistics

Data Collection		**(MDC1+peptide)**		**(MDC1)**
Space group			P1	P1
Cell dimensions				
		a (Å)	44.411	42.051
		b (Å)	62.266	44.440
		c (Å)	73.437	61.942
Cell angles		α (°)	80.379	72.947
		β (°)	85.627	87.543
		γ (°)	73.850	61.796
		λ1 (peak)	λ2 (remote)	
Wavelength (Å)			0.9795	1.0199	1.1159
Resolution range (Å)			100-2.7	100-2.7	30-1.45
Observations			368546	354512	171274
Unique reflections			19928	19537	57950
Data coverage total/final shell[1] (%)			97.9 (90.5)	98.4 (94.1)	90.8 (75.9)
<I/σI> total/final shell			13.0 (2.5)	16.6 (3.8)	14.5 (1.9)
R_{sym} total/final shell (%)[2]			13.5 (58.9)	11.3 (44.6)	4.8 (38.0)

Phasing Statistics (MDC1+peptide)	
Resolution range(Å)	30.0-2.7
No. of Selenium Sites	8/12
FOM – Solve	0.34
FOM – Resolve	0.67

Refinement Statistics		
Resolution range(Å)		30-1.5
R_{work}/R_{free} (%)[3]		0.201/0.215
No. of refined atoms	Protein	3017
	Water	269
R.m.s. deviations	Bonds (Å)	0.004
	Angles (°)	1.4
Average B-factors(Å^2)	Protein	12.1
	Water	16.5
Ramachandran	Most favored	290 (92.1%)
	Allowed	25 (7.9%)
	Generously allowed	0
	Disallowed	0

[1] Final shell: λ1: 2.80 – 2.70 Å, λ2: 2.80 - 2.70 Å, MDC1: 1.5 - 1.45 Å

[2] $R_{sym}=\Sigma |(I_{hkl}) - <I>| / \Sigma(I_{hkl})$ where I_{hkl} is the integrated intensity of a given reflection.

[3] $R_{work} = \Sigma_h|F_o(h) – F_c(h)| / \Sigma_h | F_o(h)|$, where $F_o(h)$ and $F_c(h)$ are observed and calculated structure factors. Data from wavelength λ1 were used during crystallographic refinement. R_{free} calculated with 5% of all reflections excluded from refinement stages using the native data set. No I/σI cutoff was used in the refinement.

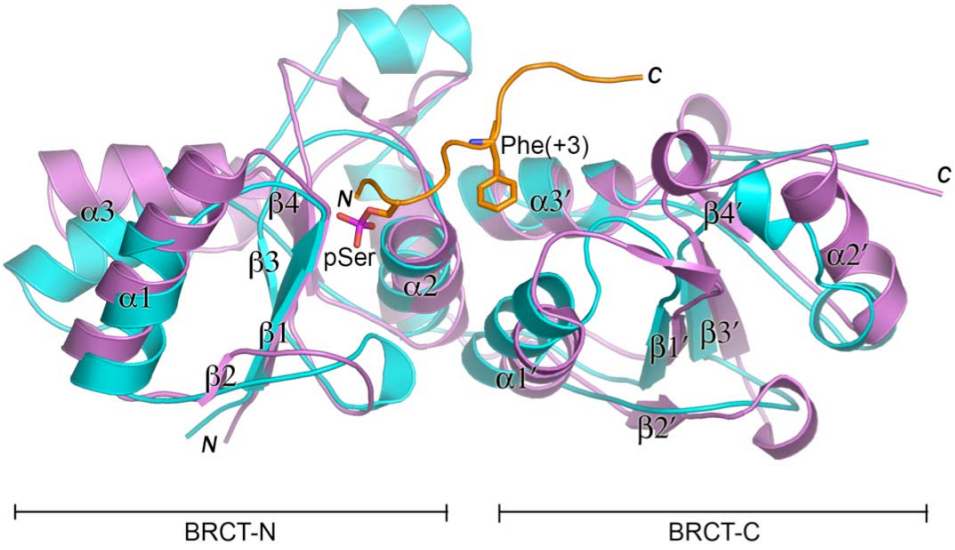
Figure 2.5. Sequence alignment of the MDC1 and BRCA1 BRCT repeats. The secondary structure of MDC1 is indicated, and residues conserved in the phosphoserine binding pocket are highlighted in *blue*. Residues involved in the head-to-tail packing of the NH2- and COOH-terminal BRCT repeats are *shaded* in *gray*.

head-to-tail manner seen in both BRCA1 (Glover, Williams & Lee 2004) and 53BP1 (Joo et al. 2002, Derbyshire et al. 2002) (Figure 2.6a).

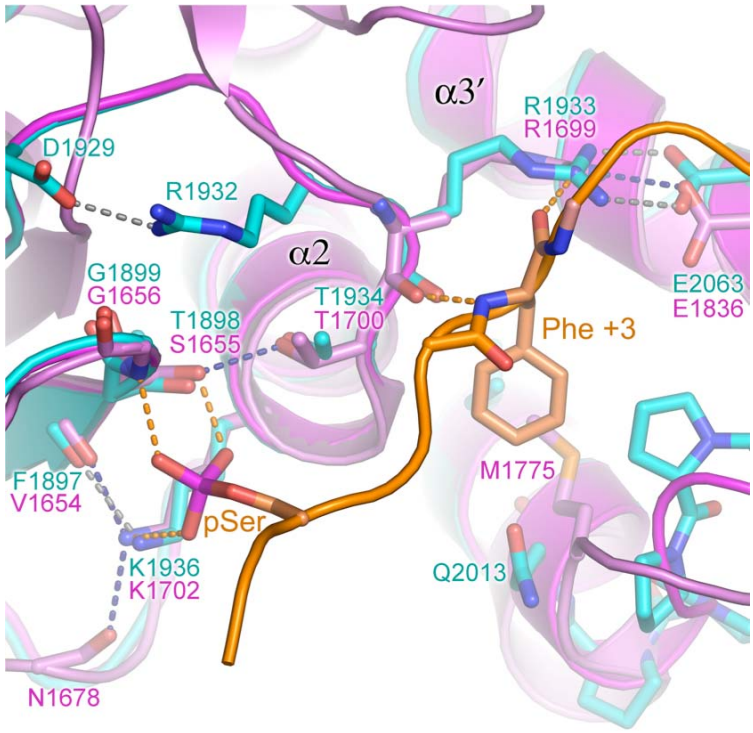
Peptide binding pocket comparison

To compare the putative peptide binding surface of MDC1 with that of BRCA1, we aligned the two structures based on a superimposition of key peptide-binding residues in BRCA1 with those in MDC1 (Figure 2.6b). The superimposition reveals that the N-terminal BRCT repeat of MDC1 contains a phospho-serine recognition pocket that is nearly identical to that of BRCA1, in spite of the fact that a critical serine in BRCA1 (Ser^{1655}) is a threonine in MDC1. The phosphate-binding ligands are the Oγ of Thr^{1898}, the main chain NH of Gly^{1899}, and the Nε of Lys^{1936}. Thr^{1898} is held in place by a hydrogen bond with Thr^{1934}, and the Nε of Lys^{1936} is held in place by a hydrogen bond with the main chain oxygen of Phe^{1897}.

In contrast, the region of MDC1 corresponding to the +3 specificity pocket in BRCA1 shows only limited conservation. Arg^{1699} is critical for phosphopeptide recognition in BRCA1, and mutations of this residue have been uncovered in breast cancer patients (Williams et al. 2004, Shiozaki et al. 2004). This residue hydrogen bonds to the main chain of the +3 residue of the peptide target in BRCA1, helping the phenylalanine side chain to dock into the specificity pocket. In MDC1, this residue is conserved (Arg^{1933}) and adopts a nearly identical conformation to that seen in BRCA1. In both BRCA1 and MDC1, the guanidinium group of the arginine



a.



b.

Figure 2.6. Structure of the tandem BRCT repeats of MDC1. a. Overview of the MDC1 BRCT structure (*cyan*) aligned with the structure of the BRCA1 BRCT (*magenta*) bound to an optimized phospho-peptide target (*orange*) (Protein Data Bank accession code 1T2V). **b.** Details of the phosphopeptide recognition surfaces of BRCA1 and MDC1, colored as in *A*. Residues involved in peptide binding are labeled and shown as *sticks*.

is held in place via a salt-bridging interaction with a conserved glutamic acid side chain (Glu^{2063} in MDC1, Glu^{1836} in BRCA1). The conservation of this critical arginine suggests that the +3 tyrosine of the γH2AX peptide will indeed bind at this site, however, the structure of the specificity pocket is otherwise quite different in both proteins. A key residue for phenylalanine recognition in BRCA1 is Met^{1775}, which forms the base of the pocket and, when mutated to arginine, blocks peptide binding and is associated with cancer (Williams et al. 2004, Shiozaki et al. 2004, Miki et al. 1994, Williams & Glover 2003). No similar hydrophobic residue is in this position in MDC1, and indeed, the loop at this position adopts a quite different and rigid conformation, due to the presence of 4 consecutive proline residues which are not conserved in BRCA1.

A comparison of the structure of the MDC1 BRCT with the structure of the BRCA1 BRCT – phospho-peptide complex suggests how the MDC1 specificity pocket may recognize the C-terminal tyrosine of γH2AX. We predict that the guanidinium group of the conserved Arg^{1933} will contact the carboxyl terminus, but this must not be sufficient, otherwise the BRCA1 BRCT would also be specific for the C-terminal carboxylate of the +3 residue. MDC1 also contains Arg^{1932}, which could also form a salt bridge to the C-terminal carboxylate of γH2AX, although this would require a re-orientation of this side chain, and a disruption of its salt bridging interactions with Asp^{1929}. Arg^{1932} could also come into proximity with the conserved glutamic acid of γH2AX at the +2 position, and could explain

the observed binding preference for glutamic acid at this position (Rodriguez et al. 2003). The specificity for tyrosine over phenylalanine at the C-terminus of γH2AX may be explained by the presence of Gln^{2013}, which is positioned to hydrogen bond with the tyrosine hydroxyl group, assuming this side chain is oriented in the pocket in a manner similar to that of the +3 phenylalanine in the BRCA1 structure. Thus, subtle differences between the specificity pockets of MDC1 and BRCA1 can explain the unique ability of the MDC1 BRCT to recognize the negatively charged C-terminus of γH2AX, and thereby be recruited to DNA double strand breaks.

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Chapter 3:

The BARD1 C-terminal domain structure and interactions with polyadenylation factor, CstF-50

Summary

The BARD1 N-terminal RING domain binds breast cancer susceptibility gene 1 (BRCA1) while the BARD1 C-terminal ankyrin and tandem BRCT repeat domains bind cleavage stimulation factor 50 (CstF-50) to modulate mRNA processing and RNA polymerase II stability in response to DNA damage. Here we characterize the BARD1 structural biochemistry responsible for CstF-50 binding. The crystal structure of the BARD1 BRCT domain uncovers a degenerate phosphopeptide binding pocket lacking the key arginine required for phosphopeptide interactions in other BRCT proteins. Limited proteolysis results indicate that a flexible tether links ankyrin and BRCT domains. Protein pull-down experiments utilizing a series of purified BARD1 deletion mutants indicate that interactions between the CstF-50 WD-40 domain and BARD1 involve the ankyrin-BRCT linker but do not require ankyrin or BRCT domains. The structural plasticity imparted by the ankyrin-BRCT linker helps to explain the regulated assembly of different protein BARD1 complexes with distinct functions in DNA damage signaling including BARD1-dependent induction of apoptosis plus p53 stabilization and interactions. BARD1 architecture and plasticity imparted by the ankyrin-BRCT linker are suitable to allow the BARD1 C-terminus to act as a hub with multiple binding sites to integrate diverse DNA damage signals directly to RNA polymerase. The majority of this chapter was originally published in the *Journal of Biological Chemistry* (Edwards et al. 2008). This chapter describes my contribution to the paper

and the additional work done by Dr. Ross Edwards is presented in the Chapter 5.

Introduction

The breast and ovarian cancer associated protein, BRCA1, together with its binding partner BARD1 (BRCA1-associated RING domain protein 1), control the cell cycle in response to DNA damage (Irminger-Finger & Leung 2002, Scully, Xie & Nagaraju 2004). Both proteins interact through N-terminal RING and adjacent helical domains to form a heterodimeric E3 ubiquitin ligase that constitutes the major catalytic activity of the BRCA1-BARD1 complex (Wu et al. 1996, Brzovic et al. 2001, Brzovic et al. 2003). While the direct targets of BRCA1-BARD1 ubiquitination are unclear, targeting likely involves conserved protein-protein interaction domains in both BRCA1 and BARD1.

Critical protein-protein interactions are mediated by a pair of sequence repeats at the C-terminus of BRCA1 called BRCT repeats (for BRCA1 C-terminal repeats) (Bork et al. 1997, Callebaut & Mornon 1997). Similar repeats are found in a number of proteins involved in the cellular response to DNA damage (Glover, Williams & Lee 2004). In BRCA1, the BRCT repeats mediate interactions with several proteins such as BACH1/BRIP (Cantor et al. 2001), CtIP (Yu et al. 1998, Li et al. 1999), and Abraxas (Kim, Huang & Chen 2007, Liu, Wu & Yu 2007). In each of these cases, the BRCA1 BRCT recognizes a phosphopeptide motif in the target protein, pSer-x-x-Phe (Manke et al. 2003, Yu et al. 2003). A series of structural studies have revealed that the N-terminal BRCT repeat contains a pocket which recognizes the phosphoserine, while the phenylalanine

residue is recognized by an adjacent hydrophobic pocket formed at the interface between the N- and C-terminal BRCT repeats (Glover, Williams & Lee 2004, Williams et al. 2004, Clapperton et al. 2004, Shiozaki et al. 2004, Varma et al. 2005, Lee et al. 2005). Cancer-associated mutations have been uncovered which specifically perturb the integrity of this phosphopeptide binding surface, demonstrating the critical importance of these interactions for the tumor suppression function of BRCA1 (Williams et al. 2003, Williams et al. 2004, Tischkowitz et al. 2008).

BARD1 also contains tandem BRCT repeats at its C-terminus, as well as a set of ankyrin repeats immediately N-terminal to the BRCT region. *In vitro* peptide binding studies suggest the BARD1 BRCT repeats may bind serine-phosphorylated peptides (Rodriguez et al. 2003), although attempts to isolate phosphorylation-dependent protein binding partners from human cells for the BARD1 BRCT region have been unsuccessful (Kim, Chen & Yu 2007). Ankyrin repeats are also well-known protein-protein interaction modules (Sedgwick & Smerdon 1999, Mosavi et al. 2004), strongly suggesting that this region could also function to recognize targets of the BRCA1-BARD1 complex. Individual ankyrin repeats consist of a helix-turn-helix followed by a β-hairpin. Multiple repeats stack together such that the loops protrude from one face of the structure to constitute the protein interaction surface. Both the ankyrin and BRCT repeat regions of BARD1 have been demonstrated to be required for chromosomal stability and homology-directed repair of DNA damage in

mammalian cells (Laufer et al. 2007). A number of missense variants within the BARD1 C-terminal regions have been isolated from breast and ovarian cancer patients, further highlighting the importance of this region for BRCA1-BARD1 function (Thai et al. 1998, Ghimenti et al. 2002, Sauer & Andrulis 2005).

A series of studies implicate the BARD1 C-terminus in the regulation of mRNA 3' processing in response to DNA damage (Kleiman & Manley 1999, Kleiman & Manley 2001, Kleiman et al. 2005, Mirkin et al. 2008). DNA damage triggers interactions between BRCA1-BARD1 and the CstF mRNA processing complex at the sites of stalled transcription (Kleiman & Manley 1999, Kleiman & Manley 2001). These interactions may regulate the inhibition of transcription through the targeted degradation of RNAP II (Kleiman et al. 2005), as well as the transient inhibition of mRNA polyadenylation. Interactions between the BRCA1-BARD1 heterodimer and the CstF complexes depend on direct interaction of the BARD1 C-terminus and the 50 kDa component of the CstF complex, CstF-50 (cleavage stimulation factor 50) (Kleiman & Manley 1999).

Here we have probed the structures and CstF-50 binding characteristics of BARD1 C-terminal regions. Limited proteolysis reveals that the BARD1 ankyrin and BRCT repeats constitute independent folded modules linked by a flexible tether. Further, the crystal structure of the BARD1 BRCT repeat uncovers a degenerate BARD1 BRCT phospho-peptide binding pocket with intact pSer interacting motifs, but which lacks

binding determinants for the pSer+3 hydrophobic specificity pocket at the inter-BRCT repeat interface. Analysis of the CstF-50 binding properties of a series of BARD1 deletion mutants maps the principle CstF-50 interaction site to the ankyrin-BRCT linker.

Experimental procedures

Protein Expression and Purification

Human BARD1 (423-777), BARD1 (423-553) and BARD1 (554-777) were expressed as GST-fusion proteins in *E. coli* strain BL21 (DE3) and purified by glutathione-affinity chromatography. The BARD1 polypeptides were cleaved from GST using PreScission protease (GE Healthcare Life Sciences) and then purified from GST by anion exchange (BARD1 (423-553)) or cation exchange chromatography (BARD1 (423-777) and BARD1 (554-777)) followed by gel filtration chromatography.

The BARD1-Q564H (G1756C) and BARD1-C557S (G1743C) derivatives were created by the two step PCR mutagenesis method (Horton et al. 1993). The presence of the mutations was verified by DNA sequencing. The derivatives were expressed in *E. coli* and purified as described for BARD1 (423-777).

Human CstF-50 (92-431) was expressed as a GST-fusion protein in *E. coli* strain BL21 (DE3) and purified by glutathione-affinity chromatography. Residues from 1 to 91 were excluded from the construct to limit possible self-association (Takagaki & Manley 2000). The protein was then purified by anion exchange followed by gel filtration chromatography.

Proteolytic mapping of the BRCT domain

Purified BARD1 (423-777) at 2 mg/mL was digested with 5 µg/mL trypsin for 0-60 min. The reaction was terminated with phenylmethanesulfonyl fluoride (PMSF), and the reaction products were separated by SDS-PAGE and stained with Coomassie blue. Electrospray mass spectrometry was used to identify the masses of tryptic fragments.

Multi-angle laser light scattering

100 µL of BARD1(423-777) at 4 mg/mL in 50 mM tris-HCl pH 7.5, 100mM NaCl was injected on to a Superose 12 10/300 size exclusion column at 0.2 mL/minute. In-line with the column were DAWN EOS MALLS and Optilab rEX differential refractive index detectors (Wyatt Technologies, Santa Barbara, CA). The MALLS data were analyzed using the program ASTRA 4.90.

Crystallization

Crystals of BARD1 BRCT (554-777) crystal form I (CFI) were grown by vapor diffusion in hanging drops at 4°C by mixing 2 µl of 25 mg/mL BRCT domain in protein solution (100 mM NaCl, 5 mM Tris HCl, pH 7.5, 1 mM dithiothreitol) with 2 µl of well solution (12% PEG 8000, 0.1 M citrate, pH 3.9).

BARD1 BRCT crystal form II (CFII) was grown by vapor diffusion in hanging drops at room temperature. The crystals were grown by mixing 1 µL of 6 mg/mL BRCT domain with a 1.5-fold molar excess of a Ac-pSDDE-NH2 peptide in protein solution (100 mM NaCl, 5 mM Tris HCl pH 7.5, 1 mM dithiothreitol) with 1 µL of well solution (20% PEG 3350, 0.2 M

ammonium chloride pH 6.3). No evidence of bound peptide was found in $2F_o$-F_c or F_o-F_c electron maps. For cryopreservation, single crystals were soaked in the appropriate well solution supplemented with 26% (v/v) glycerol and then flash-frozen in liquid nitrogen.

Data Collection

All data were collected at beamline 8.3.1 of the Advanced Light Source, Lawrence Berkeley National Laboratory, using an ADSC Q210 detector and a wavelength of 11111 eV. Data were collected on CFI to 2.6 Å from a single, elongated orthorhombic rod crystal. A total of 180° of data were collected using five second 1° oscillations at a crystal-to-detector distance of 220 mm. The crystal was translated twice at 60° and 120° to minimize radiation damage. Data were indexed as primitive orthorhombic and scaled in the space group $P2_12_12_1$. The post-refined unit cell dimensions were a=55.5 Å, b=67.9 Å, and c=120.4 Å, and the mosaicity was 0.24°. Data were collected on CFII to 2.1 Å. Crystals formed in the same space group as CFI, but with a different unit cell, a = 56.8 Å, b = 75.6 Å and c = 118.0 Å (see Table 3.1).

Structure solution and refinement

A structure-based sequence alignment was made from the superposition of BRCT repeats from BRCA1 (PDB code 1JNX) and 53BP1 (PDB code 1KZY). The amino acid sequence of the BARD1 BRCT repeat was manually aligned to this structural alignment (Figure 3.1). Molecular

Table 3.1. Crystallographic data collection, phasing and refinement statistics

		CFI	CFII
Space group		$P2_12_12_1$	$P2_12_12_1$
Cell dimensions	a (Å)	55.477	56.821
	b (Å)	67.906	75.550
	c (Å)	120.398	117.967
Wavelength (Å)		1.1159	1.1159
Resolution range (Å)		50 -2.6 (2.69-2.60)	28.4-2.1 (2.18-2.10)
Observations		200053	231155
Unique reflections		13944	28825
Completeness		95.3% (68.2%)	94.9% (70.0)
$<I/\sigma I>$		31.5 (2.8)	35.5 (2.2)
Linear R-factor		0.052 (0.282)	0.032 (0.437)

Resolution range	28.4-2.1 Å	
R-factor/R-free	0.223/0.262	
No. of refined atoms	Protein	3312
	Water	118
	Other	12
R.m.s. deviations	Bonds (Å)	0.010
	Angles (°)	1.221
Average B-factors(Å^2)	Main chain	46.6
	Side chain and waters	47.2
	All atoms	46.9
Ramachandran	Most favored	333 (92.8%)
	Allowed	24 (6.7%)
	Generously allowed	1 (0.3%)
	Disallowed	1 (0.3%)

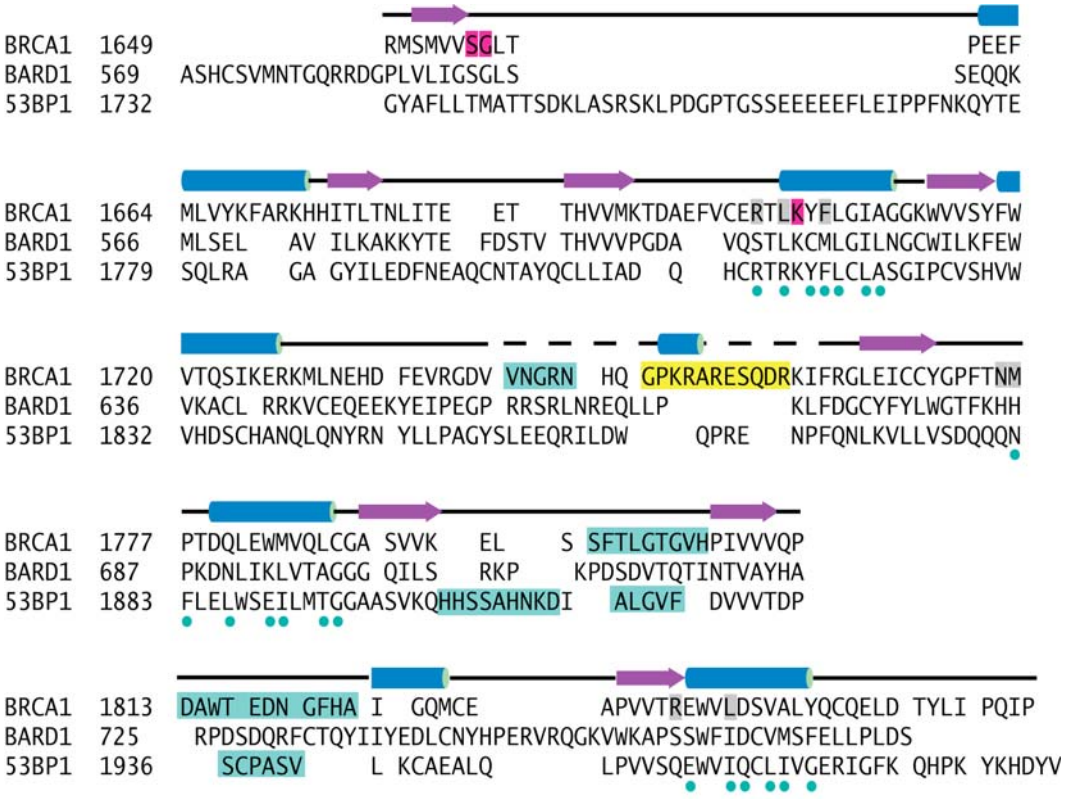


Figure 3.1. A structure-based sequence alignment of BRCT domains from BRCA1, 53BP1 and BARD1. Poorly conserved, structurally divergent loops are indicated (*green box* – loop, *yellow box* – helix + turn). Residues that contact the pSer and Phe at the +3 position of the phospho-peptide target are highlighted (magenta and grey, respectively). BRCT dual-repeat-interacting residues are marked by green dots.

replacement trial models were constructed based on the main-chain of the BRCT repeat from 1JNX with side-chains mutated to conform to the BARD1 sequence based on the structural alignment. Extended loops and the C-terminal extension were removed from the model. Using this model a dyad search was preformed on data from CFI in MOLREP (Vagin & Teplyakov 1997), searching for two copies of the model simultaneously. The rotation function was further constrained to search only therotations determined from a self-rotation function, also calculated in MOLREP. A solution was found having an initial R-factor and correlation coefficient of 0.561 and 0.229 respectively. Rigid body refinement in REFMAC (Murshudov, Vagin & Dodson 1997, Winn, Isupov & Murchudov 2001) yielded an R-factor of 0.557 and R-free of 0.575. Rigid-body refinement in CNS gave 0.551/0.545 for R and R-free. Further simulated annealing refinement with torsion angle molecular dynamics in CNS (Brunger et al. 1998) followed by 10 cycles of restrained refinement on maximum likelihood targets gave an R-factor of 0.425 and R-free of 0.469. The model was further refined in CNS to an R-factor of 0.335/0.339 with NCS restraints imposed. The model was further refined against the 2.1 Å CFII dataset in REFMAC utilizing two-fold NCS restraints and TLS group anisotropic B-factor refinement. The final model contains 3312 protein atoms, 118 waters and two glycerol molecules. The final R-factor and R-free were 0.223 and 0.262. We were unable to model the N-terminal residues 554-568 in either chain, nor residues 742-748 in chain A (part of

the extended loop between β3' and β4' in the C-terminal BRCT domain), and we presume these regions are disordered in the crystals. All model building was carried out in COOT (Emsley & Cowtan 2004). An asymmetric 7σ peak near His 686 in chain A could not be satisfactorily modeled. The Ramachandran plot contained 92.8% of all residues in the favored regions and 99.7% of all residues were in allowed regions (Table 3.1). The only residue in disallowed region, Glu655, is located within a very constrained turn, at the top of a helix. The main chain density in chain A and B appears reasonable and the psi angle in within allowed range; however, the phi angle is slightly out of allowed range. Due to two-fold NCS restraints imposed during refinement, we cannot verify that Glu655 in chain A and B independently adopted the same confirmation. Atomic coordinates and structure factors have been submitted to Protein Database (RCSB accession code 2R1Z).

GST pull-down assays

15 µg of GST or GST-CstF-50 (92-431) was incubated with 125 µg of the indicated BARD1 constructs for 30 min at 30°C in 25 µL final volume wash buffer (400 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 0.5% NP-40, 1 µg/µL leupeptin, 0.7 µg/µL pepstatin and 25 µg/µL PMSF). Binding reactions were incubated with glutathione-agarose beads for 90 min at 4°C and the beads were washed four times with the wash buffer. Bound proteins were then eluted and separated by SDS-PAGE, and stained with SYPRO® Orange (Sigma).

Results and Discussion

Mapping structural domains within the BARD1 C-terminal region

Sequence analysis of BARD1 C-terminal to the RING domain suggested the presence of three to four ankyrin repeats followed by a pair of tandem BRCT repeats. To better understand BARD1 domain architecture, we first employed limited proteolytic mapping to locate folded protein domains within a purified C-terminal fragment of human BARD1 (423-777) which contains both the ankyrin and BRCT repeat domains. Tryptic digestion rapidly converted BARD1 (423-777) into two protease stable fragments (Figure 3.2). Electrospray mass spectrometry and N-terminal sequencing of the large trypsin fragment identified a protein with molecular weight 25736 ± 5 Da and an N-terminal alanine, corresponding to BARD1 residues 554-777. This fragment contains both BARD1 BRCT repeats, indicating that the two BRCT repeats form a stable structural unit similar to those of BRCA1 and MDC1 (Lee et al. 2005, Williams, Green & Glover 2001). Mass spectrometry of the small fragment revealed a mass of 14301 ± 1 Da matching the BARD1 ankyrin repeat region spanning residues 423-553. This analysis shows the ankyrin repeat regions and the tandem BRCT repeats each adopt stably folded structures linked by a protease sensitive linker peptide.

BARD1 C-terminal region in solution

To verify the monomeric state of the BARD1 C-terminal region in

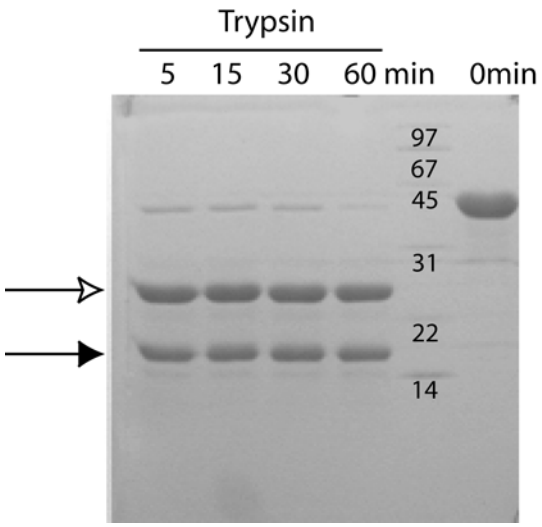


Figure 3.2. The BARD1 ankyrin and BRCT repeats are tethered by a flexible linker. BARD1(423-777) was digested with trypsin for the times indicated, and the products were analyzed by SDS-PAGE. The open arrow indicates the BARD1-BRCT fragment, and the closed arrow indicates the BARD1-ankyrin fragment.

solution, we used multi-angle laser light scattering (MALLS) under similar experimental conditions to those used in the limited proteolysis experiment (Figure 3.3). We determined our construct, BARD1 (423-777) to be monomeric with no trace of higher order oligomeric species.

X-ray crystal structure of the BARD1 BRCT repeats

To probe the structure of the BARD1 C-terminus, we crystallized and determined the X-ray structure of the tandem BARD1 BRCT repeat domain (554-777). The BARD1 BRCT repeat structure was solved using molecular replacement methods and refined to 2.1 Å resolution (see Experimental Procedures). The structure of BARD1 (554-777) is reminiscent of the BRCT repeats in BRCA1 and MDC1 with the two BRCT domains packing in the same head-to-tail manner (Figure 3.4a). The structure determined here is also essentially identical to the structure of a smaller BARD1 BRCT fragment recently determined by Birrane et. al. (Birrane et al. 2007).

The N-terminal domains of BRCT repeats whose structures are known are structurally well conserved relative to their C-terminal BRCT domains, which tend to have larger and more diverse loops. A major difference in the N-terminal BRCT domain of BARD1 occurs in the loop between helix α3 and the linker helix. In both BRCA1 and MDC1, a 10-13 amino acid variable loop caps the N-terminal end of the linker helix. This loop is absent in BARD1. Residues in this loop make no direct contacts to either the phosphoserine or phenylalanine binding sites. Likewise the

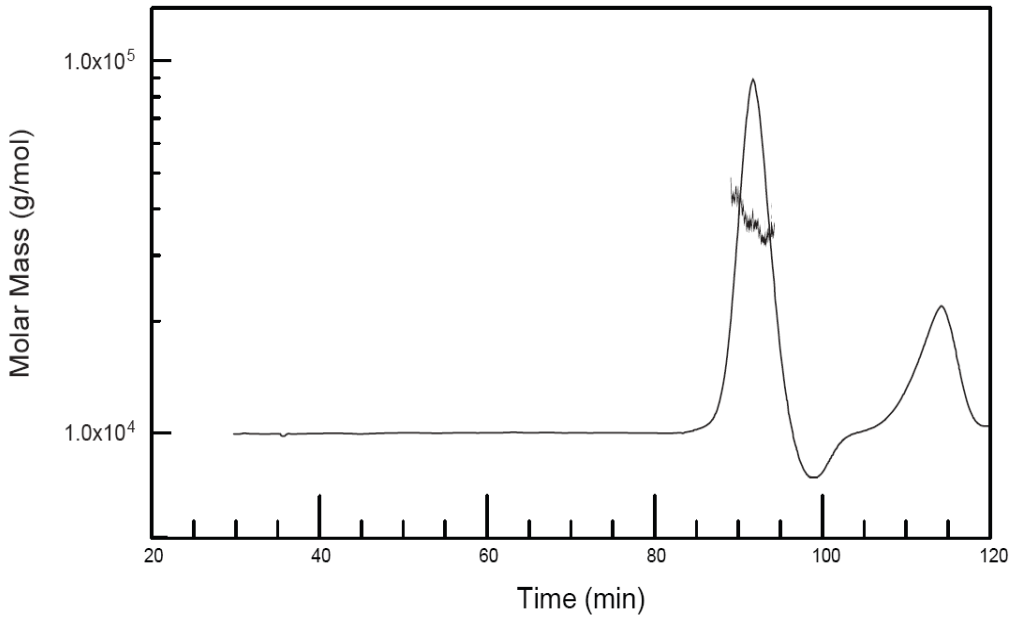
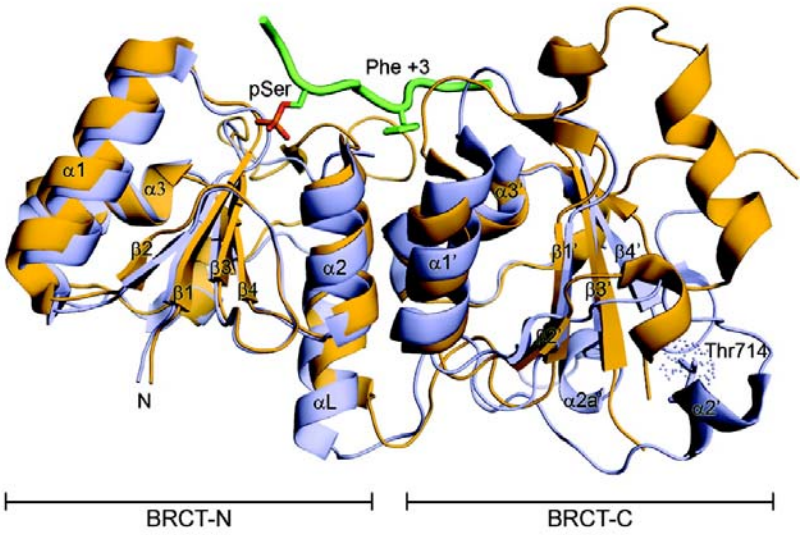


Figure 3.3. BARD1(423-777) is a monomer in solution. BARD1(423-777) eluted as a monomer with molar mass moments Mn, Mw and Mz of 3.67e+04 (2.5%), 3.70e+04 (2.6%) and 3.73e+04 (5%) g/mol. The previously calibrated void volume on this column elutes at 40 minutes. A plot of molar mass vs time over the BARD1 peak is shown superimposed on the differential refractive index trace beween 30 and 120 minutes.

a.



b.

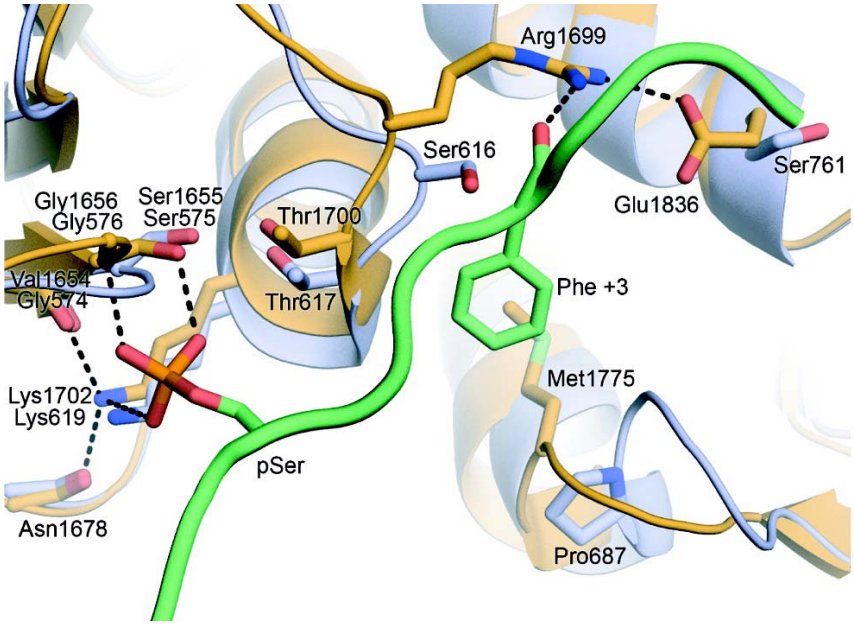


Figure 3.4. Structure of the tandem BRCT repeats of BARD1. a. Overview of the BARD1 BRCT structure (orange) aligned with the structure of the BRCA1 BRCT (blue) bound to an optimized phosphopeptide target (green) (PDB accession code 1T2V). **b.** Details of the phosphopeptide recognition surfaces of BRCA1 and BARD1, colored as in (a). Residues involved in peptide binding are labeled and shown as sticks.

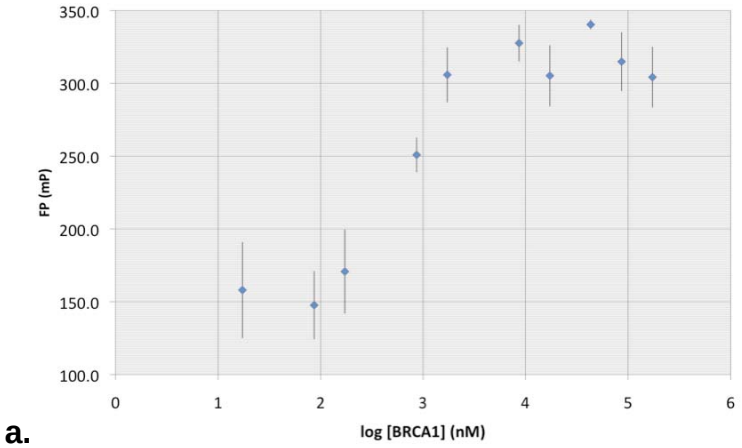
topology and core of the C-terminal domain is conserved. However the loop connecting β2' and β3' is significantly longer in BARD1 covering the base of the C-terminal domain core, distal to the peptide binding face.

An amino acid sequence comparison of BARD1 with BRCA1 and MDC1 revealed that the residues involved in directly binding to the phosphoserine in the N-terminal BRCT domain are conserved in BARD1 (Ser1655, Gly1656, Thr1700 and Lys1702) and functionally conserved in MDC1 (Thr1898, Gly1899, Thr1934 and Lys1936) (Williams et al. 2004). Superposition of the BARD1 BRCT structure on that of BRCA1 and MDC1 further shows that the arrangement of these residues are spatially conserved, consistent with the hypothesis that this surface may also act as a phosphate binding site in BARD1 (Figure 3.4b). Indeed, a previous peptide selection study suggested that the tandem BARD1 BRCT repeats preferred phosphoserine containing peptides (Yu et al. 2003).

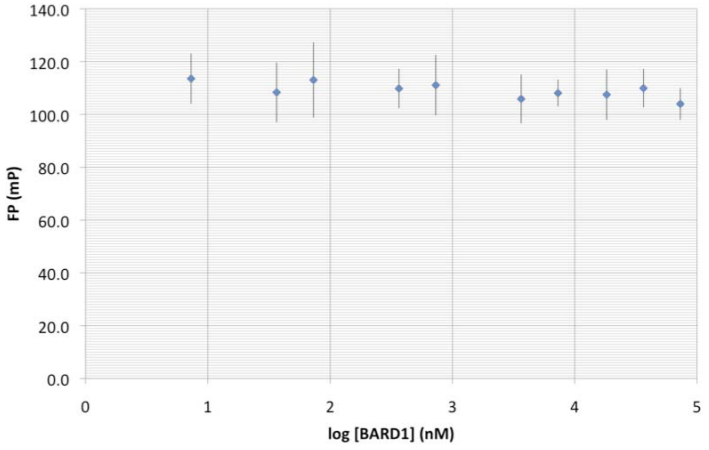
In contrast to the phosphoserine recognition motifs, the pSer +3 specificity pocket of BARD1 is quite different from that of BRCA1 (Williams et al. 2004) and MDC1 (Lee et al. 2005, Stucki et al. 2005). Arg1699, one of the critical phosphopeptide recognition residues in this region of BRCA1, hydrogen bonds to the main chain of the +3 residue peptide targets, and orients the phenylalanine side chain to dock into the +3 specificity pocket (Williams et al. 2004). A cancer-associated missense mutation of this residue (R1699W) in BRCA1 disrupts protein folding (Williams et al. 2003) and phosphopeptide binding (Williams et al. 2004)

underscoring the importance of this residue in mediating phosphopeptide interactions in the BRCT protein family. In MDC1, the corresponding residue, Arg1933, adopts a similar conformation to Arg1699 in BRCA1 (Lee et al. 2005). This arginine forms a strong, dual salt-bridging interaction with the negatively charged carboxyl terminus of its interacting peptide, γ-H2AX (Stucki et al. 2005). Interestingly, BARD1 contains a serine instead of an arginine in the +3 specificity pocket, suggesting that interactions with the peptide mainchain at +3 may be very different from that of BRCA1 or MDC1.

Oriented peptide library analysis indicated that the BARD1 BRCT repeat specifically recognizes pS-[D/E]-[D/E]-E (Rodriguez et al. 2003). To probe the affinity of the BRCT domain of BARD1 for the predicted peptide substrates, we monitored the change of BARD1 intrinsic fluorescence upon binding to peptide substrates. Binding affinities were measured for the phosphopeptide (fluorescein-pSDDE-CONH2) as well as for the nonphosphorylated counterpart (fluorescein-SDDE- CONH2). BARD1 failed to show any binding to either peptide in these studies, while binding was demonstrated between the BRCA1 BRCT domain and its phospho-peptide target from BACH1 (fluorescein-GGSRSTpSPTFNK-CONH2) (Figure 3.5). The interaction may be transient during DNA damage repair, suggested by the low BARD1-BRCT specificities for the phosphopeptide residues at positions from 0 to +3 (Rodriguez et al. 2003).



a.



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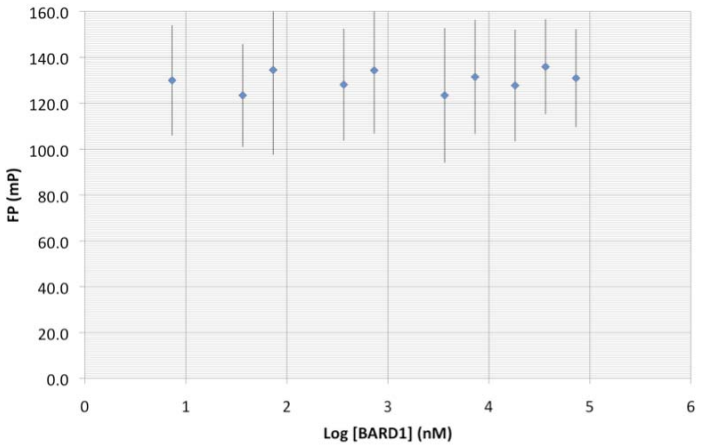

c.

Figure. 3.5. The BARD1-BRCT domain showed no binding to the predicted phosphoepitopde SDDE. Fluorescence polarization was performed with fixed peptide and increasing protein concentrations. **a.** (*control*) The BRCA1-BRCT domain showed binding to the BACH1 phosphopeptide. **b.** The BARD1-BRCT domain showed no binding to the pSDDE peptide. **c.** The BARD1-BRCT domain showed no binding to the SDDE peptide.

A previous report has suggested that Thr714 and Thr734 in BARD1 may be important DNA damage phosphorylation sites (Kim et al. 2006). Our structure shows that Thr734 is buried within a BRCT repeat whereas Thr714 is exposed to solvent. Therefore it is unlikely that Thr734 is a true phosphorylation site, possibly explaining why phospho-specific antibody raised against p-Thr734 peptide failed to recognize BARD (Kim et al. 2006). Inhibition of 3' mRNA cleavage after DNA damage and preferential degradation of RNAP IIO in BARD1 T734A mutant may be due to its negative effects on integrity of the BRCT repeat structure. In contrast, Thr714 is positioned within a large loop on the surface of the C-terminal BRCT repeat between α1' and β2'.

The BARD1 ankyrin-BRCT linker is critical for interactions with CstF-50

To probe the potential functional implications of BARD1 C-terminal domain flexibility for its interactions with its protein partners, we mapped physical interactions between BARD1 and CstF-50. CstF-50 (92-431), lacking the 91 amino acid tail N-terminal to its WD-40 domain, was previously shown to be necessary and sufficient for BARD1 interactions by two-hybrid studies (Kleiman & Manley 1999). To confirm this interaction, GST-CstF-50 (92-431) and BARD1 (423-777) were expressed separately in *E. coli* and tested for interaction by glutathione-affinity chromatography (Figure 3.6a). BARD1 (423-777) bound to GST-CstF-50 (92-431), but not to GST alone, revealing a specific interaction between the two proteins.

Two BARD1 cancer-associated missense variants, Q564H and C557S, map to the ankyrin-BRCT linker (Thai et al. 1998, Ghimenti et al. 2002). To test the effects of these mutations on interactions with CstF-50, we expressed BARD1 (423-777) containing either the Q564H or C557S mutation for binding assays with GST-CstF-50 (92-431) (Figure 3.6a). BARD1 (Q564H) reproducibly bound to GST-CstF-50 (92-431) as well as to GST alone, indicating that this mutation decreased the specificity of BARD1 protein-protein interactions. Conversely, the BARD1 variant, C557S, bound to GST-CstF-50 (92-431), but not to GST, suggesting that this mutation does not affect the BARD1 - CstF-50 interaction. Interestingly, a very recent case-control study has found no association between the C557S variant and breast or ovarian cancer (Johnatty et al. 2008).

To map the region of BARD1 required for interaction with CstF-50, we compared binding of CstF-50 to the isolated ankyrin domain (BARD1 (423-553)), the linker-BRCT construct (BARD1 (554-777)), the BRCT repeat alone (BARD1 (569-777)), and the ankyrin-linker-BRCT construct (BARD1 (423-777)). The BRCT repeat alone exhibited weak CstF-50 binding and the ankyrin domain in isolation did not bind to CstF-50, suggesting these regions make minimal contributions to high affinity CstF-50 interaction (Figure 3.6c). In contrast, the ANK-linker-BRCT and linker-BRCT displayed robust CstF-50 binding (Figure 3.6a, b) implicating the flexible ankyrin-BRCT linker as the core CstF-50 interaction region.

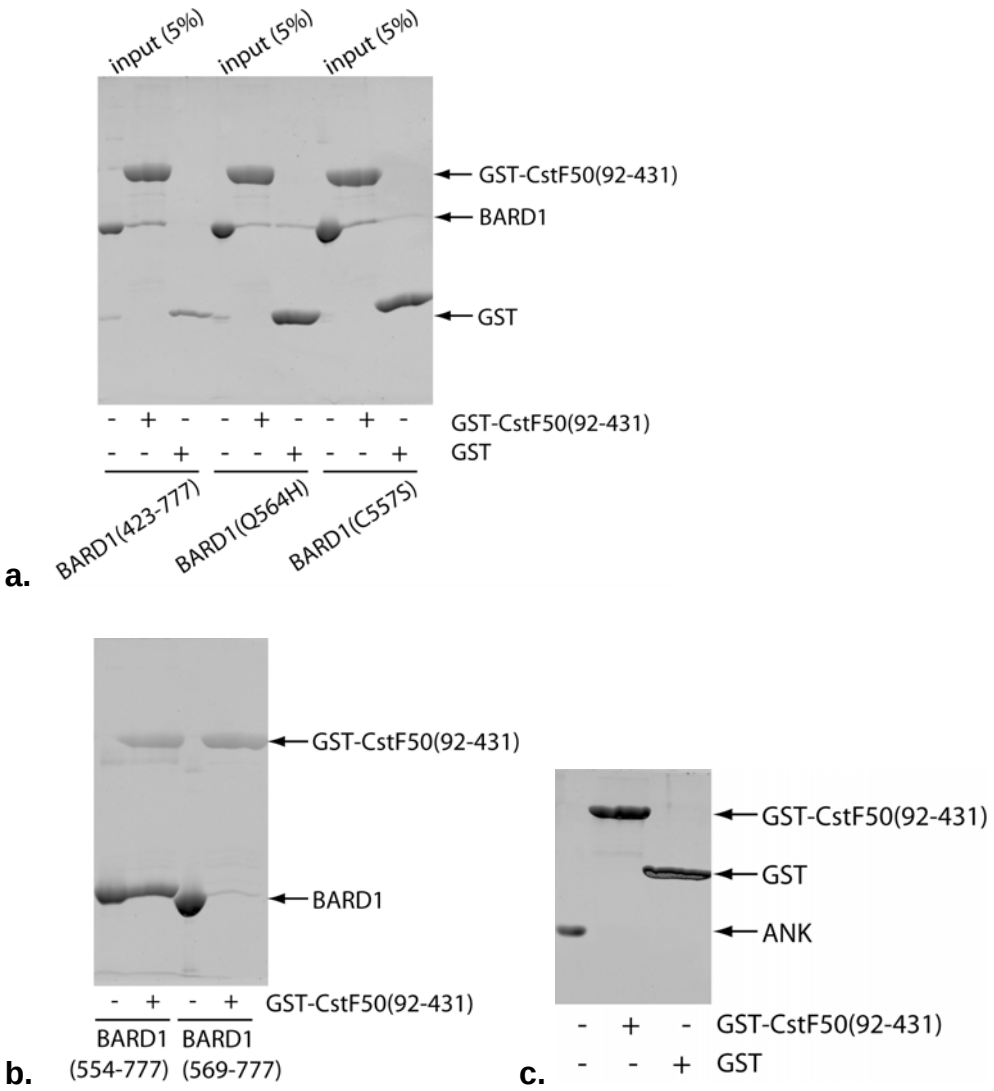

Figure 3.6. The BARD1 ankyrin-BRCT linker is critical for interactions with CstF-50. **a.** Interaction of GST-CstF50 (92-431) and BARD1 (423-777). The BARD1 construct containing both ankyrin repeats and BRCT repeats was incubated with purified GST or GST-CstF50 (92-431). Bound proteins were eluted and resolved by SDS-PAGE. Five percent of the GST-CstF50 (92-431) used in binding reactions is shown. **b.** Requirement of the BARD1 linker for CstF-50 interaction. BARD1-BRCT derivatives with or without the BARD1 linker were used in binding reactions with GST-CstF50 (92-431). Five percent of each of the GST-CstF50(92-431) used in binding reactions is shown. **c.** Lack of interaction between BARD1-ankyrin and GST-CstF50 (92-431). BARD1-ankyrin was incubated with purified GST or GST-CstF50 (92-431). Five percent of the BARD1 ankyrin used in binding reactions is shown.

Discussion

Our biochemical and crystallographic data indicate that the C-terminal region of BARD1 consists of a two-domain structure connected by a flexible peptide linker. In addition, a recent NMR spectroscopic study failed to uncover any interactions between the BARD1 ankyrin and BRCT domains (Fox et al. 2008). Thus, the protein interaction surfaces on the ankyrin and BRCT domains are not pre-aligned to form a contiguous recognition surface.

CstF-50 is the best-characterized binding partner for the BARD1 C-terminal region. Our pull-down data demonstrate that specific interactions between the CstF-50 WD-40 domain and BARD1 critically depend on the BARD1 interdomain linker (Figure 3.6). Structural studies of WD-40 containing protein complexes indicate that proteins of this family interact with protein partners at the depression formed at the centre of the WD-40 β-propeller structure. These interactions can then form an anchor for assembly of larger protein complexes. Such interactions were first structurally characterized for heterotrimeric G-proteins where the β-subunit is a WD-40 protein (Gaudet, Bohm & Sigler 1996, Wall et al. 1995). It is possible that interactions between CstF-50 and the BARD1 linker could rigidify the BARD1 C-terminal region, reducing the relative flexibility of the ankyrin and BRCT domains, thereby facilitating interactions with other factors.

CstF-50 is implicated in the direct recognition of the C-terminal domain (CTD) of RNA polymerase II, potentially providing a critical interaction to recruit the CstF complex to the transcribing RNA polymerase holoenzyme (Kleiman & Manley 1999, Fong & Bentley 2001, McCracken et al. 1997). This interaction does not require the WD-40 domain of CstF-50, but relies on an N-terminal region that we have shown is not required for interactions with BARD1 (Fong & Bentley 2001). Since the minimal binding regions do not overlap, the RNAP II CTD, CstF-50, and the BARD1 C-terminal domains could potentially all interact within the elongating transcriptional complex. The proximity of the BARD1 BRCT to the CTD would allow for additional interactions between the phosphorylated CTD and the BARD1 BRCT. Since the BARD1 BRCT preferentially binds phospho-peptides with negatively charged side chains at the +2 and +3 positions *in vitro* (Rodriguez et al. 2003), CTD binding by the BARD1 BRCT might be facilitated by phosphorylation at both the position 2 and 5 serines of the CTD.

In addition to interactions with CstF-50, the BARD1 C-terminal region has also been implicated in interactions with p53. These interactions lead to the phosphorylation and stabilization of p53 and have been implicated in facilitating apoptosis in response to DNA damage (Fabbro et al. 2004, Feki et al. 2005, Irminger-Finger et al. 2001). The ankyrin repeats, linker and a portion of the N-terminal BRCT repeat contain the minimal region of BARD1 required for p53 interactions (Feki et

al. 2005). The Q564H BARD1 mutation attenuates BARD1-dependent induction of apoptosis, p53 stabilization, and interactions with p53, suggesting that the BARD1 linker region is critical for a functional interaction of BARD1 with p53 (Irminger-Finger et al. 2001). Thus, the C-terminal region of BARD1 can interact with diverse partner proteins to regulate different aspects of the DNA damage response. The structural plasticity of this region, imparted by the ANK-BRCT linker, is therefore likely key to the regulated assembly of different protein complexes with distinct functions in DNA damage signaling.

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Chapter 4:

Analysis of missense variations in the BRCT domain of BRCA1 by structural and functional assays

Summary

Genetic screening of the breast and ovarian cancer associated gene BRCA1 has uncovered a large number of variants of undefined clinical significance. Here we use biochemical assays to assess the structural and functional defects associated with a large set of 117 distinct BRCA1 missense variants within the essential C-terminal domain of the BRCA1 protein (BRCT). In the first method, we used limited proteolysis to assess the protein folding stability of each of the mutants compared to the wild type. In the second method, we used a phospho-peptide pull-down assay to assess the ability of each of the variants to specifically interact with a peptide containing a pSer-X-X-Phe motif, a known functional target of the BRCA1 BRCT domain. Taken together, the results reveal that 65% of these variants show significant structural and/or functional defects. None of the variants that cause a significant unfolding of the BRCT domain were found to be active in peptide binding assays, indicating the folded 3D structure of the BRCT domain is essential for its function. A subset of well-folded variants was defective for phospho-peptide interactions and many of these variants could be rationalized in terms of specific disruption of the phospho-peptide binding groove. The excellent agreement between the structural and functional effects of these mutations and clinical data available for some of the variants suggests that this data could be used to help assess the cancer risks posed by these variants.

Introduction

Since the cloning of BRCA1 in 1994, a large effort has been made to sequence the BRCA1 genes of women who are at increased risk for early onset breast and ovarian cancers (Miki et al. 1994). This effort has uncovered a number of mutations, which are strongly linked to cancer. Unfortunately, a large number of rare BRCA1 variants have been uncovered in the human population for which risk assessment has been problematic, due to a lack of pedigree data linking each mutation with disease risk. BRCA1 encodes a large, 1863 amino acid nuclear protein, which plays a critical role in the response of cells to genotoxic stress (Scully & Livingston 2000, Venkitaraman 2002). BRCA1 is thought to act as an essential mediator protein in DNA damage induced nuclear signaling events, where it interacts with phosphorylated partner proteins such as the DNA helicase, BACH1, the nuclease CtIP, and another signaling protein, Abraxas, to relay signals generated from chromatin surrounding the damage to downstream targets such as DNA repair proteins and factors involved in cell cycle regulation (Yu et al. 1998, Rodriguez et al. 2003, Botuyan et al. 2004, Yu, Chen 2004, Varma et al. 2005, Kim, Huang & Chen 2007, Wang et al. 2007). Interactions with phosphorylated partner proteins are mediated by a pair of tandem repeats at the C-terminus of BRCA1, termed BRCT repeats (Clapperton et al. 2004, Shiozaki et al. 2004, Williams et al. 2004). The BRCT repeat region can also act as a transcriptional activation domain when linked to a

sequence-specific DNA binding module and this activity may contribute to the ability of BRCA1 to regulate the expression of genes such as p21, GADD45 and ER (Chapman & Verma 1996, Monteiro, August & Hanafusa 1996). The N-terminal region of the protein contains a RING domain which forms a heterodimeric complex with the RING domain of BARD1 to form a ubiquitin ligase (Wu et al. 1996, Brzovic et al. 2001, Brzovic et al. 2003). Cancer-associated mutations tend to cluster in the RING and BRCT repeat regions, demonstrating the critical role of these domains in BRCA1 tumour suppression.

The BRCA1 BRCT domain specifically interacts with phophorylated protein targets containing the motif pSer-x-x-Phe. Structural studies have revealed that recognition involves a conserved phospho-serine recognition pocket in the N-terminal BRCT repeat, composed of Ser1655, Gly1656 and Lys1702, which each supply ligands to recognize the phosphate (Clapperton et al. 2004, Shiozaki et al. 2004, Williams et al. 2004). The phenylalanine residue at the +3 position of the peptide target is recognized by a largely hydrophobic groove formed at the interface between the repeats. Phospho-peptide recognition is likely a common function of BRCT domains in many proteins associated with DNA damage-mediated signal transduction. For example, the phosphorylated form of the histone variant, H2AX, which serves as a critical chromatin mark of DNA double strand breaks, is specifically bound by the tandem BRCT repeats of MDC1

to initiate DNA double strand break signaling (Lee et al. 2005, Stucki et al. 2005).

A number of studies have attempted to predict the cancer risks associated with unclassified missense variants in BRCA1 through bioinformatics analysis based on multiple sequence alignment data and protein structure prediction, as well as analyses of the clinical and family history data available for some of these mutations (Miki et al. 1994, Chasman & Adams 2001, Abkevich et al. 2004, Chenevix-Trench et al. 2006, Lovelock et al. 2006). These studies indicate that the RING and BRCT domains, which are the most highly conserved regions of BRCA1, likely contain the vast majority of cancer-associated mutations. Here we utilize in vitro protein folding and phospho-peptide binding to assess the structural and functional consequences of a set of 117 distinct missense variants, which have been uncovered in the BRCA1 BRCT domain in the human population. Our results indicate that the majority of the BRCT missense variants result in significant structural and/or functional defects, which are likely linked to an increased cancer risk. We use this data set to train a bioinformatics approach to better predict cancer risks for variants where there are no functional, biological or patient data.

Experimental procedures

Mutagenesis

All the BRCA1 BRCT missense variants recorded (as of Novermber, 2008) in the Breast Cancer Information Core (BIC) and eleven additional patient-derived variants were generated. The eleven additional variants are: T1700A (Abkevich et al. 2004), A1708V (Lovelock et al. 2007), D1739V, R1753T (Carvalho et al. 2007), M1775K (Tischkowitz et al. 2008), D1778Y, M1783I, Q1785H (Carvalho et al. 2007), E1794D (Carvalho et al. 2007), H1805P and R1835P.

Coding sequences for the tandem BRCT repeats of BRCA1 were generated with PCR primers FT7 (5'-gga cga gaa ttc tta acc agg gag ctg att atg gtg aac aaa aga atg tcc atg-3') and CD6 (5'-gat ctg gga tcc tca ggg gat ctg ggg tat cag-3'). The 5' primer FT7 includes a ribosome binding site and an EcoRI restriction site for cloning. The 3' primer CD6 includes stop codons and a BamHI restriction site for cloning. The mutant PCR products were then cloned into the T7 promoter based expression vector, pLM1-BRCA1-BRCT (1646-1858), as previously reported (Williams et al. 2003). For P1859R, P1856S, L1854P, Y1853C, D1851E, L1844R, A1843P, S1841R, S1841N, V1838E, W1837C, W1837G, W1837R, E1836K, R1835P and V1833M, coding sequences were amplified using FT7 and modified CD6 primers that incorporated relevant mutations. For N1647K, S1651F, M1652T, M1652I, V1653M, S1655F, G1656D, F1662S, M1663L,

M1663K, L1664P, V1665M and A1669S, coding sequences were amplified using CD6 and modified FT7 that included relevant mutations. All other missense substitutions were engineered using PCR splicing methods (Horton et al. 1993). All the vectors were sequenced to confirm presence of desired mutations.

Proteolysis assays

0.25 μg of pLM1 plasmid encoding the BRCT variants was used directly as the template for protein synthesis reactions with the TNT-Quick *in vitro* transcription/translation system (Promega). Both wild type and mutant proteins were produced by *in vitro* transcription/translation because many mutants could not be expressed in soluble form in *E. coli.* Immediately prior to proteolytic digestion, proteins were translated and labeled with [35S] methionine at 30 °C for 2 h. The reticulocyte lysates were then centrifuged for 2 min at 10,000 x *g* to remove insoluble material, and 3 μl of the lysate supernatants containing the labeled translation products were added to 12 μl of digestion buffer (150 mM NaCl, 50 mM potassium phosphate, pH 7.5) containing increasing concentrations of trypsin (Sigma). After digestion at 20 °C for 12 min, the reactions were stopped with phenylmethylsulfonyl fluoride. The digestion products were electrophoresed on 15% SDS-PAGE gels and visualized with a phosphorimaging plate and a Molecular Dynamics Typhoon scanner. A local average background correction was used during quantification of the

reaction products with ImageQuaNT (Amersham Biosciences). The measurements were normalized against the internal background and the control measurements taken on the same day. The missense variants were tested in triplicate.

Functional assays

The BRCT variants were translated and labeled as in proteolysis assays. The ability of the BRCA1 BRCT mutants to bind pSer-containing peptides was tested using peptide-binding assays, as previously reported (Williams et al. 2004).

Bead-immobilized peptide affinity resin was prepared in binding buffer (150mM NaCl, 50mM TrisHCl, pH 7.5, 0.1% (v/v) NP-40, 1mM EDTA, 1mM DTT) by incubating a ten-fold molar excess of a biotinylated phosphopeptide (SRSTpSPTFNK) and the corresponding unphosphorylated peptide (SRSTSPTFNK) with streptavidin agarose beads (Sigma-Aldrich) for 50 min at 4°C. Excess peptide was removed by washing five times with ten bed volumes of binding buffer. [35S]methionine-labeled BRCT variant containing lysate (1μl) was added to 20μl of affinity resin in binding buffer supplemented with 0.3 mg/ml BSA, which was used to reduce background resin binding. After incubation for 2 hr at 4°C, the resin was washed three times with 200 μl binding buffer. Seven mutants, with the wild type BRCA1-BRCT (1646-1858) as a positive control and M1775R and A1708E as negative controls, were

examined at a time. Bound BRCT variants were eluted from the resin with the addition of 20 ul SDS-PAGE loading buffer, run on a 15% (w/v) SDS-PAGE gel, and visualized using a Molecular Dynamics Typhoon phosphorimager. The intensities of the bands were measured and the backgrounds were corrected using ImageQuaNT (Amersham Biosciences). To compensate for pipetting errors, radioactivity decay and gel exposure time difference, the measurements were normalized against the wildtype measurement taken on the same day. The binding assays were performed in triplicate.

Results

Generation of a large set of BRCA1 BRCT missense variants

Many of the BRCA1 variants that have been uncovered through world-wide sequencing efforts are now deposited within the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/), as well as the Kathleen Cuningham Foundation Consortium for research into Familial Breast Cancer (kConFab) database (http://www.kconfab.org) (Figure 4.1). To facilitate the assessment of the structural and functional defects associated with these mutations, we cloned all the currently available missense variants into an *E. coli* expression vector, which we used previously for our structural and functional studies of the BRCA1 BRCT domain. This vector places the BRCA1 BRCT coding sequence (corresponding to residues 1646-1859) under control of a T7 promotor. Using this plasmid system, recombinant protein can be expressed in *E. coli* and purified using established procedures for detailed biochemical and structural studies. The T7 promoter can also be utilized for *in vitro* transcription/translation of the recombinant protein. The *in vitro* transcription/translation system is rapid and allows the generation of multiple proteins in parallel and, since the recombinant protein is specifically labeled with ^{35}S-methionine, no purification is required. All variants can be successfully made in this system, regardless of their folding stability. This is a further advantage over *E. coli* expression, where we have found that only well-folded variants can be expressed in soluble

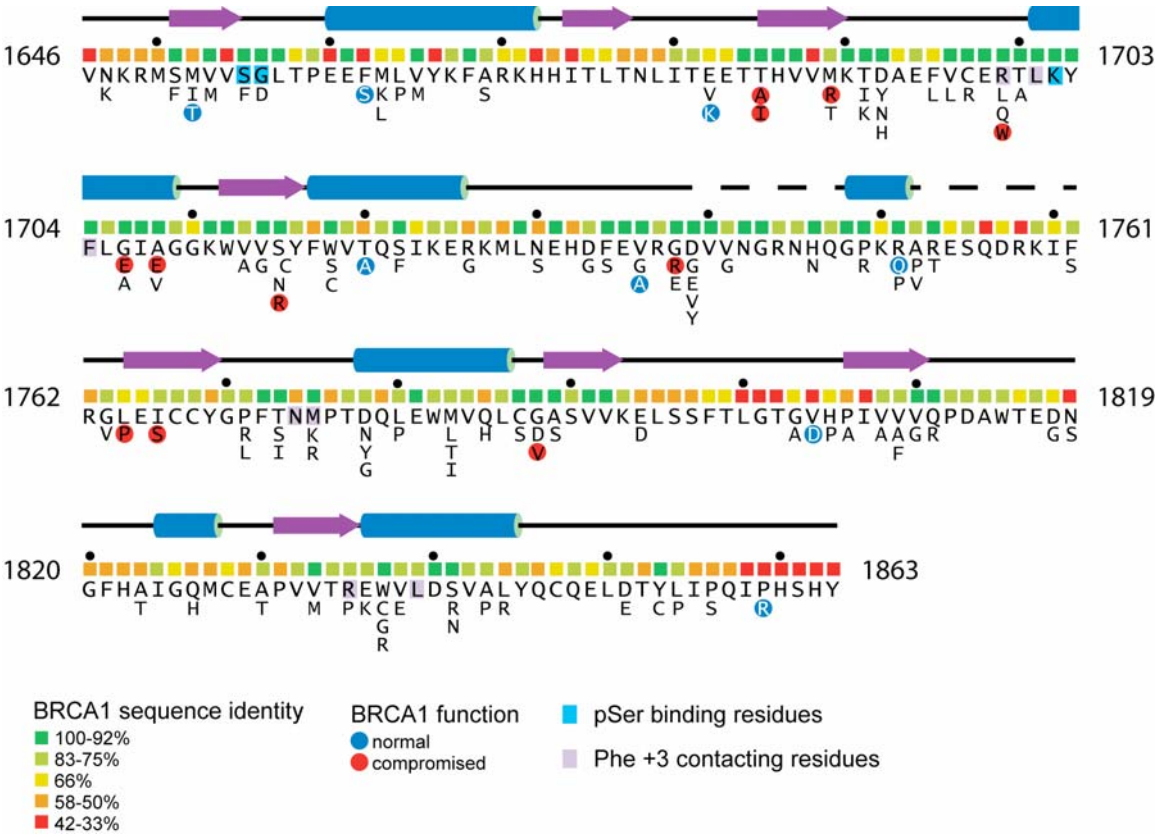


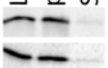
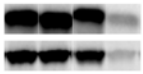
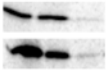
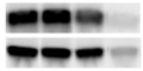
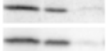
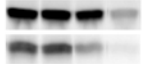
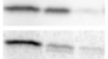
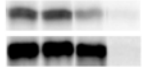
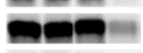
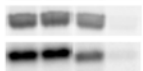
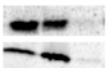
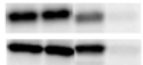
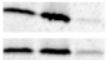
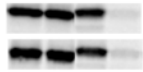
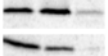
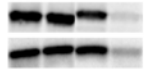
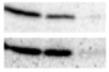
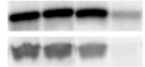
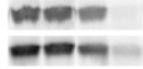
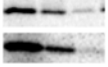
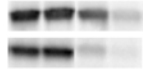
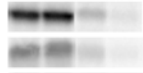
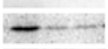
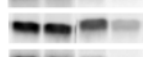
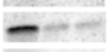
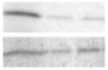
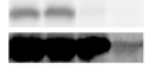
Figure 4.1. The complete BRCA1 BRCT missense variant set. Shown is the sequence of the human BRCA1 BRCT domain with the protein secondary structure indicated. Below the sequence are colored boxes indicating the level of sequence identity of each residue within a 13 species alignment (http://agvgd.iarc.fr/alignments.php). Neutral and disease-associated variants (Easton et al. 2007) are highlighted, as are residues that contact the pSer and Phe at the +3 position of the phospho-peptide target.

form and purified. Thus, we have used *in vitro* transcribed/translated material for our structural stability and peptide binding assays described below.

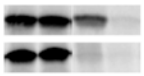
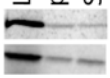
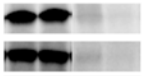
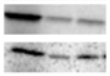
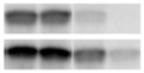
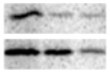
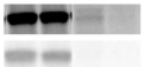
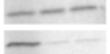
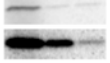
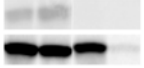
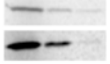
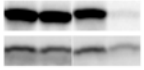
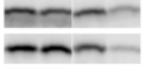
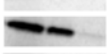
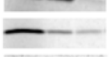
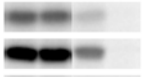
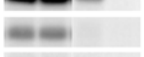
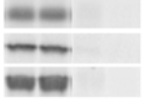
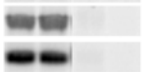
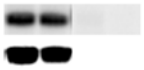
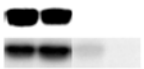
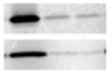
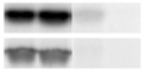
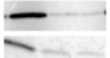
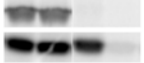
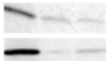
Determination of the protein folding defects associated with BRCT missense variants

We used limited proteolysis to determine the stability of the protein fold in the set of BRCA1 BRCT missense variants produced by *in vitro* transcription/translation (Figure 4.2). The wild type protein is highly resistant to digestion by trypsin, elastase or chymotrypsin, indicating that the *in vitro* produced BRCT protein is stably folded (Williams, Green & Glover 2001). On the other hand, amino acid substitutions in the BRCT domain of BRCA1 have previously been shown to destabilize its structure, leading to increased susceptibility to trypsin-mediated proteolysis (Williams et al. 2003, Williams & Glover 2003, Williams et al. 2004). Mutations that occur in non-surface areas may destabilize the hydrophobic BRCT core or the hydrophobic interrepeat interface, leading to the unfolding of the structure (Williams, Green & Glover 2001). Therefore, in the cases where a substitution is not expected to introduce a new trypsin clevage site, the enhanced trypsin sensitivity is an indication of a subtle structural change in a given mutant. The level of sensitivity of the BRCA1 BRCT mutants to proteolytic degradation should indicate a degree of

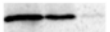
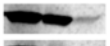
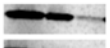
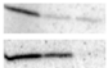
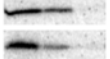
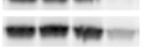
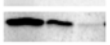
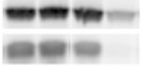
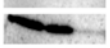
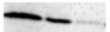
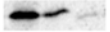
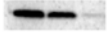
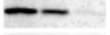
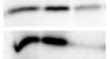
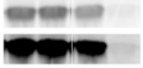
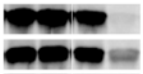
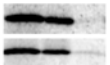
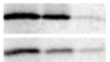
a.

Variant	Structural assay		Functional assay			Clinical data	Interspecies sequence variation[13]
	Trypsin 1 2 3 4	Structural stability(%)	Load pSPTF SPTF	Binding activity(%)	Binding specificity(%)		
WT		100		100	100		
N1647K		123±4		93±27	78±24		
S1651F		84±2		118±23	112±28		
M1652T		84±7		79±22	83±10		
M1652I		79±4		75±24	100±7	-[1]	
V1653M		40±6		57±10	47±19		
S1655F		113±8		9±2	36±11		
G1656D		88±4		3±1	8±2		
F1662S		121±8		67±33	76±18		
M1663L		87±4		56±17	98±14		
M1663K		87±10		94±25	102±19		
L1664P		93±10		103±31	139±13		
V1665M		82±4		77±5	72±3		
A1669S		70±2		108±18	66±7	-[2]	-
E1682K		92±5		76±11	87±15		
E1682V		66±3		107±8	55±1		
T1685I		13±1		32±10	11±6		+
T1685A		18±3		16±0	14±1		+
M1689T		91±3		31±17	22±8		
M1689R		20±22		N/A	N/A		+
T1691K		3±0		10±4	2±1		
T1691I		3±1		68±37	5±2		
D1692H		62±3		48±14	32±15		
D1692N		108±5		88±27	130±12	+[3]	
D1692Y		54±3		83±17	45±8	+[3]	
F1695L		64±10		113±10	59±3		-
V1696L		44±5		4±2	15±2		+
C1697R		3±3		37±0	22±11	+[4]	
R1699W		90±2		3±2	8±3	+[5]	
R1699Q		96±2		9±3	10±2	+[6]	+
R1699L		80±2		9±4	5±1		+
T1700A		85±5		3±2	5±2		+
G1706A		65±8		108±21	108±12		+
G1706E		2±0		5±0	14±2	+[7]	+
A1708V		58±3		24±3	16±10		
A1708E		4±1		24±5	9±4	+[5]	

b.

Variant	Structural assay		Functional assay			Clinical data	Interspecies sequence variation[13]
	Trypsin 1 2 3 4	Structural stability(%)	Load pSPTF SPTF	Binding activity(%)	Binding specificity(%)		
V1713A		61±3		25±7	3±3		
V1714G		4±1		13±3	9±1		
S1715R		1±0		10±4	9±1	+[7]	+
S1715N		22±7		42±25	7±3		+
S1715C		59±1		91±28	39±27		+
W1718C		4±0		97±3	11±1		
W1718S		5±1		18±5	8±5		+
T1720A		74±1		87±4	56±4	-[7]	
S1722F		3±3		19±2	12±6		
R1726G		100±1		84±11	126±27		-
N1730S		91±5		114±43	94±37		
D1733G		95±9		113±15	99±5		
F1734S		20±2		55±16	20±5		
V1736A		22±2		72±5	49±3		+
V1736G		8±1		14±1	5±1		
G1738E		3±0		71±3	3±3	+[8]	+
G1738R		2±1		15±10	3±0	+[5]	+
D1739Y		2±1		10±8	5±1		
D1739V		0±1		5±4	11±2		
D1739E		11±2		6±2	20±5		+
D1739G		N/A		16±7	1±0		
V1741G		97±3		5±3	8±9	-[9]	
H1746N		55±4		9±7	10±1		+
P1749R		3±1		29±18	18±9	+[10]	
R1751P		0±1		6±3	3±1		+
R1751Q		59±4		76±12	58±10	+[11]	
A1752V		0±0		89±7	31±8		
A1752P		N/A		16±12	5±1		
R1753T		0±1		42±9	16±9		
F1761S		18±1		N/A	N/A		
G1763V		41±1		19±1	15±7		
L1764P		18±0		23±9	19±6	+[7]	
I1766S		0±1		94±11	5±1	+[7]	+
P1771L		74±5		75±15	87±38		
P1771R		75±5		97±31	85±16		
T1773I		78±5		63±5	15±7		

c.

Variant	Structural assay		Functional assay			Clinical data	Interspecies sequence variation[13]
	Trypsin 1 2 3 4	Structural stability(%)	Load pSPTF SPTF	Binding activity(%)	Binding specificity(%)		
T1773S		70±3		68±7	71±25		
M1775R		48±5		3±1	21±10	+[12]	
M1775K		103±7		19±3	12±0		
D1778G		103±6		87±21	88±15		
D1778Y		99±8		82±23	80±8		
D1778N		90±6		10±19	92±12		–
L1780P		9±2		29±5	2±0		+
M1783I		112±2		84±16	92±12		
M1783T		75±10		48±7	59±2		+
M1783L		106±0		139±16	96±27		
Q1785H		101±7		98±3	66±22		
C1787S		108±7		113±2	72±8		+
G1788V		0±0		107±6	16±3	+[7]	+
G1788D		100±6		66±2	32±10		+
A1789S		87±4		56±7	63±13		
E1794D		93±2		112±26	109±25		
G1803A		84±5		92±5	103±10		
V1804D		89±9		119±10	53±1	-[7]	
H1805P		70±2		102±21	75±16		
P1806A		74±2		79±15	106±13		
V1808A		95±5		68±12	58±10		
V1809F		12±2		72±22	39±8		
V1809A		84±4		66±13	49±8		
V1810G		83±2		97±9	69±3		
Q1811R		51±3		37±2	20±0		+
D1818G		57±6		104±6	88±10		
N1819S		90±6		98±1	82±6		
A1823T		112±2		141±2	105±9		
Q1826H		88±3		52±6	59±25		
A1830T		100±1		85±9	49±2		
V1833M		55±4		96±22	80±8		
R1835P		106±14		N/A	N/A		
E1836K		67±8		2±0	2±1		
W1837R		3±0		18±3	10±4		+
W1837G		2±1		24±9	39±3		+
W1837C		2±2		7±3	1±0		

d.

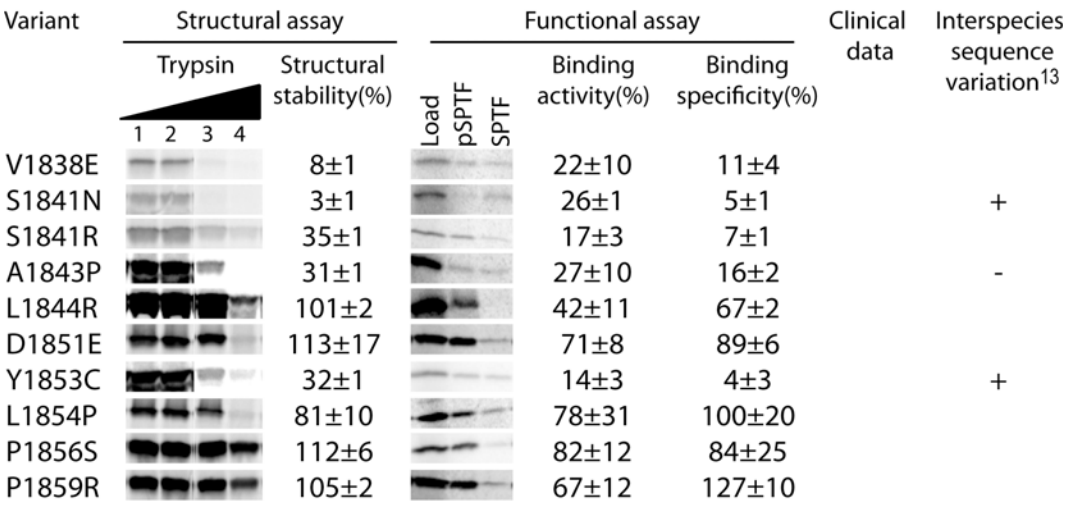


Figure 4.2. Evaluation of BRCA1 missense variants. *Structural assay:* Destabilization of the BRCT domains by missense mutations. The indicated mutations were digested with increasing concentration amounts of trypsin. Lanes 1-4: 0.1, 1, 10 and 100 μg/ml trypsin. *Functional assay:* Ability of the missense variants to bind pSer-containing peptide and to distinguish between the phosphopeptide and the nonphosphopeptide. The far left lane shows 100% of the load material. The ability of the wt BRCA1 to discriminate phosphopeptide over nonphosphopeptide was expressed as 100%, with the other results placed on this scale. *Clinical data:* Many studies characterized missense variants based on histopathological features of tumours and other various clinical data (refer to Figure References). -, neutral; +, clinically deleterious. *Interspecies sequence variation:* Abkevich *et al.* (Abkevich et al. 2004) classified 314 BRCA1 missense variants via a combination of a multiple sequence alignment of orthologous BRCA1 sequences and a measure of the chemical difference between the amino acids at individual residues in the sequence alignment. -, neutral; +, clinically deleterious.

structural defect caused by substitution. The assay is also sufficiently sensitive to discriminate wild-type protein from the strongly destabilized variant A1708E, as well as the moderately destabilized variant, M1775R. Proteolysis of each of the missense variants was carried out at a series of trypsin concentrations, and the amount of full length protein remaining was visualized by SDS-PAGE. The results were compared to those of the wild-type protein as well as the M1775R and A1708E variants carried out in parallel. We quantified the percentages of full-length protein remaining following digestion at different concentrations of trypsin and determined the severity of the destabilizing effects by comparing the percentage of protein remaining following digestion at 10 μg/mL trypsin to the controls (Figure 4.3). At 1 μg/mL and 100 μg/mL, the differences of percentages were insignificant amongst the mutants. We also noted that C1697R mutant showed particularly low protein expression levels.

Analysis of the phospho-peptide binding activity of BRCT missense variants

The tandem BRCT repeats of BRCA1 function as phosphopeptide-binding modules (Manke et al. 2003, Rodriguez et al. 2003, Yu et al. 2003). The BRCA1 BRCT domain interacts with BACH1 and several other proteins through the selective recognition of the peptide motif pSer-x-x-Phe (Rodriguez et al. 2003, Botuyan et al. 2004, Shiozaki et al. 2004, Clapperton et al. 2004, Williams et al. 2004, Yu et al. 2003). In addition,

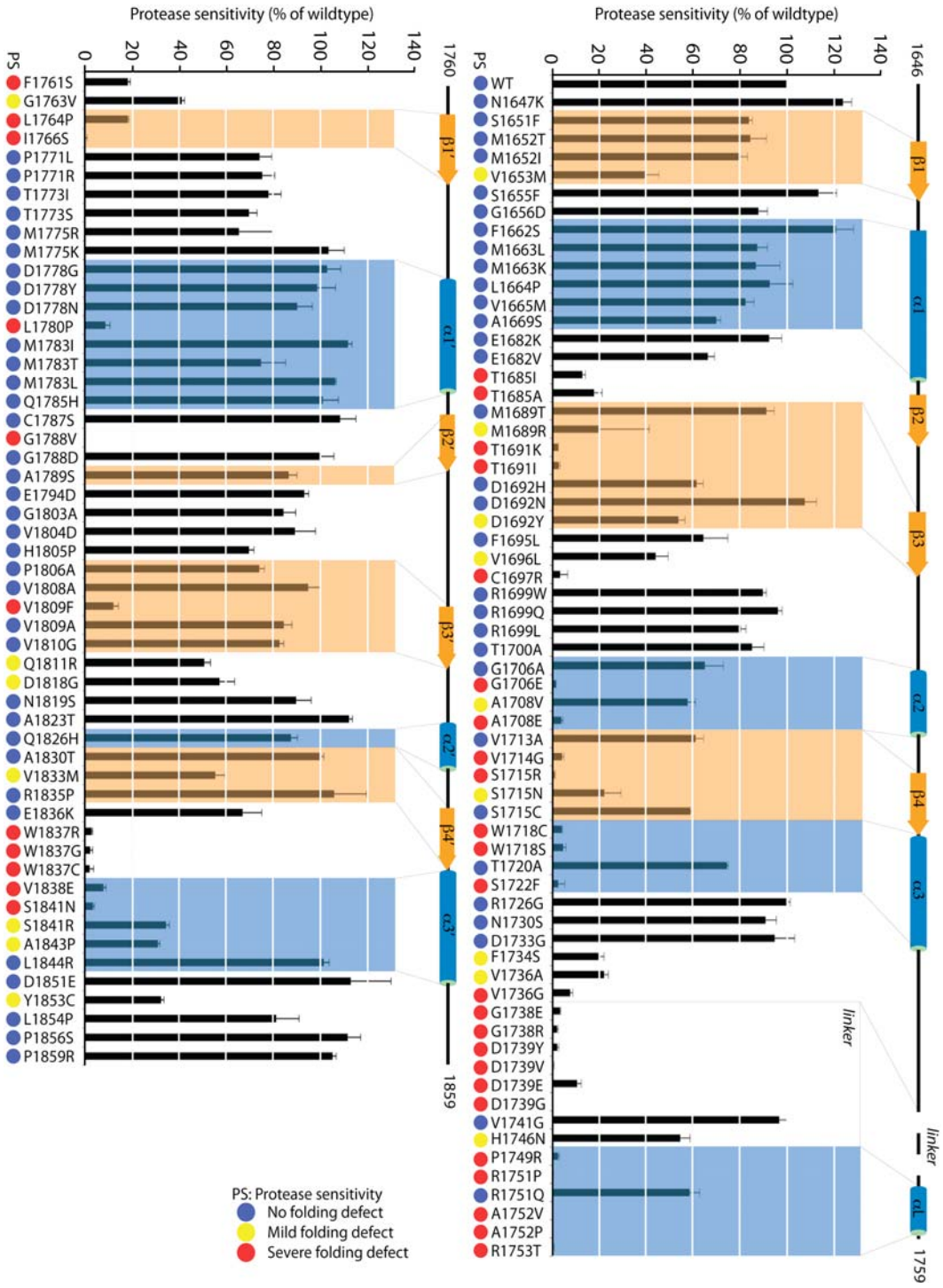


Figure 4.3. Analysis of the proteolytic stabilities of the BRCA1 BRCT missense variant set. The proteolytic stabilities of each variant were determined as described in the text and plotted relative to the stability of the wild type domain. Error bars represent standard deviations derived from the results of at least 3 independent experiments. The variants are clustered into three groups based on the level of folding defect observed.

the mutations linked to disease disposition, such as A1708E and M1775R, fail to bind specifically to the phosphorylated motif in BACH1 (Williams et al. 2004). This indicates the functional importance of the BRCA1 BRCT domain as a phosphopeptide-binding module in tumor suppression.

We examined the ability of the BRCT missense variants to bind peptides derived from BACH1 using a pull-down assay in which biotinylated peptide was bound to streptavidin beads (Figure 4.2). We tested binding to a phosphorylated version of the peptide containing the BRCA1 target sequence pSer-Pro-Thr-Phe, or an unphosphorylated version as a control. The results were compared to experiments with the wild type BRCA1 BRCT, as well as with the peptide-binding deficient variants, A1708E and M1775R carried out in parallel. We present two analyses of the data. In the first, we define “binding activity” as the fraction of the input protein which bound to the phosphorylated peptide, normalized against the binding of the wild type protein (Figure 4.4). In the second, we define “binding specificity” as the ratio of the variant bound to the phosphopeptide compared to the nonphosphorylated counterpart, normalized to the wild type control (Figure 4.5). As expected, none of the highly misfolded variants were found to specifically bind the phospho-peptide target. However, five of the severely destabilized variants (T1691I, W1718C, A1752V, I1766S, G1788V) showed significant non-specific binding to the peptides, independent of phosphorylation. It is likely that the unfolded state of these variants exposes hydrophobic residues that are

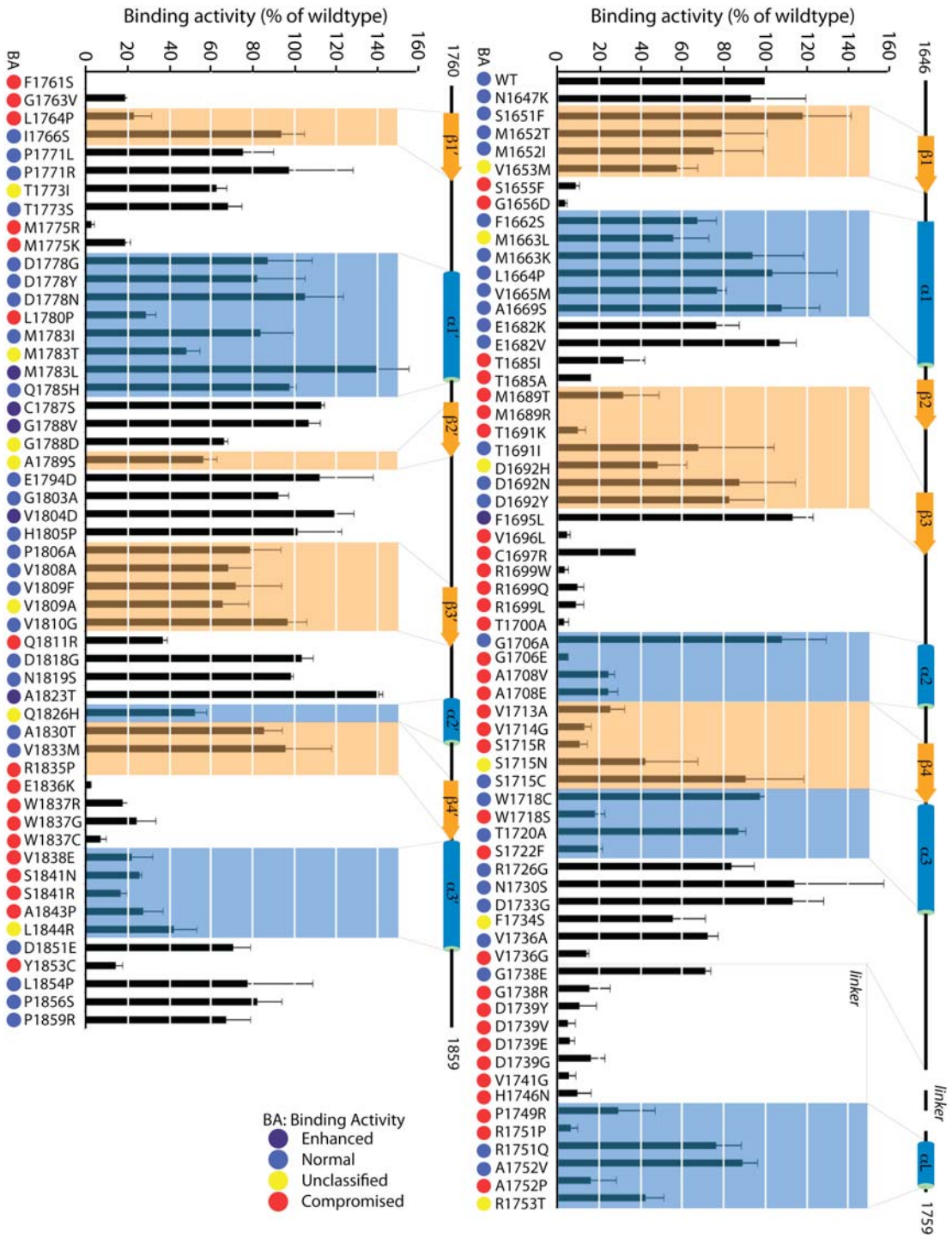


Figure 4.4. Analysis of the phospho-peptide binding activities of the BRCA1 BRCT missense variant set. The overall binding of the variant to the phospho-peptide was measured in a pull-down assay and normalized to the wild type protein control. Error bars represent standard deviations derived from the results of at least 3 independent experiments. The variants are clustered into three groups based on the level of binding defect observed.

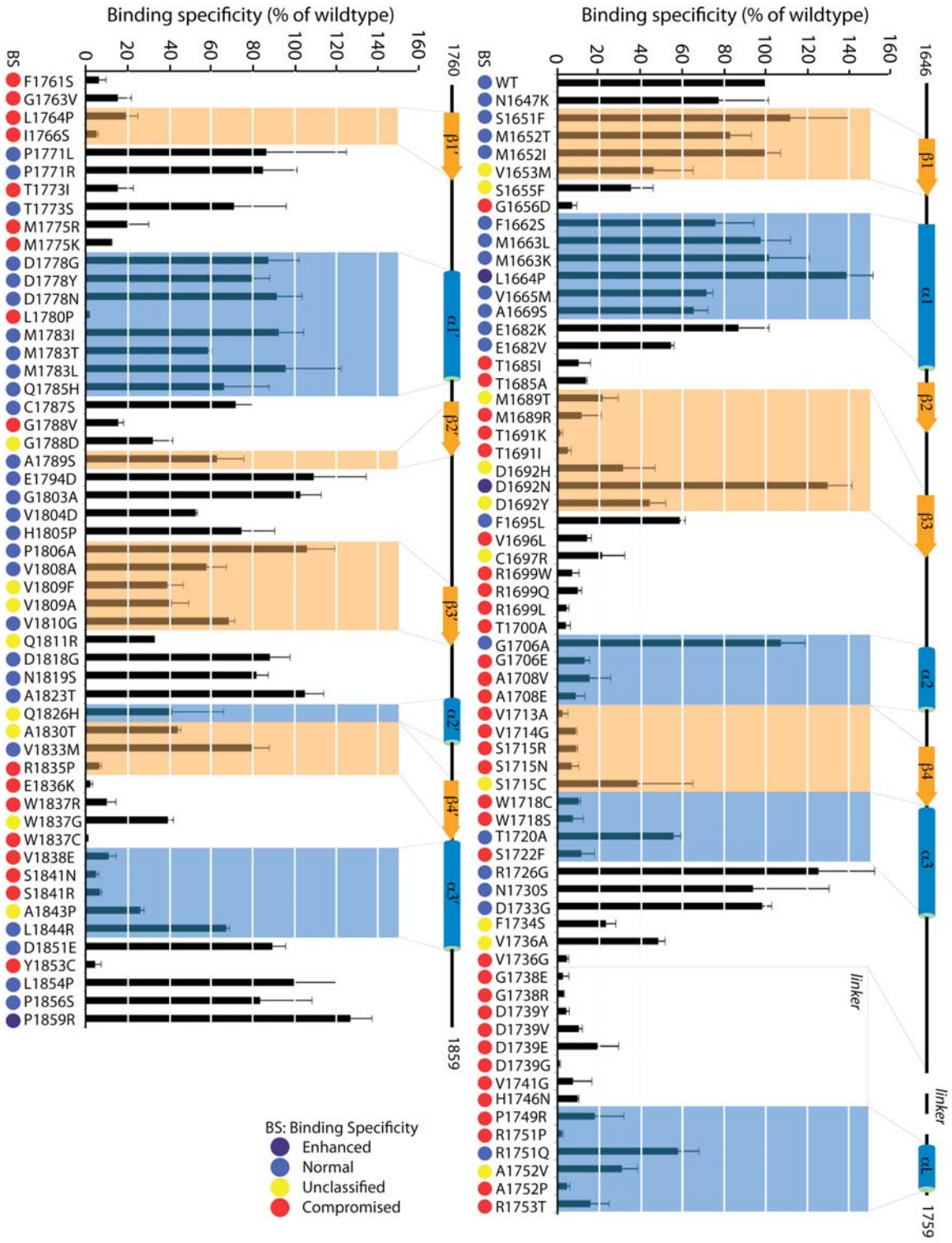


Figure 4.5. Analysis of the phospho-peptide specificity of the BRCA1 BRCT missense variant set. The level of binding of the variant to the phospho-peptide relative to the non-phosphorylated version of the peptide was measured in a pull-down assay and normalized to the wild type protein control. Error bars represent standard deviations derived from the results of at least 3 independent experiments. The variants are clustered into three groups based on the level of binding specificity.

responsible for these non-specific interactions, as suggested previously (Williams et al. 2004). Intriguingly, a number of structurally stable variants showed significant phospho-peptide binding defects, suggesting that in these variants a localized structural perturbation, and not a global unfolding of the BRCT domain, is responsible for the loss of phosphopeptide binding (see below).

Correlation of structural and functional data with family history and clinical data

Easton *et al.* have used a combination of data, including co-occurrence with known deleterious mutations, personal and family history of patients carrying the variant, as well as co-segregation of the variant with disease within pedigrees, to assign an overall risk score, termed 'combined $\log_{10}$-likelihood-ratio (LLR) score' for several of the BRCA1 BRCT variants. Sufficient data was available to assign 11 of the BRCA1 BRCT variants as strongly associated with disease, while 8 of the variants were predicted to be neutral. To compare the structural and functional data to this model of risk assessment, we plotted the protein stability and peptide binding data for these 19 variants against their combined LLR scores as calculated by Easton et al. (Easton et al. 2007) (Figure 4.6). In general, this analysis revealed a clear correlation between structural/functional defects and cancer risk. Only one of the variants that are strongly associated with disease, R1699W, is structurally stable.

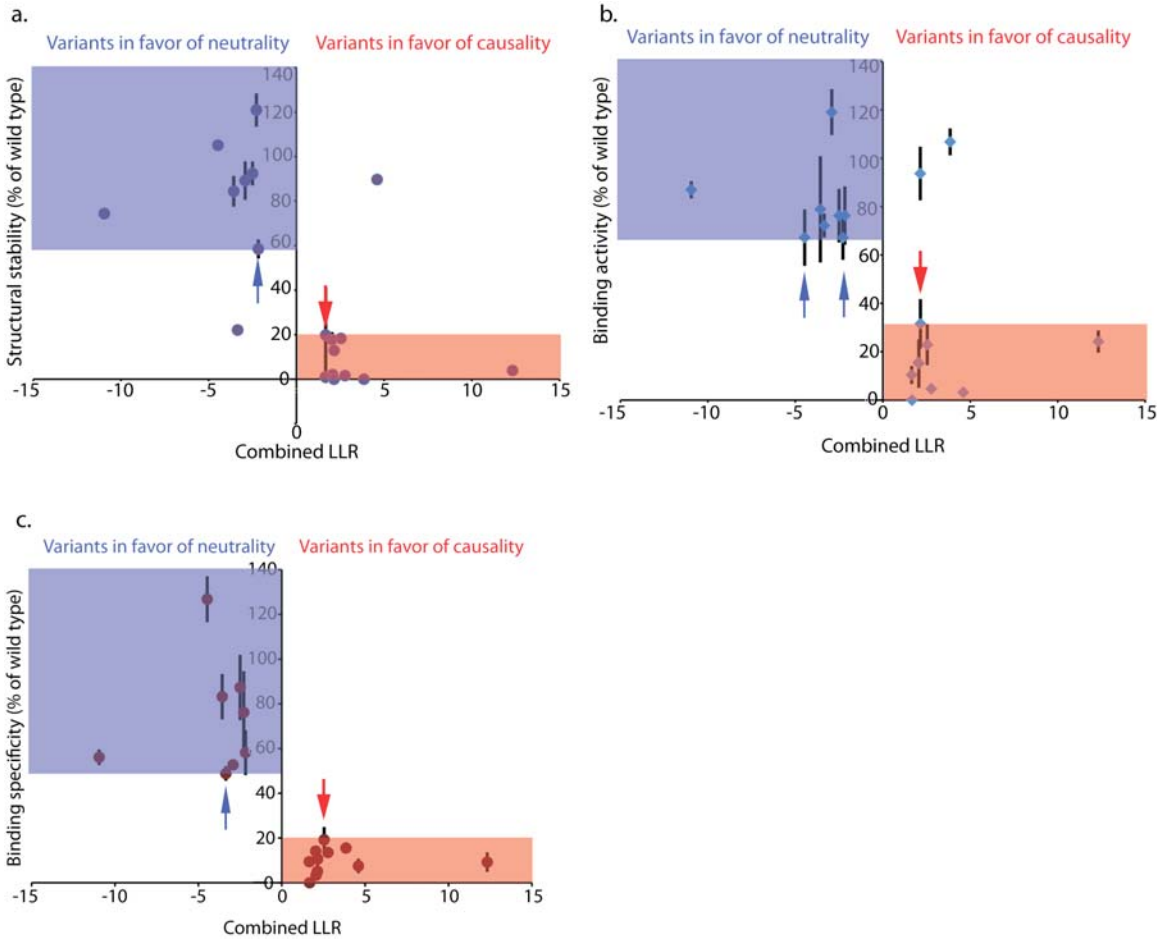


Figure 4.6. Correlation of structural and functional data with family history and clinical data. Analyses of 19 variants were plotted against the combined $\log_{10}$-likelihood-ratio score. The average binding activity of each the variant as percent of wild type activity is indicated with standard deviation indicated by bars. a. Structural stability. b. Phosphopeptide binding activity. c. Phosphopeptide binding specificity. *Blue* and *red arrows* - neutral variant with lowest activity and deleterious variant with highest activity, respectively.

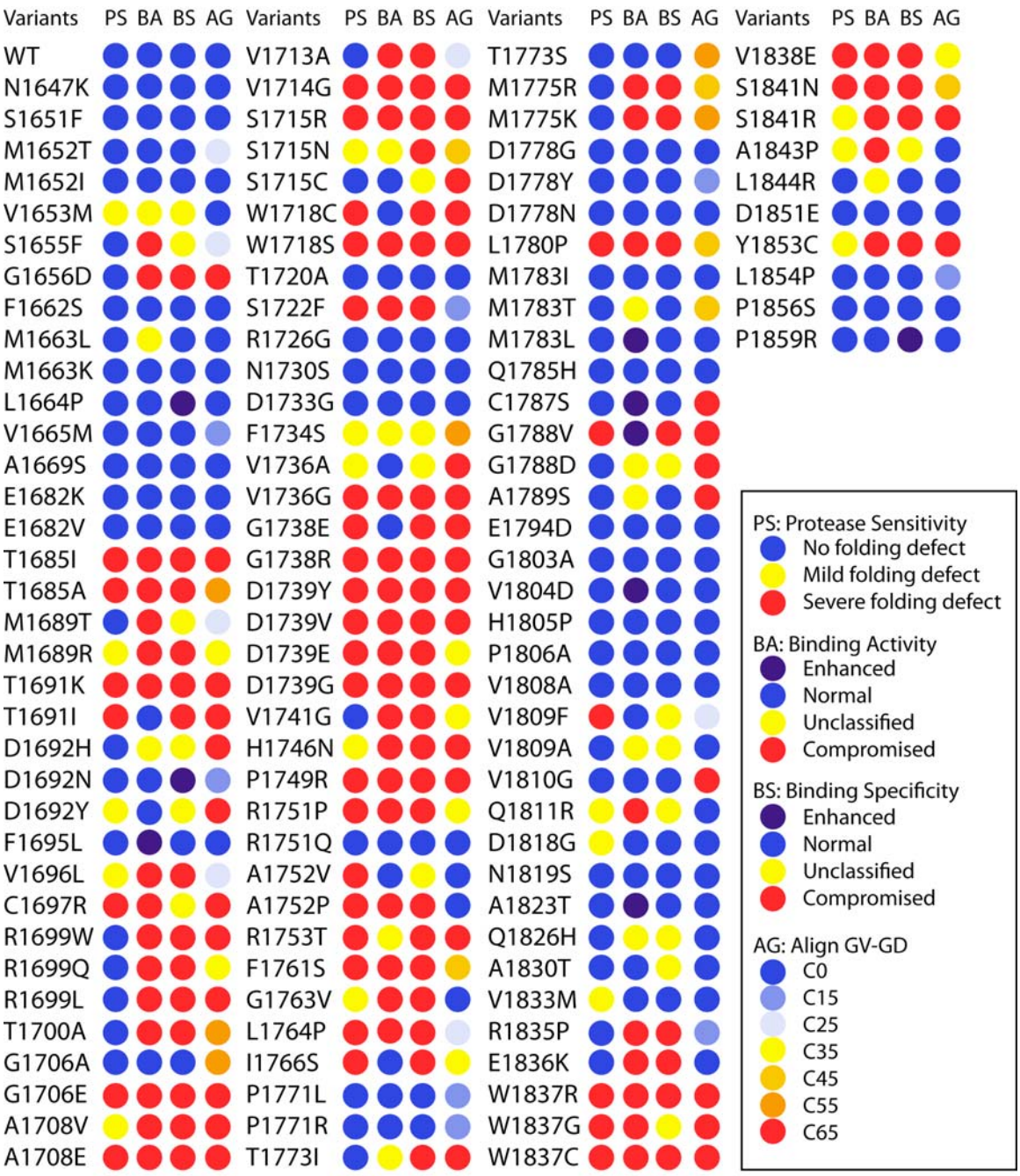


Figure 4.7. Cross validation of the structural and functional assays with align-GVGD grades. The Align-GVGD method uses the Grantham Deviation and the Grantham Variation scores to classify missense variants into seven categories, with C0 being the class of the lowest risk to cause deleterious mutation and C65 being the one of the highest risk.

However it is highly defective for specific phospho-peptide recognition, suggesting a subtle structural perturbation in this variant. Only one of the neutral variants, V1736A, showed significant sensitivity to proteolysis, yet this variant nevertheless demonstrated strong phosphopeptide interaction.

Using these correlations, we have defined limits to predict disease association for the uncharacterized variants based on the structural and biochemical assay data (Figure 4.7). The cancer-associated variants in general exhibited protein stability levels <20% of the wild type (Figure 4.6a), phosphopeptide binding activities <32% of wild type (Figure 4.6b), phosphopeptide specificity binding activities <20% of wild type (Figure 4.6c). Conversely, the neutral variants in general had protein stability levels >59% of the wild type (Figure 4.6a), phosphopeptide binding activities >67% of wild type (Figure 4.6b), phosphopeptide specificity binding activities >49% of wild type (Figure 4.6c).

Based on these criteria, 35/117 of the misssense mutations showed high sensitivity to tryptic digestion, 16/117 showed intermediate sensitivity, and 66/117 showed a stability that was indistinguishable from the wild type. We also determined that 49/117 showed no defects in binding activity, while 49/117 showed a significant defect, and 6 variants actually showed a small but significant enhancement in peptide binding activity. Similar numbers were obtained for peptide binding specificity: 45/117 showed comparable levels of specificity to wild type, 51/117 showed a

significantly decreased level of peptide binding specificity, while 3 of the variants showed enhanced peptide binding specificity.

Based on all the criteria taken together, 25 variants are predicted to be strongly associated with disease while 42 variants are predicted to be neutral. Several mutants are structurally stable yet show significant peptide binding and transcriptional defects and these likely have very specific structural rearrangements which target the peptide binding groove of the BRCT (see Discussion). These variants have been assigned as associated with an increased disease risk. The remaining 50 variants fall into the grey area between strong disease risk and neutrality and may represent a class of low penetrance variants.

Effects of sequence conservation

The effect of missense changes on phenotype strongly correlates with the chemical difference between amino acids (Abkevich et al. 2004). Therefore we postulated that a high degree of chemical difference between the two amino acids would negatively affect the BRCA1 phosphopeptide binding function. We chose to use two scores, Grantham Deviation (GD) and Grantham Variation (GV), for each amino acid residue substitution to measure the degree of chemical difference (Tavtigian et al. 2006, Grantham 1974). GV measures the degree of biochemical variation among amino acids found at a given position in the multiple sequence alignment and GD measures the 'biochemical distance' of the mutant

amino acid from the observed amino acid at a particular position (Tavtigian et al. 2006). The method is an extension of the original Grantham difference, which is based on a multivariate combination of residue side chain composition, polarity and volume (Grantham 1974).

We plotted the GD and GV scores of the mutant against their phosphopeptide binding function (Figure 4.8). We observed a small positive correlation between the binding specificities of the mutants and their GV scores (r = 0.59) (Figure 4.8a). In addition, a majority of the mutations of invariant residues (GV score = 0) showed low binding specificity (<20%), indicating they may be deleterious mutations. As expected, we observed a negative correlation between the binding specificities of the mutants and their GD scores (r = -0.61) (Figure 4.8c), confirming that the positions of functionally defective mutants are strongly biased toward invariant residues. However, the correlations between the binding activities and their GV and GD scores were weaker (r = 0.44 and -0.47 respectively) (Figure 4.8b and d).

Effects of solvent accessibility

The residues that form the hydrophobic core of a protein are essential for its stability. Many studies have suggested that the hydrophobic core residues are likely the sites of deleterious mutations (Hecht, Nelson & Sauer 1983, Loeb et al. 1989, Rennell et al. 1991, Sandberg et al. 1995, Suckow et al. 1996). Based on the theory that

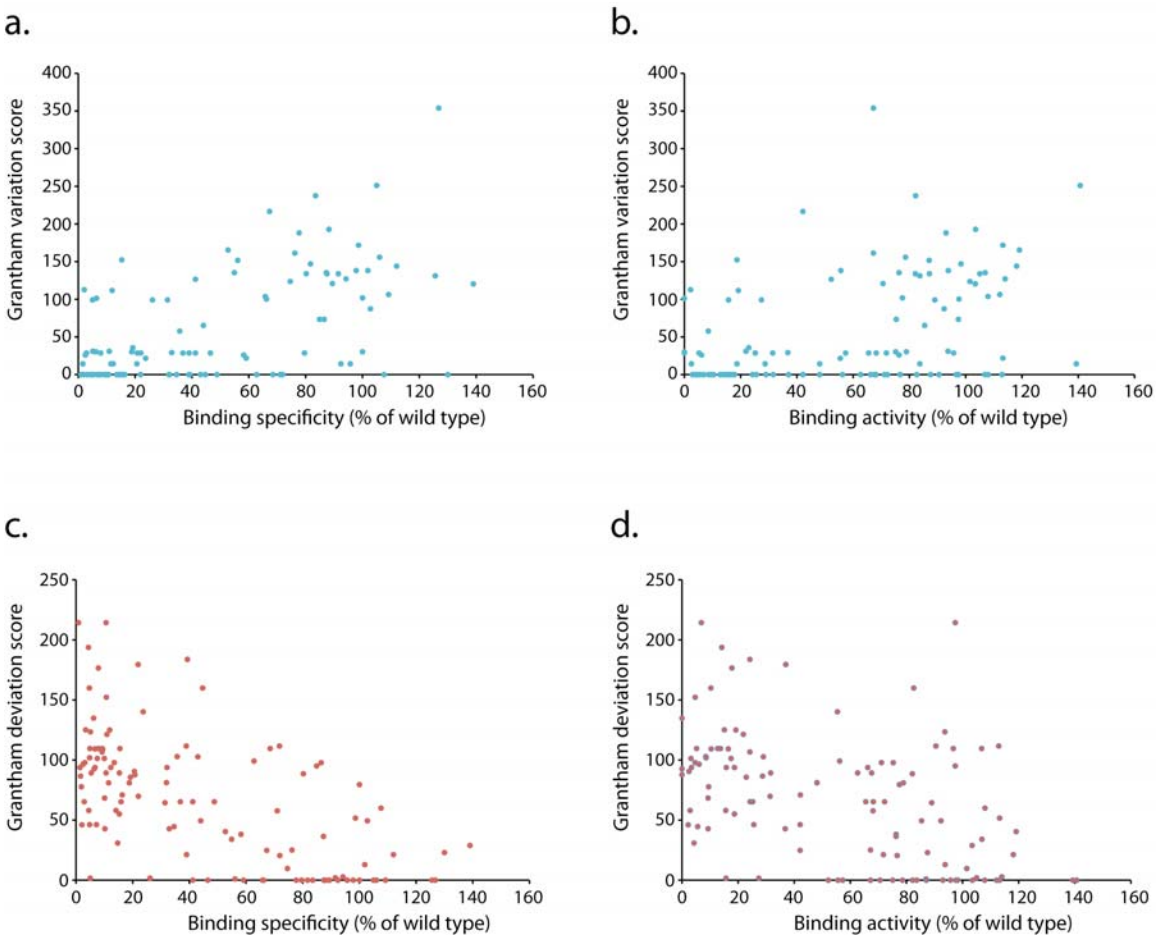


Figure 4.8. Effect of sequence conservation on ability to bind phosphopeptides. **a & b.** Analyses of the binding specificities and activities of the variants in context of their Grantham variation scores. **c & d.** Analyses of the binding specificities and activities of the variants in context of their Grantham deviation scores.

among many structural characteristics, the hydrophobic core stability parameters are the most paramount predictors of disease mutations (Ramensky, Bork & Sunyaev 2002), we proposed to identify buried residues of the BRCA1 BRCT repeats and investigate the peptide binding function of missense mutations at those sites. We used the program, Naccess v.2.1.1 (http://www.bioinf.manchester.ac.uk/naccess/), by the Lee & Richards method (Lee & Richards 1971) to calculate residue solvent accessibilities (Figure 4.9). Notably, many mutants with low binding activities (<20%) clustered around 0% solvent accessibility. However, there was no strong correlation between the peptide binding function and the solvent accessibility (r = 0.33 for binding activites and r = 0.52 for binding specificities).

Effects of distance from the BRCA1 BRCT phosphopeptide pocket

A conserved BRCT phophoserine-binding pocket is located between the BRCT repeats (Williams et al. 2004) and the mutations of residues that line the binding pocket resulted in loss of function (Figure 4.1 & 4.2). Therefore we postulated that the distance between the mutated residue and the peptide binding pocket could indicate the degree of the peptide binding function of the variant. The distances between the Cα of each mutated residue and the pSer and Phe (+3) of the phosphopeptide were measured (PDB ID 1T2V). Then the variants were divided into two groups depending on which residue of the peptide the variant was closer

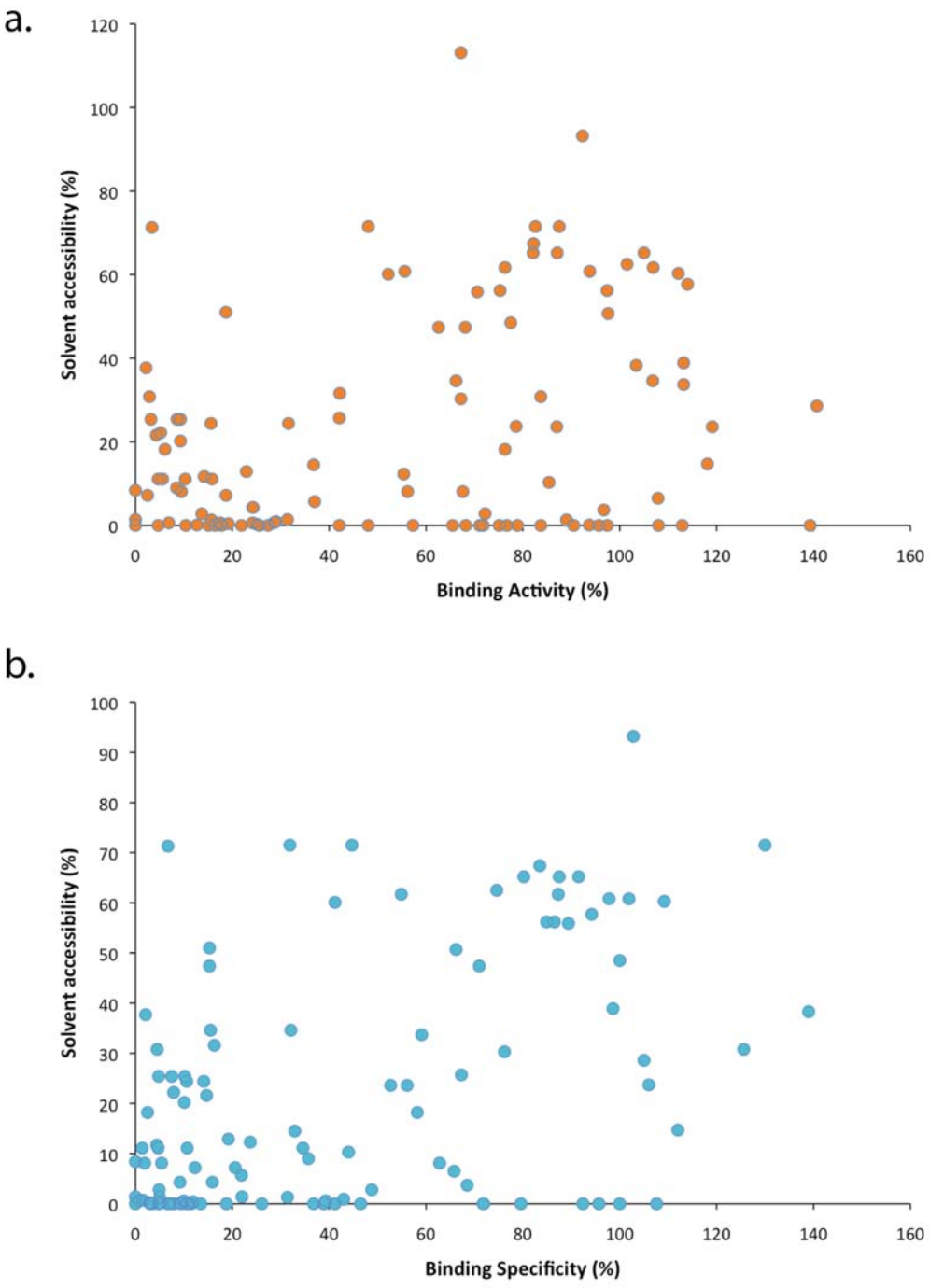


Figure 4.9. Effect of solvent accessibility on ability to bind phosphopeptides. The phosphopeptide binding (a) activities and (b) specificities of mutations are examined in context of their solvent accessibility.

to 49 variants were closer to the pSer (Figure 4.10a) and the other 65 variants were closer to the Phe (+3) (Figure 4.10b). Our analyses indicated no strong correlation between the peptide binding specificity and the distance of the mutation from the phosphoserine binding pocket (r=0.24) nor the Phe (+3) binding pocket (r=0.49).

Interestingly, the correlations between the protein function and the distances improved when the highly destabilizing mutants were excluded from the analysis (r=0.42 for the pSer pocket and r=0.52 for the Phe(+3) pocket). This may signify that the distance from the peptide binding pocket does not play any role in protein function once the protein structure is highly destabilized.

Splicing abnormalities due to missense mutations

Evidence suggests that missense modification can be deleterious by affecting normal pre-mRNA splicing via disruption of the consensus sequences (i.e., the 5' donor and 3' acceptor splice sites and the branch site) or creation of cryptic sequences (Cartegni, Chew & Krainer 2002, Zatkova et al. 2004). To determine whether any missense variant affected the normal splicing pattern and possibly resulted in functional changes, we used splice junction scores by a systematic analysis with the NNSplice 0.9 algorithm (Reese et al. 1997) to measure the theoretical effect on splicing (Table 4.1). The software's default thresholds (0.4) were used for finding

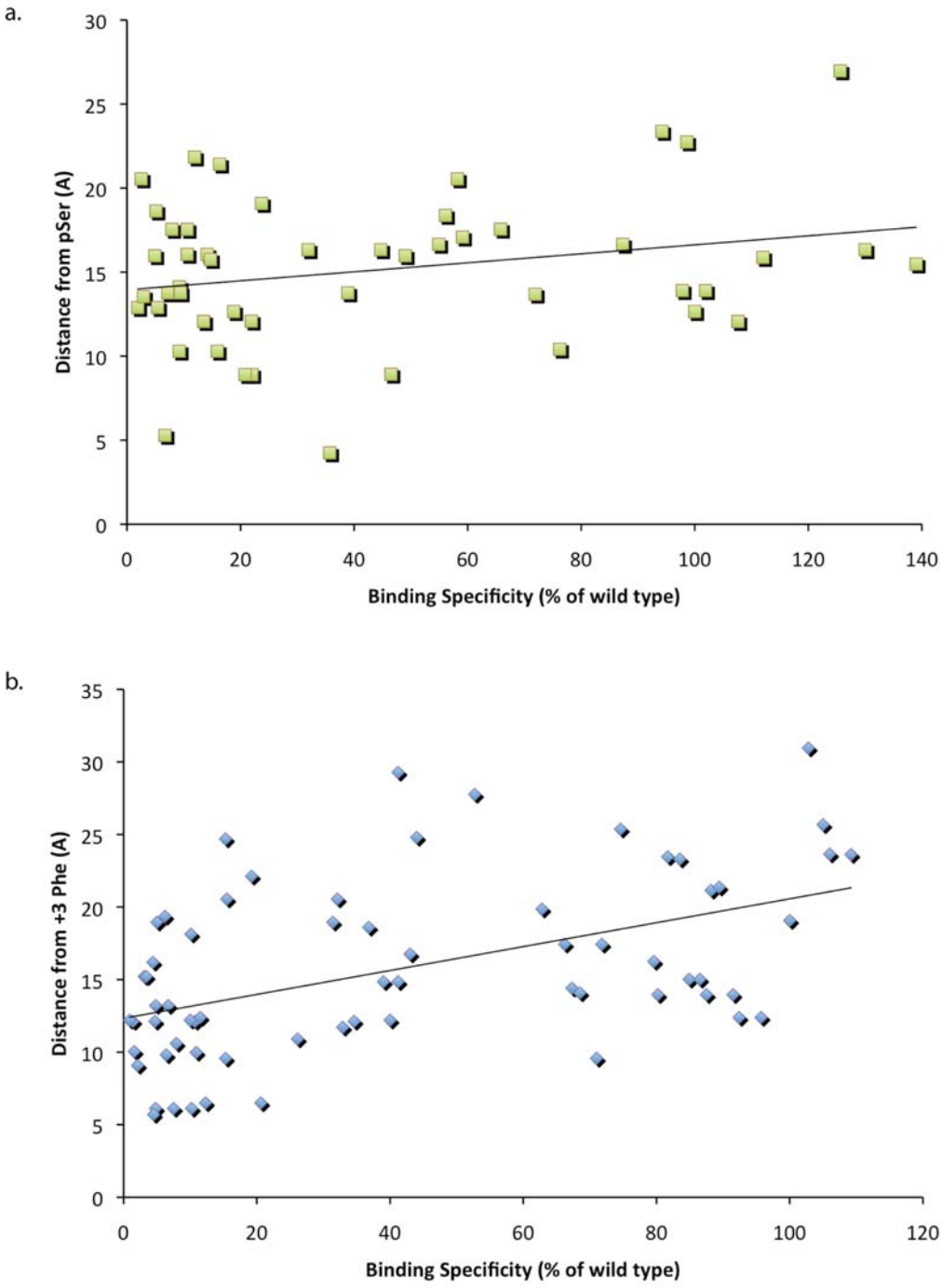


Figure 4.10. Effect of distance from the peptide binding pocket on ability to bind phosphopeptides. a. The binding activities of the 49 missense variants were plotted against the distances from the pSer of the phosphopeptide. **b.** The binding activities of the 65 missense variants were plotted against the distances from Phe (+3) of the phosphopeptide.

Missense mutation	Location	Splice junction scores (wt>mutant)
M1663L(A5106T)	first nt of exon 17	0.61>0.34
M1663K(T5107A)	2nd nt of exon 17	0.61>0.24
D1692H(G5193C)	last nt of exon 17	0.92>0.13
D1692N(G5193A)	last nt of exon 17	0.92>0.03
D1692Y(G5193T)	last nt of exon 17	0.92>0.02
V1714G(T5260G)	11nt upstream from 5′ splice site	0.24>0.97
S1715R(C5264G)	7nt upstream from 5′ splice site	0>0.64
W1718C(G5273T)	2nd nt of exon 19	0.01>0.51
A1752V(C5374T)	22nt upstream from 5′ splice site	0>0.53
M1775R(T5443G)	8nt upstream from 5′ splice site	0.72>0.21
M1775K(T5443A)	8nt upstream from 5′ splice site	0.72>0.21
D1778Y(G5451T)	last nt of exon 21	0.72>0
D1778N(G5451A)	last nt of exon 21	0.72>0
V1809F(G5544T)	18nt downstream from 3′ splice site	0.07>0.47

Table 4.1. BRCA1 BRCT pre-mRNA splicing abnormalities due to missense mutations. The scores indicated are assigned by the web-based tool (NNSplice) following analysis of the input genomic sequence. Default thresholds were used for finding 5' and 3' splice sites. Nucleotide positions are numbered on the basis of the genomic sequence.

splice sites and we arbitrarily specified that the mutant score should be at least 20% lower or higher that the wild type score to consider the prediction as positive and deleterious as previously reported (Houdayer et al. 2008).

Fourteen of the tested mutants showed splice defects (Table 4.1). Nine mutations (M1663L, M1663K, D1692H, D1692N, D1692Y M1775R, M1775K, D1778Y ad D1778N) induced a dramatic reduction in consensus scores. These nine mutations could result in skipping an exon and this can be verified only by analysis by RNA analysis. Interestingly, M1663K, D1692N, D1778Y and D1778N bound specifically to phosphopeptides (Figure 4.2). Five mutations (V1714G, S1715R, W1718C, A1752V and V1809F) created cryptic splice sites with high scores and they all displayed defective peptide binding function (Figure 4.2).

Recent work by Toulas et al. provided additional evidence that the variant A1708E should be considered as deleterious due to exon skipping (Millevoi et al. 2009). With A1708E, an increase of binding activity by the mutated RNA to *cis*-regulatory elements known as Exonic Splicing Silencer (ESS) was observed and the sequencing of RT-PCR products of the carriers showed the presence of heterozygosity of exon splicing. The mutated exon was predominantly but not completely skipped, suggesting that a fraction of mutated mRNAs can avoid the aberrant splicing and encode a misfolded protein with defective functional properties.

Reclassification of the BRCT missense mutations

Our previous study classified the BRCT missense mutations into four categories based on their distribution in the BRCT domain structure: surface, BRCT interface, BRCT fold and BRCA1 fold mutations (Williams et al. 2003). However, the classification was prior to the findings that the BRCA1 BRCT domain functions as a phospho-peptide binding domain (Yu et al. 2003, Manke et al. 2003). Thus, several residues need to be reclassified into a new category, peptide-binding pocket mutation. This category should include S1655F, G1656D, R1699W, R1699Q, R1699L, T1700A, M1775R, M1775K and R1835P. S1655, G1656 and T1700 make contact with the phosphate and R1699, M1775 and R1835 are positioned in a groove that interacts with Phe (+3).

Discussion

Here we present the first comprehensive assessment of the structural and functional consequences of the complete set of BRCA1 BRCT missense variants detected to date in the human population. Our protease sensitivity and peptide binding assays show a remarkable degree of agreement, and give us confidence in the validity of these results. Correlation of this experimental data with the available clinical and family history data for a subset of these variants defines limits, which can be used to tentatively predict the disease risk associated with these variants. In this way, we predict that 31% of the variants are associated with an increased cancer risk, while 36% are predicted to be neutral, and the remaining 33% are intermediate, and perhaps represent low penetrance variants.

The total number of unique BRCA1 mutations recorded in the BIC at this time is 1643 and 570 of them are missense mutations. Of those 570 mutations, 56 occur in the BRCA1 RING domain (fragment 1-109, (Brzovic et al. 2001)) and 109 occur in the BRCA1 BRCT domain. Only seven of the 109 mutations are considered as cancer-associated by the BIC steering committee and the rest remain unclassified. Unlike truncation mutations where impact on function can be clearly inferred, missense mutations continue to be difficult to characterize due to low frequency in the general population. This poses a real threat in breast and ovarian cancer risk assessment. Our study results in a prediction of cancer

association of the BRCA1 BRCT domain mutations that cannot be classified by other traditional approaches. As expected, the mutations that significantly destablize the protein fold abolish peptide binding activity. On the other hand, the missense mutations that cause little or no folding defects demonstrated a range of peptide binding activities (Figure 4.11). We drew a hypothetical trend line, assuming a perfect correlation between the structural stability and functional activity in the BRCA1 BRCT domain. A majority of the mutants either fall on or are above the trend line, indicating that the protein cannot function without a proper structure.

Structurally stable variants with defective function

Mutations affecting the phosphopeptide binding pocket led to defective peptide binding function. S1655 supplies a critical ligand for phosphoserine recognition and this interaction is supported by hydrogen-bonding interactions with T1700. The mutations, S1655F and T1700A, abolished the phosphopeptide interaction to the BRCT repeats and gross structural destabilization is a doubtful cause for their defective protein function. It is likely that a lack of hydroxyl group in the mutant S1655F and T1700A and sterical interference by the phenyl ring in S1655F led to defective peptide binding.

The main chain amide of G1656 residue also supplies a key ligand for recognition of the phosphate group of the phosphopeptide target. The

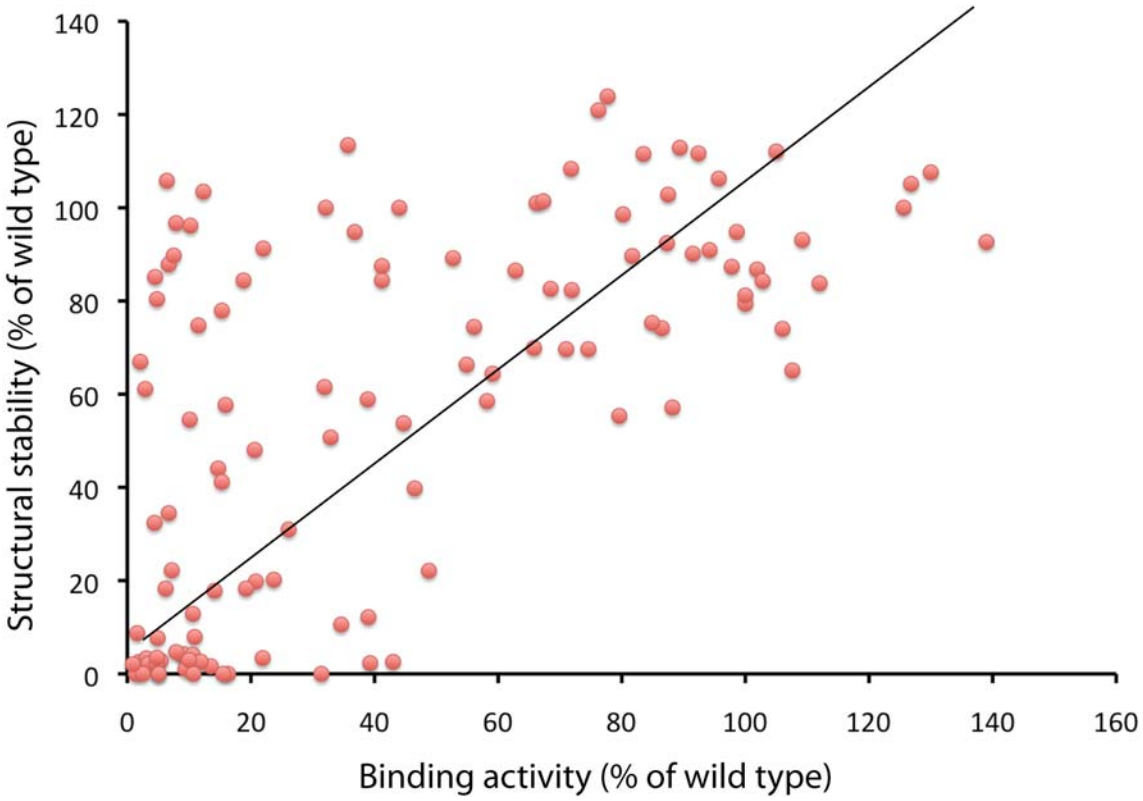


Figure 4.11. Structural stability and functional activity of 117 BRCA1 BRCT missense mutations. Values are the mean of the triplicate proteolysis and peptide binding assays. A theoretical trendline is drawn, intercepting 0 and 100.

mutation from Gly to Asp introduces a negative charge, which will likely result in electrostatic repulsion with the negatively charged phosphoserine and may also perturb the local structure, reducing the strength of the mainchain hydrogen bond.

M1689 is buried within the BRCT structure and packs against S1655. While the mutation M1689T does not destabilize the overall BRCT fold, it is likely that the mutation to the smaller Thr sidechain influences the orientation of S1655 and thereby contributes to a peptide binding deficiency.

V1696 is located near the BRCT surface and distant from the phosphopeptide binding surface. The mutation V1696L decreases the overall protein fold stability, perhaps through a destabilization of a salt-bridge network involving R1744, D1692 and E1694.

R1699 plays a critical role in phosphopeptide binding through interactions with the mainchain of the peptide, which orients the Phe(+3) sidechain into the specificity pocket. Three distinct variants of this residue have been identified: R1699W, R1699Q and R1699L. The mutation R1699W has been shown to be cancer-linked by blocking the protein's interaction with BACH1 (Shiozaki et al. 2004, Clapperton et al. 2004) and our assays showed that the mutant lost peptide binding activity and phospho-specificity. The mutant R1699L also showed decreased peptide binding function despite the presence of possible main chain hydrogen bonds. R1699W and R1699L are predicted to disrupt the critical

recognition of the mainchain carbonyl of the Phe(+3) residue. Although the glutamine side chain in R1699Q may be expected to form a main chain hydrogen bond to the peptide as arginine does in the wild-type (Clapperton et al. 2004, Williams et al. 2004), the mutant showed equally defective peptide binding function. R1699Q would be unable to make salt bridging interactions with E1836 and D1840 that stabilize the orientation of R1699. It is therefore possible that R1699Q is more flexible than R1699, thus weakening recognition of the peptide and explaining the peptide binding defects associated with this variant.

Two distinct missense variants have been uncovered at residue 1708: A1708E and A1708V. Pedigree analysis shows that A1708E is associated with an increased cancer risk, and this substitution results in a profound destabilization of the BRCT structure, likely because the negatively charged glutamic acid side chain cannot be accommodated within the tight, hydrophobic environment of the wild type alanine residue. A1708V is a conservative substitution, which results in a more subtle structural perturbation and a significant phospho-peptide binding defect. It is possible that the larger Val side chain may be poorly accommodated within the tight interface between the two BRCT repeats. A subtle destabilization of the head-to-tail packing of the BRCT repeats may be enough to perturb the structure of the peptide binding groove.

The conservative substitution, V1713A, within the hydrophobic core of the protein resulted in a modest decrease in protein stability but a

dramatic reduction in phosphopeptide binding. V1713 is located in the four-stranded parallel β-sheet in the N-terminal BRCT. A sequence alignment of 13 BRCA1 orthologs showed that hydrophobic, β-branched amino acid residues are conserved at this position (Carvalho et al. 2007). Hence V1713 is most likely critical to the stability of the BRCT domain fold. Although Ala is hydrophobic, is not β-branched. In addition, V1713 contacts M1689, which in turn supports S1655 and the pSer binding pocket. It is possible that the substitution may destablize the conformation of M1689 and thereby the pSer binding pocket.

A series of mutations affecting S1715 showed interesting binding differences. When Ser was mutated to Arg, the binding activity and the specificity were the worst of the three. And the mutation to Cys affected the binding activity and the specificity the least. Normally S1715 is located in a tight space, therefore it is presumably the differences in size of the mutant side chain that caused the differences in binding activity and specificity.

V1741, located in the inter-BRCT linker, makes van der Waals contact with both the peptide C-terminal to the +3 residue, as well as to R1699. As regions in the peptide C-terminal to the +3 residue contribute little to overall BRCT binding, it is unlikely that this direct interaction explains the dramatic peptide binding deficiency observed in V1741G. Instead, we suggest that packing of the Val against R1699 may help to stabilize its orientation, thereby strengthening its interactions with the

peptide backbone. Interestingly, F1979 in MDC1 packs against and likely stabilizes the orientation of R1933 in a similar manner.

H1746 is also in the inter-BRCT linker and is buried in the interface between the two repeats and participates in a long, buried hydrogen bond with Y1703. The H1746N substitution would disrupt the hydrogen bond to Y1703 without a significant rearrangement of the backbone structure. Interestingly, the structural stability of this variant is indistinguishable from the wild type protein, however, peptide binding is severely destabilized. We therefore suggest that a subtle conformational rearrangement occurs in this variant that perturbs its peptide binding properties.

A1752 is in the BRCT linker α helix, buried within the protein. Why the conservative substitution, A1752V, results in a profound destabilization of the protein fold and a significant loss in phospho-peptide binding activity is unclear.

G1763 makes a β-turn to initiate β1 of the second BRCT repeat. Mutation of this residue to the more conformationally restricted Val is predicted to disrupt the structure of this turn, thereby explaining the moderate conformational destabilization detected in the proteolysis assay.

The three variants, T1773I, Q1811R and R1835P, all occur in the same region of the protein and show similar structural and functional defects. T1773 is located in the specificity loop of the second BRCT repeat which is critical for recognition of the Phe side chain at the +3 position of the phospho-peptide target. Hydrogen bonding interactions involving the

buried hydrophilic residues R1835 and Q1811 likely help to stabilize the loop conformation. Mutation of the Thr to Ile would reduce hydrogen bonding to Q1811 and thereby could destabilize the conformation of this loop. This could explain the significant loss of specific phospho-peptide binding activity associated with this mutation. Similarly, Q1811R shows a moderate destabilization of the protein fold and a significant loss in peptide binding activity. This substitution is predicted to disrupt hydrogen bonding interactions with T1773 and also introduce charge repulsion with the adjacent R1835. The R1835P substitution would also disrupt these interactions, as well as perturb the structure of the α3' helix.

As described previously, M1755R and M1775K are associated with a mild protein folding defect and a strong peptide binding defect due to a specific disruption of the Phe(+3) binding pocket. The crystal structure of M1775K revealed that the side chain of the substituted Lys sterically clashes with the phenyl ring of Phe(+3) of the peptide, in a manner similar to M1775R (Tischkowitz et al. 2008, Williams et al. 2004).

G1788 is highly conserved in the BRCT protein family and helps to define a sharp turn in the polypeptide backbone between α1' and β2' in the C-terminal BRCT repeat. While this turn might be predicted to be disrupted in either the G1788V or G1788D variant, our results indicate a significant difference in their structural stabilities; while G1788V is strongly destabilized, G1788D exhibits a similar stability to the wild type protein. A possible reason for this may lie in the close proximity of K1759 to G1788.

While the K1759 amino group does not contact any residue in the wild type structure, it is positioned to potentially form a salt bridge with D1788, thereby stabilizing the structure of the G1788D variant.

The V1809F variant shows a significant destabilization of the protein fold and a loss in specific peptide binding activity. We previously determined its crystal structure, which revealed an intriguing concerted, long range conformational change induced by the substitution of the Val side chain for the larger Phe residue which results in a perturbation of the +3 specificity pocket. In contrast, the V1809A variant shows no significant structural perturbation by the proteolysis assay, however, this variant still displays a small but significantly reduced peptide binding specificity.

E1836 creates salt-bridging interactions with R1699 and the Lys(+5) side chain of the phosphopeptide target. When Glu was mutated to Lys, our assays showed that the protein failed to interact with the phosphopeptide, supporting the previous spot blot and peptide library experiments (Rodriguez et al. 2003, Manke et al. 2003). The E1836K substitution introduces a potential repulsive charge interaction with Arg 1699, likely destabilizing its conformation and explaining the profound peptide binding defects associated with this variant.

A1843 is located on α3' in the second BRCT repeat and is buried within the protein core. An Ala for Pro substitution would be predicted to disrupt the α-helical structure of α3', explaining the structural destabilization and peptide binding defects associated with this variant.

Y1853 is located in the C-terminal tail of the second BRCT repeat and is largely packed within this repeat. A nonsense mutation at this position, resulting in the loss of the C-terminal tail, is associated with and increased cancer risk. The Y1853C variant shows significant protein folding defects and a loss of peptide binding activity. The reduced hydrophobicity of the introduced Cys, and its inability to recapitulate the hydrogen bonding interactions with D1840, an interacting partner of R1699, may contribute to these deficiencies.

Sequence conservation in BRCA1

BRCA1 contains a RING-finger motif at its N-terminus and two BRCT domains at its C-terminus and with the exception of these domains, BRCA1 shares no sequence similarity with other known proteins. Our approach is currently limited to the BRCT domain and cannot be applied to the entire protein, until further structural or functional data emerge for other regions of the protein. However, it should be noted that only the amino and carboxy termini of BRCA1 are highly conserved. The residues in the N-terminal BRCT repeat are >85% similar among human, canine and murine orthologs (Szabo et al. 1996). This high degree of conservation is maintained in the C-terminal BRCT repeat as well and the three residues affected by unquestionably disease-associated missense mutations (M1775R, A1708E and P1749R) are completely conserved among human, canine and murine BRCA1. At the amino end of the protein, the first 200 resides are 90% identical between human and canine

and 90% similar between human and murine (Szabo et al. 1996). On the other hand, the central portion of BRCA1 is rather divergent with 70% identity between human and canine and 53% identity between human and murine (Szabo et al. 1996). Taken together, these data indicate that the central portion of BRCA1 may be less sensitive to missense mutations than the BRCT domain.

Evaluation of the assays

Our method of direct transcription/translation from the PCR product, followed by protein digestion and phosphopeptide binding, will provide a fast and cost-effective method for mutant BRCT assessment (Figure 4.12). Some of our intermediate mutations may actually be associated with a moderate risk of cancer, possibly acting additively or multiplicatively to increase cancer risk (Johnson et al. 2007). In the case of rare variants, no single data source can unambiguously classify them into neutral or deleterious. However, our analysis can act as a monitor for the integrity of the BRCA1 BRCT domain.

The final predictions of pathogenicity are driven by the availability of information on tumour phenotype and segregation. Thus, we cannot be sure that all of the neutral missense changes we propose are actually neutral. Nor can we be sure that the deleterious missense changes we propose are actually deleterious. However, we believe that our method is

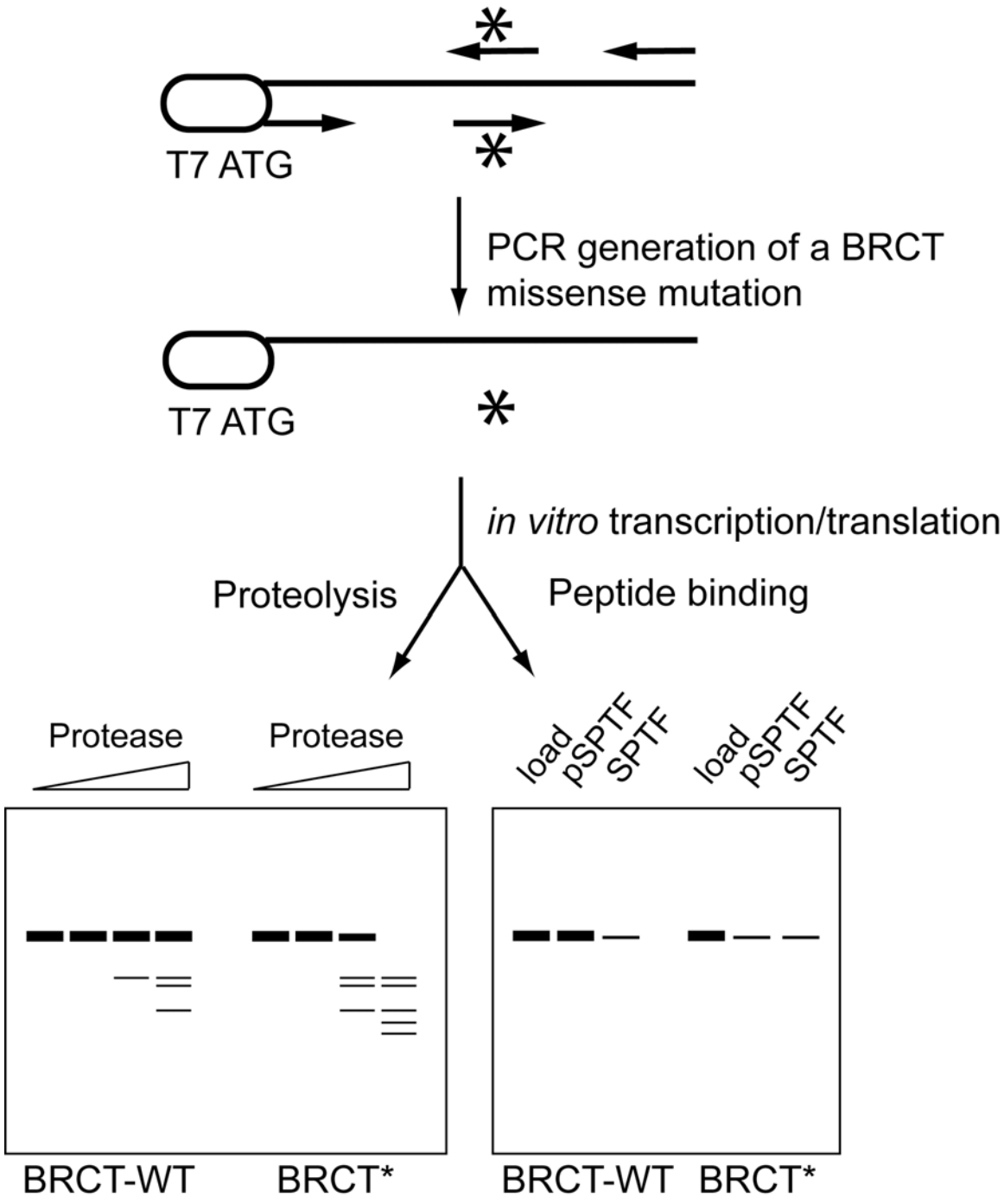


Figure 4.12. Schematic diagram of the protocol for classification of missense variants. BRCT missense mutations identified by sequencing are generated by PCR. The mutations are then transcribed and translated for proteolysis and peptide binding assays. In the proteolysis assay, the disappearance of protein species is monitored. In peptide binding assay, the presence of protein species is monitored. Adapted from Williams et al. 2003.

validated on two counts: first, our experimental design and protocols, such as performing all reactions in triplicate and using internal controls, ensured strong consistency in our experimental data. Second, our assessment of the BRCA1 BRCT missense variants is in good agreement with the clinical data and the predictions from other reported methods.

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Chapter 5:

General discussion and Conclusions

MDC1: Recruiting DNA damage response machinery to broken chromosomes

The X-ray structure of the MDC1 BRCT domain complexed with the phosphopeptide (KKApTQApSQEY) derived from the γH2AX C-terminus defined our model of phosphopeptide recognition (Stucki et al. 2005). The structure of the complex indeed confirmed our speculation that the organization of the MDC1-BRCT-γH2AX complex is astonishingly similar to that of the BRCA1-BRCT-BACH1 complex. The structure also showed that the conserved arginine residue (Arg1933) in the MDC1 BRCT domain formed a strong, dual salt-bridging interaction with the negatively charged carboxyl terminus of γH2AX, whereas in BRCA1, the analogous arginine (Arg1699) contacts the main-chain carbonyl group of the BACH1 peptide at the +3 position from the pSer residue via an uncharged hydrogen bond (Figure 5.1). Selection for tyrosine rather than phenylalanine at the +3 position is explained by a water-mediated hydrogen-bonding interaction with the side chain amide group of Gln2008 (Figure 5.2). In addition, the glutamic acid at the +2 position forms a water-mediated contact with Arg1933 via its carboxylate group, van der Waals interactions with Arg1932 and Thr1934 and electrostatic interactions with the basic region around Arg1932 (Stucki et al. 2005). The side chain of the Gln at the +1 position is directed away from the MDC1-BRCT surface toward bulk solvent. The +1 position most likely plays only a minor role in determining MDC1 binding specificity, however is a highly conserved, crucial

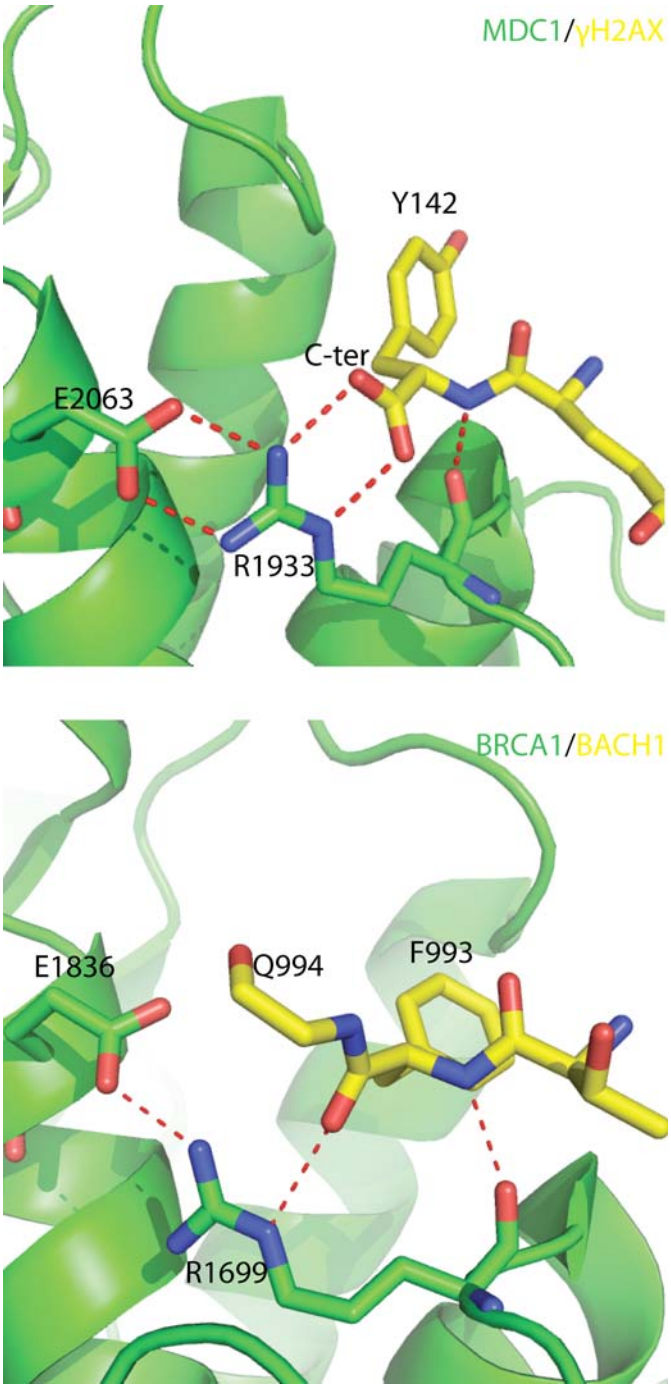


Figure 5.1. Comparison of the structures of MDC1-γH2AX (top, pdbID-2AZM) and BRCA1-BACH1 (bottom, pdbID-1T15) on recognition of the C-terminus. In MDC1, Arg1933 is positioned optimally to provide hydrogen-bonding to the charged H2AX C-terminus.

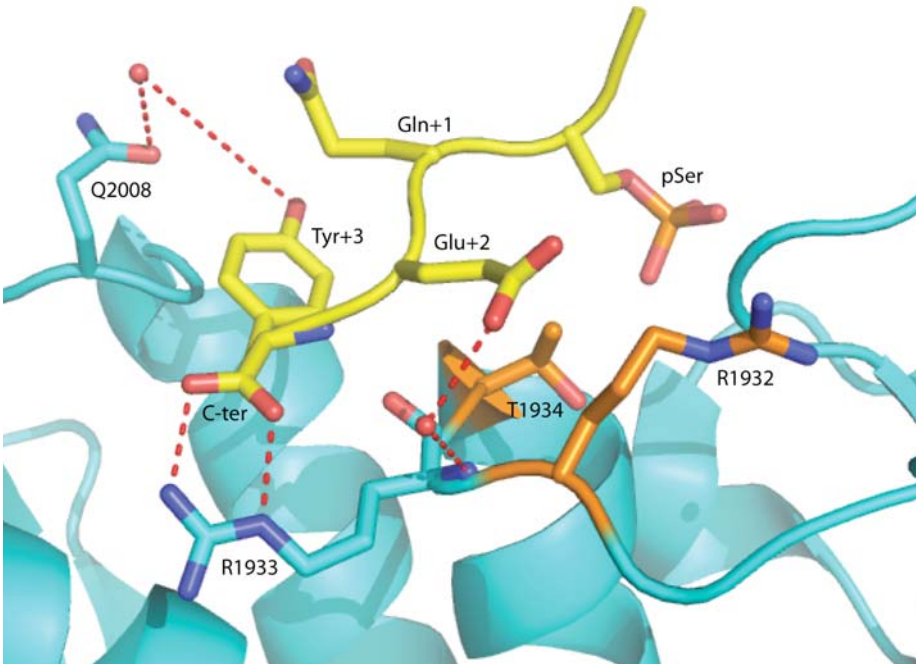


Figure 5.2. Structure of the MDC1 BRCT-γH2AX tail complex. The γH2AX peptide (shown in yellow) binds at the interface between the two BRCT repeats (shown in blue). Hydrogen bonds and water molecules are denoted by red dashed lines and red circles, respectively. The residues involved in van der Waals interactions are shown in orange.

determinant of H2AX phosphorylation by members of the phosphoinositide-3-kinase-related protein kinase family (Stucki et al. 2005).

Studies support the functionally important connection between H2AX and MDC1 in the context of the whole living organism (Lou et al. 2006). 53BP1, NBS1 and the activated, phosphorylated form of ATM fail to accumulate in cells lacking the MDC1-γH2AX interaction (Stucki et al. 2005). Furthermore, MDC1-deficient mice show many phenotypes of H2AX deficiency, such as growth retardation, immune defects, chromosome instability and DNA repair defect. Although MDC1 may indeed mediate γH2AX-dependent chromatin retention of many DDR factors, certain observations have not been explained.

NBS1: Spreading phosphorylation of histone H2AX

The initial redistribution of NBS1, BRCA1 and 53BP1 to sites of DSBs does not require H2AX and NBS1 and 53BP1 can be recruited to sites of damage in the absence of MDC1 (Celeste et al. 2003, Lukas et al. 2004, Bekker-Jensen et al. 2005, Lou et al. 2006). However, the efficient accumulation and sustained retention of NBS1 and 53BP1 at the sites of DNA damage does require MDC1 and γH2AX (Celeste et al. 2002, Stewart et al. 2003, Ward et al. 2003, Lukas et al. 2004, Bekker-Jensen et al. 2005, Stucki et al. 2005, Lou et al. 2006). Taking the current data together, a model for γH2AX formation, DDR factor recruitment and DDR

factor retention at sites of chromosomal DSBs has been proposed (Figure 5.3) (Stucki & Jackson 2006). The MRN complex directly recognizes unpaired DSBs and recruits ATM to the free DNA ends. This interaction is due to a direct interaction between ATM and the NBS1 C-terminus (You et al. 2005). The activated ATM phosphorylates H2AX molecules that are located proximal to the DNA damage and the γH2AX molecules are then recognized by MDC1 via its tandem BRCT domain. At this point, MDC1 not only helps to counter γH2AX dephosphorylation by phosphatases but also acts as an adaptor protein for more MRN complexes to bind. Phospho-dependent interactions between NBS1 and MDC1 mediate chromatin retention of the MRN complex at DSBs and the interactions appear to be dependent on both the FHA and tandem BRCT domains of NBS1 (Chapman & Jackson 2008). Whether the proximity of the FHA and BRCT domains of NBS1 causes them to be conformationally inter-dependent or both domains are indeed required for the interaction is unknown. A high-resolution structure of the NBS1, in a complex with the phosphorylated MDC1, should resolve the above issue. Accumulation of MRN complex can in turn recruit more autophosphorylated ATM molecules (Stucki et al. 2005, Lou et al. 2006, Chapman & Jackson 2008, Melander et al. 2008, Spycher et al. 2008), which can lead to the phosphorylation of additional H2AX molecules located more distal to the original lesion. This positive feedback loop initiated by MDC1 propagates the DNA damage signal.

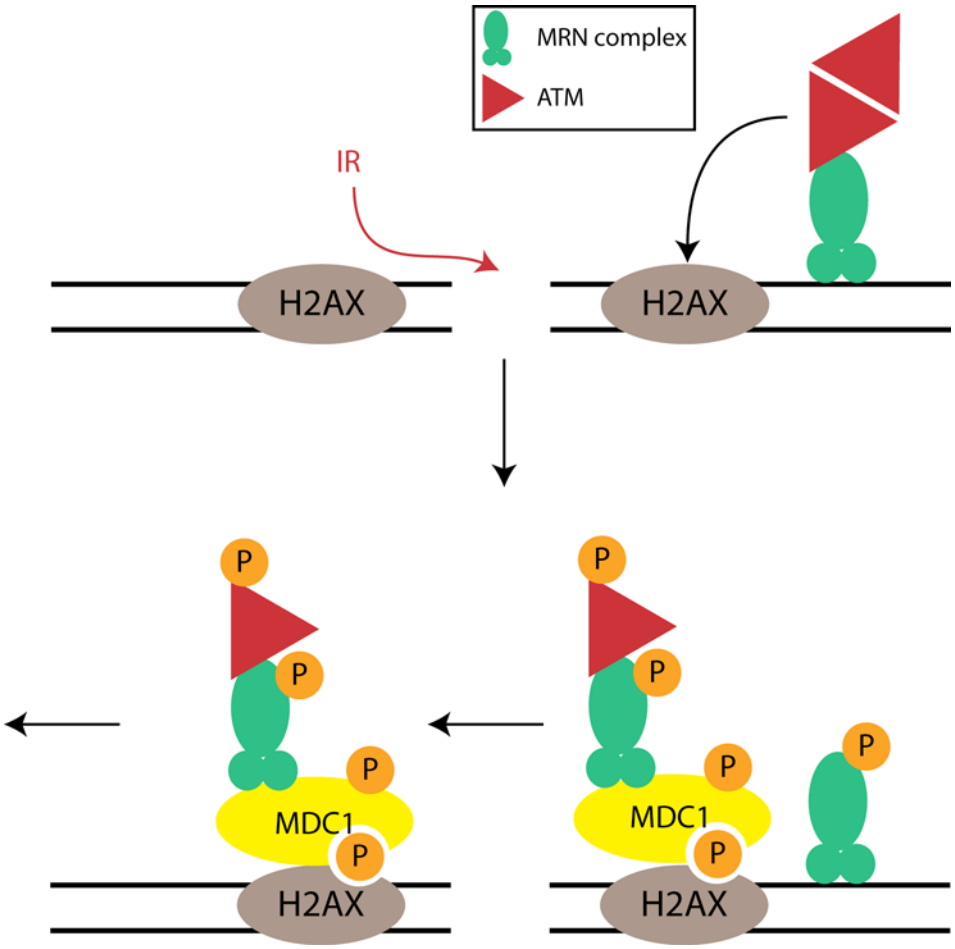


Figure 5.3. Model for γH2AX formation. Un-paired DSBs are recognized by the MRN complex. The MRN complex then recruits ATM through a direct interaction between ATM and the NBS1 C-terminus. ATM becomes activated and phosphorylates H2AX molecules at the site of the DNA damage. Next, MDC1 recognizes the phosphorylated H2AX and acts as a mediator protein, thus recruiting more MRN complexes to bind. This leads to the recruitment of further activated ATM molecules to the chromatin nearby the damage site, thereby creating a positive feedback loop.

53BP1

53BP1 can be recruited to sites of damage in the absence of MDC1. Yet, the efficient accumulation and sustained retention of 53BP1 at the sites of DNA damage does require MDC1 and γH2AX (Celeste et al. 2002, Stewart et al. 2003, Ward et al. 2003, Lukas et al. 2004, Bekker-Jensen et al. 2005, Stucki et al. 2005, Lou et al. 2006). In addition, the recruitment of 53BP1 to sites of DSBs requires the two consecutive Tudor domains of 53BP1 (Ward et al. 2003, Iwabuchi et al. 2003, Huyen et al. 2004) and the Tudor domains were shown to bind directly both to histone H3 methylated on K79 and histone H4 methylated on K20 (Huyen et al. 2004, Botuyan et al. 2006, Yan et al. 2007, Schotta et al. 2008). The fact that the tandem BRCT repeats of 53BP1 are dispensable in recruitment to DNA DSBs (Ward et al. 2003, Iwabuchi et al. 2003, Huyen et al. 2004, Pryde et al. 2005) and they do not bind to γH2AX (Stucki et al. 2005) raises a question: how does H2AX phosphorylation and MDC1 regulate 53BP1 indirectly? A flurry of recent work has delineated a novel regulatory ubiquitylation pathway downstream of MDC1 and provided some answers (Figure 5.4). Once bound to γH2AX, MDC1 recruits the ubiquitin ligase RNF8 via a phospho-dependent interaction that is essential for formation of 53BP1 IRIF (Huen et al. 2007, Kolas et al. 2007, Mailand et al. 2007, Wang & Elledge 2007). RNF8 physically interacts with Ubc13 and catalyzes K63-linked H2A/H2AX ubiquitylation (Plans et al. 2006, Huen et al. 2007, Mailand et al. 2007). Remarkably, two independent studies

discovered RNF168, an ubiquitin ligase downstream of RNF8, as a new factor imperative for 53BP1 focus formation and amplification of ubiquitylation signals created by RNF8 (Stewart et al. 2009, Doil et al. 2009). It is unclear how ubiquitylation can be so crucial for 53BP1 recruitment to DNA damage sites since 53BP1 does not contain any known ubiquitin-binding motifs and it requires dimethylated form of lysine 20 of histone H4 (H4K20me2) for its recruitment (Botuyan et al. 2006). One possible explanation is that ubiquitylation adjusts the nucleosome to expose normally buried H4K20me2 mark to the 53BP1 Tudor domain (Figure 5.4). Structures of the nucleosome, before and after RNF168 ubiquitylation, would provide researchers with useful tools to prove or disprove the 53BP1 recruitment model.

BRCA1-Abraxas pathway

BRCA1 accumulates into IRIF in a γH2AX and MDC1-dependent manner (Celeste et al. 2002, Stewart et al. 2003). Recently, several groups have identified RAP80 and Abraxas as proteins that stimulate relocalization of BRCA1 to DSB sites. The BRCA1 BRCT domain interacts with Abraxas via a phosphorylation-dependent interaction (Kim, Chen & Yu 2007, Sobhian et al. 2007, Wang et al. 2007, Liu, Wu & Yu 2007) to form the BRCA1-Abraxas-RAP80 complex and to promote the G2/M checkpoint. RAP80 is the key mediator of relocalization of the complex as it binds to polyubiquitin chains created by RNF8 (Yan et al. 2007, Kim,

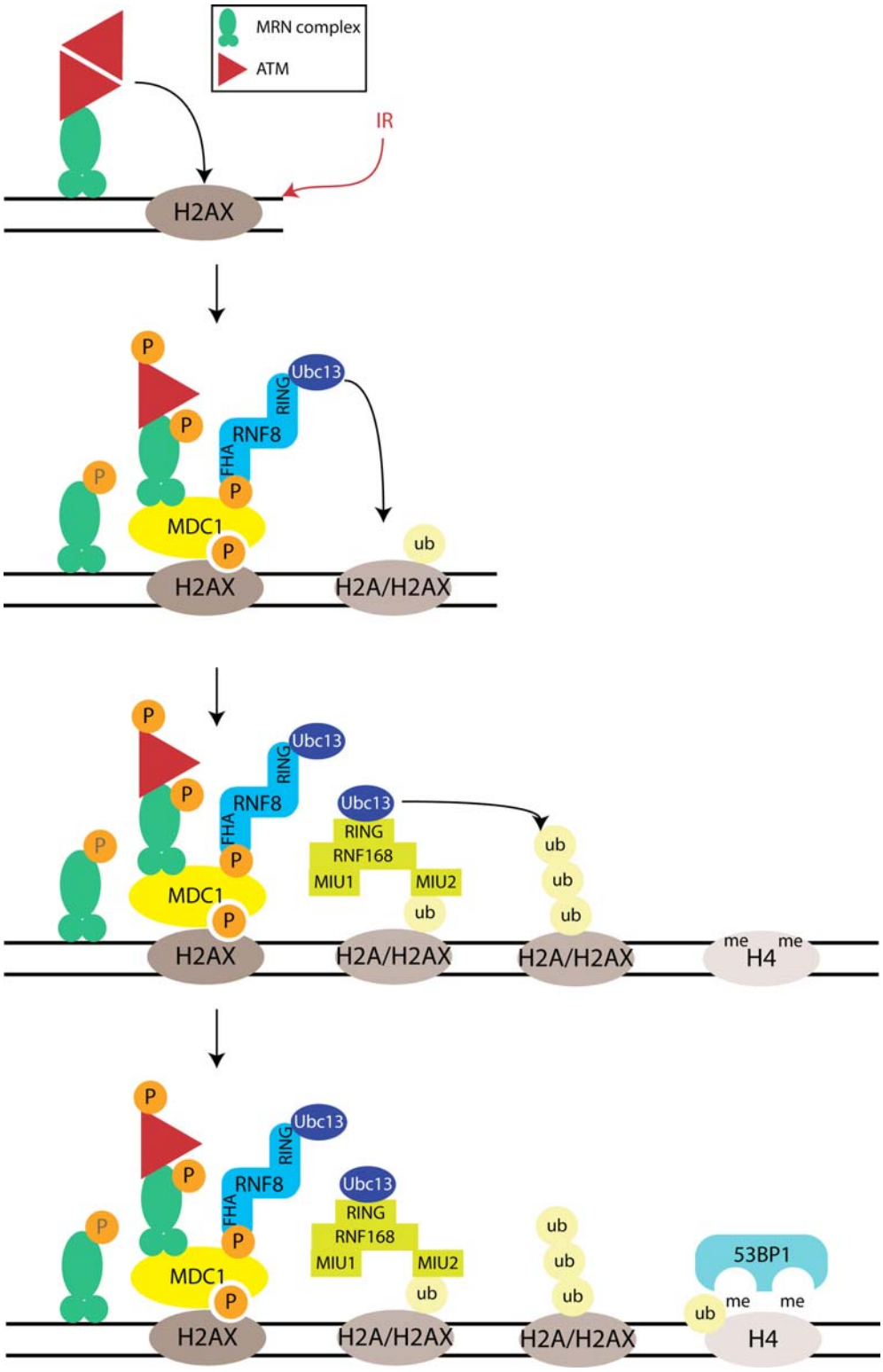


Figure 5.4. Model of RNF8/RNF168-dependent ubiquitylation amd 53BP1 recruitment. See main text for details.

Chen & Yu 2007, Sobhian et al. 2007, Wang et al. 2007) and Abraxas functions as an adaptor holding BRCA1, RAP80 and BRCC36 proteins together (Figure 5.5). In the complex, the presence of BRCC36, a deubiquitylating enzyme, hints a possibility that this complex may modulate BRCA1-BARD1 heterodimer-dependent ubiquitylation at DSBs (Dong et al. 2003). However, the BRCA1-BARD1 E3 ligase substrates relevant to DNA damage repair or tumour suppression are unknown. Surprisingly, a recent study showed that cells lacking the ubiquitin ligase activity of BRCA1 are viable and are not hypersensitive to the DNA cross-linking agent mitomycin C (Reid et al. 2008). This suggests that the E3 ligase activity of BRCA1 may not be relevant to key aspects of BRCA1 function in genome maintenance.

γH2AX

Another issue to be addressed is the precise mechanism by which γH2AX is removed from chromatin in mammalian cells once the damage is repaired. Does H2AX become dephosphorylated directly in situ or does histone exchange precede dephosphorylation of H2AX? In *S. cerevisiae*, it appears that protein phosphatase Pph3 dephosphorylates γH2AX after it has been removed from chromatin (Keogh et al. 2006). In mammalian cells, γH2AX phosphatase, PP2A, is shown to accumulate in IRIF, however, PP2A is not the direct Pph3 orthologue (Chowdhury et al. 2005). Conflicting study has shown that in mammalian cells, the direct Pph3 orthologue, PP4 contributes to the dephosphorylation of γH2AX, both at

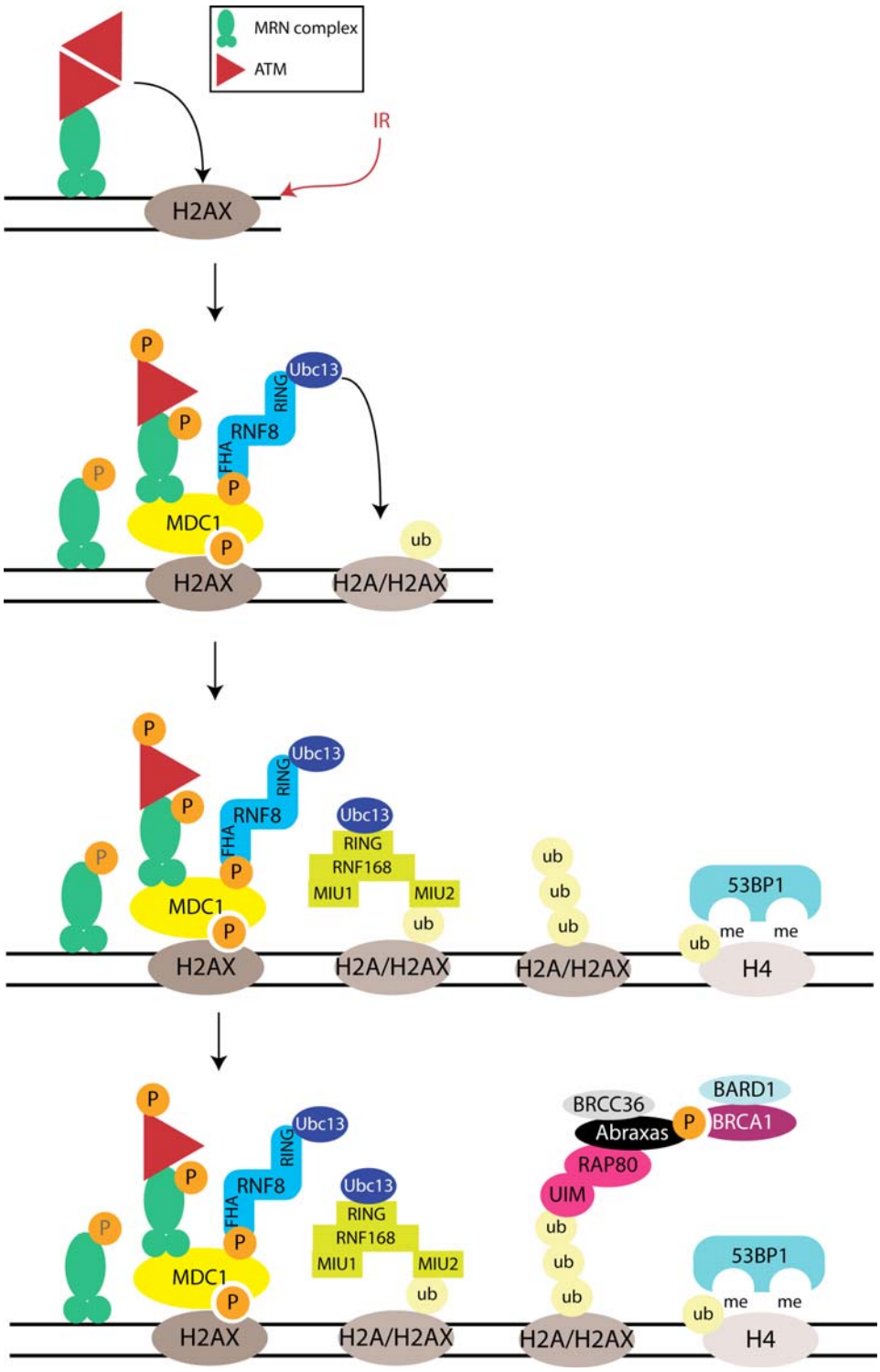


Figure 5.5. Recruitment of the BRCA1-BARD1 complex. The recruitment occurs via a ubiquitin-dependent interaction between the UIM motifs of RAP80 and the ubiquitylated histones by RNF168. RAP80 then mediates the accumulation of the BRCA1-BARD1-Abraxas complex to the sites of DNA damage. BRCC36, a BRCA1-associated deubiquitylating enzyme, may modulate the pathway.

the sites of DNA damage and in undamaged chromatin (Nakada et al. 2008). Furthermore, depletion of PP4C leads to a prolonged checkpoint arrest, most likely due to the persistence of MDC1 at the sites of damage. The study also indicated that PP4 counteracts γH2AX primarily in the chromatin rather than in the nucleoplasm, suggesting histone recycling into chromatin may be an important step prior to dephosphorylation. It is possible that PP4 and PP2A might respond to different types of DNA damage.

In addition to having a role in DNA damage repair, nuclear phosphorylation of H2AX has been shown to be a critical component of apoptosis (Lu et al. 2006). A single post-translational modification, phosphorylation of c-terminal residue of H2AX (Tyr142) controls the balance between apoptosis and survival (Cook et al. 2009). In the presence of Tyr142 phosphorylation, binding of repair factors, such as MDC1, to pSer139 is inhibited, whereas recruitment of pro-apoptotic factors is stimulated. EYA, a protein tyrosine phosphatase, dephosphorylates H2AX Y142 and modulates apoptotic and repair responses to genototix stress (Cook et al. 2009).

BARD1

Our initial attempts to crystallize the BARD1 ankyrin-BRCT region failed, likely due to the fact that the proteolytically labile ankyrin-BRCT linker (BARD1 (554-568)) is flexible, thus hindering crystallization.

Small angle X-ray scattering (SAXS) supports our biochemical and X-ray crystallographic structural data and indicates that the ankyrin and BRCT repeats of BARD1 do not adopt a fixed orientation with respect to one another (Edwards et al. 2008). The ensemble optimization method (Bernado et al. 2007) was used to analyze the solution scattering profile of the BARD1 ankyrin-BRCT region (Edwards et al. 2008). In this method, an ensemble was selected from a pool of 10000 randomly generated C-terminal domain conformers. Then values of D_{max} (maximum dimensions of the structure) and R_G(radius of gyration) of the ensemble, optimized against the experimental scattering curve, were compared against those calculated from the pool. If the molecule in question adopts a limited number of conformations in solution, the D_{max} and R_G of the ensemble would be in a narrow range, in comparison to the values of the pool. However, if the molecule is flexible in solution, the ensemble would sample a broad range of D_{max} and R_G, similar to the values of the pool (Bernado et al. 2007, Bernado et al. 2008). The BARD1 ankyrin-BRCT domain demonstrated conformational heterogeneity in solution (Figure 5.6a and b). SAXS data shows that the solution structure is most consistent with an ensemble of ankyrin-BRCT models suggesting that the two domains sample a wide range of orientations with respect to one

another (Fig 5.6c). This supports our speculation that these protein-protein interaction motifs do not form a contiguous rigid surface, but rather alter their structure depending on binding partners of the linker region of BARD1.

BRCA1-independent functions of BARD1

Unfortunately the search for novel binding partners of the BARD1 BRCT and ankyrin domains remains in its infancy. The only BARD1 function that is independent of the presence of BRCA1 as yet identified is a p53-dependent proapoptotic activity (Feki et al. 2005, Irminger-Finger et al. 2001). The studies showed that BARD1 binds to both p53 and phospho-p53; the region of BARD1 required for binding is the region sufficient for apoptosis induction, although the specific binding region on p53 has not been determined. Furthermore, BARD1 interacts with the DNA-dependent protein kinase (DNA-PK) regulatory subunit, Ku-70, which suggests that the mechanism of p53-induced apoptosis requires BARD1 for the phosphorylation of p53 (Feki et al. 2005). The region of BARD1 sufficient for the p53 interaction and apoptosis induction comprises the ANK domain, the ANK-BRCT linker, and a part of the BRCT domain. Therefore, it is possible that the BRCT domain binds to the kinase and that the kinase targets bind to the adjacent region (Feki et al. 2005). If that is the case, the function of the BARD1 BRCT domain may be to induce apoptosis as the last resort to avoid proliferation of cells containing abnormal DNA. Interestingly, the 53BP2 protein, which contains an SH3

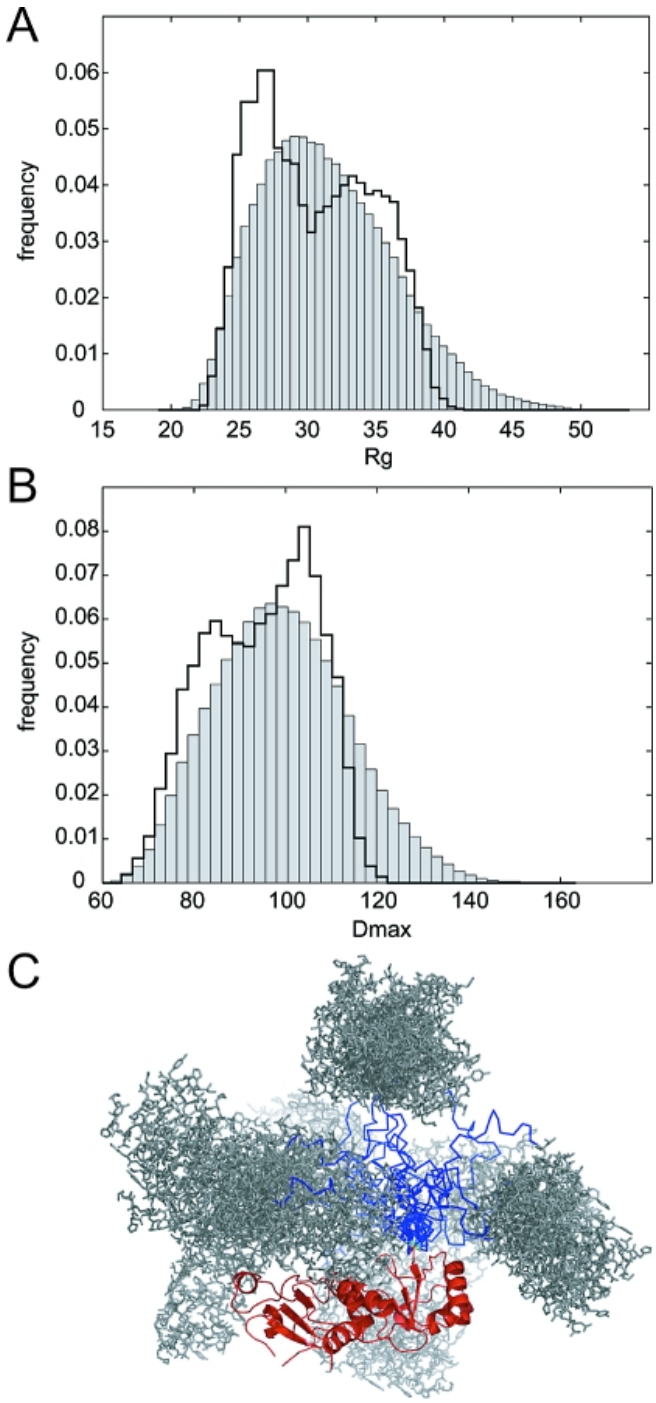


Figure 5.6. BARD1 ANK-BRCT conformations in solution. Frequency of occurance of (a) R_G and (b) D_{max} values for the optimized ensemble (empty boxes) in comparison to those of the pool of 10000 randomly generated conformations (filled boxes). (c) The optimized ensemble that best represents the experimental SAXS curve. All models in the optimized ensemble are shown aligned on their BRCT domains. *Red* – the BRCT repeat, *blue* – the flexible linker and *gray* – ankyrin domains. Edwards, et al. 2008.

(Src homology 3) domain and four ankyrin repeats, binds the p53 DNA binding domain (Gorina & Pavletich 1996). BARD1 also contains four ankyrin repeats, therefore we can speculate a possible interaction between p53 DNA binding domain and BARD1. However, our preliminary GST pull-down experiment with the p53 DNA binding domain and our BARD1 constructs failed to show any binding (data not shown). Moreover, BARD1 lacks a SH3 domain that participated in interaction between 53BP2 and p53. Therefore it is possible that the interaction between p53 and BARD1 does not involve the DNA binding domain of p53 and the ankyrin repeats of BARD1. In addition, Ku-70 contains a 'SEEE' (aa 458-461) motif, which is the BARD1-BRCT phosphoepitope motif proposed by Rodriguez et al. (Rodriguez et al. 2003). Yet, the major phosphorylation site on Ku-70 by DNA-PK is serine 6 (Chan et al. 1999). Therefore, it is unlikely that Ku-70 interacts with the BARD1 BRCT domain in a phospho-dependent manner. Identifying interaction partners of the BARD1 ankyrin and BRCT domains will provide researchers a valuable tool to understand precise functions of BARD1 that are independent of BRCA1.

Alternative mode of BRCT domain binding

Structure of BRCA1 and its BACH1 phosphopeptide showed that, in addition to the dominant interactions provided by pSer and Phe(+3), other residues of the phosphopeptide provided additional hydrogen bonds and van der Waals contacts to recognize the BRCT repeats (Figure 5.7) (Shiozaki et al. 2004). Rodriguez et al. found possible *in vivo* binding

partners for the proteins such as BRCA1 and MDC1, however not for BARD1 (Rodriguez et al. 2003). The failure to identify the possible binding partners of BARD1 could be due to relatively weak selectivity level at the +3 position from pS. Then an intriguing question arises: could the BARD1 BRCT domain recognize its phosphoepitope via an alternative binding mode? Unlike MDC1 or BRCA1, could the peptide specificity of the BARD1 BRCT domain be mediated by pS(-1) to (-5) positions? G1656, V1696, E1698 and V1740 in BRCA1 contribute to the recognition of the residues N-terminal to pSer (Figure 5.7). Their corresponding residues in BARD1, G576 and Q615 respectively (there are no structurally corresponding residues for V1696 and V1740 in BARD1) and P610 is located at the peptide binding surface (Figure 5.8a). These three residues are conserved among human, chimpanzee, dog, mouse and rat BARD1 (Figure 5.8b). This may indicate that there is another conserved surface region in the phosphopeptide binding pocket in BARD1, pointing to a different peptide binding mode.

Assessment of functional effects of variants of tumor suppressors

The use of functional assays is based on the principle that the detection of a decrease in activity of a tumour suppressor represents increased cancer predisposition. Therefore, functional assays that can quantify alterations in the activity of a tumour suppressor variant are often used to predict whether the variant is cancer-associated. Currently,

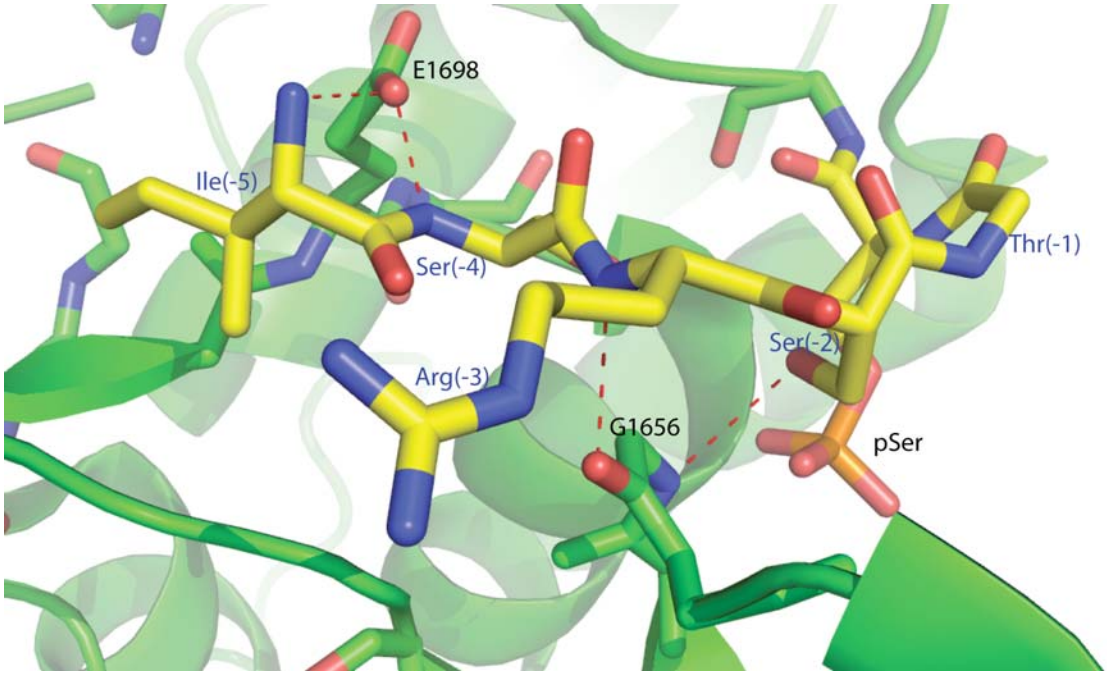
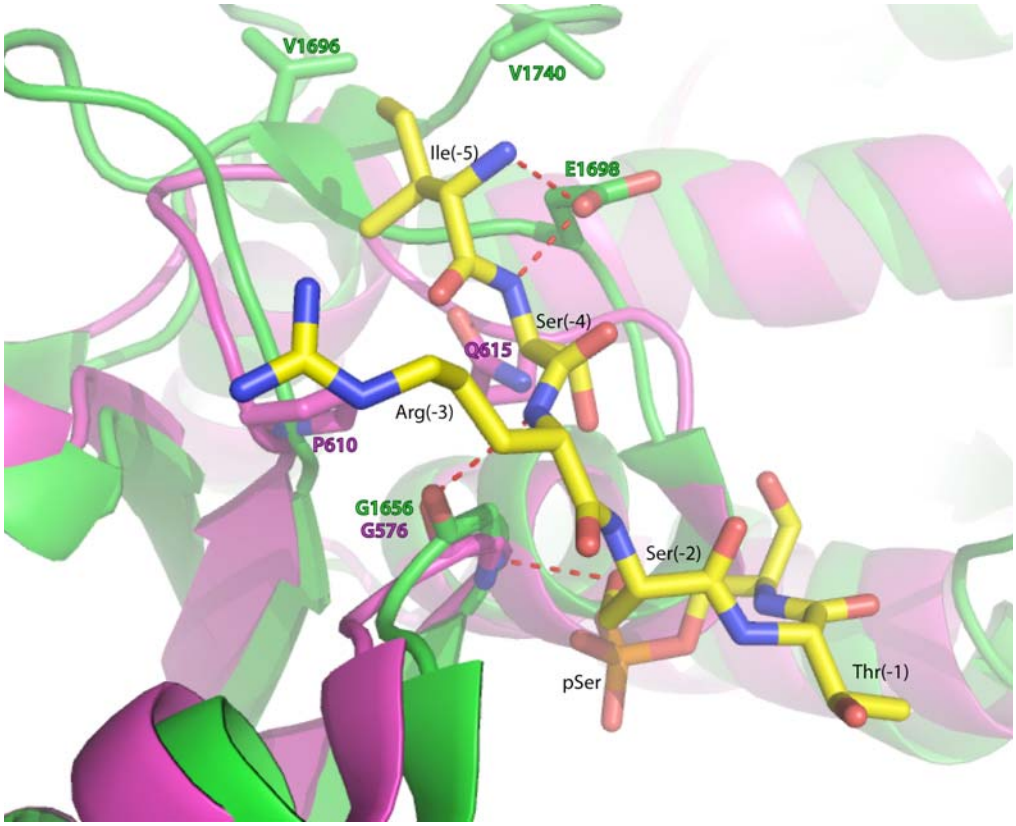


Figure 5.7. The contribution of BACH1 residues to the binding of the BRCA1 BRCT repeats. The hydrogen bonds are represented by red dashes (pdbID-2NTE, Shiozaki, et al. 2004).

a.



b.

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human          566  RDGPLVLIGSGLSSEQQKMLSELAVILKAKKYTEFDSTVTHVVVPGDA- 614
chimpanzee     422  RDGPLVLIGSGLSSEQQKMLSELAAILKAKKYTEFDSTVTHVVVPGDA- 470
dog            712  RDGPLVLIGSGLSSEQQKMLSELAAILKAKKCAEFDNTVTHVIVPGDT- 760
mouse          554  KNGPLVFIGSGLSSQQQKMLSKLETVLKAKKCMEFDSTVTHVIVPDEE- 602
rat            557  KSGPLVLIGSGLSSQQQKLLSKLETVLKAKKCAEFDNTVTHVIVPDEE- 605
BRCA1         1646  VNKRMSMVVSGLTPEEFMLVYKFARKHHITLTNLITEETTHVVMKTDAE 1694

human          615  --VQSTLKCMLGILNGCWILKFEWVKACLRRKVCEQEEKYEIPEGPRRS 660
chimpanzee     471  --VQSTLKCMLGILNGCWILKFEWVKACLRRKVCEQEEKYEIPEGPRRS 110
dog            761  --VQSTLKCMLGILNGCWILKFEWVKACLQRKACEQEEKYEIPEGPHRS 116
mouse          603  --AQSTLKCMLGILSGCWILKFDWVKACLDSKVREQEEKYEVPGGPQRS 98
rat            606  --AQSTLKCMLGILNGCWVLKFDWVKACLDSQEREQEEKYEVPGGPQRS 111
BRCA1         1695  FVCERTLKYFLGIAGGKWVVSYFWVTQSIKERKMLNEHDFEVRGDVVNG 1743
```

Figure 5.8. Alignment of BARD1 and BRCA1. a. Structural comparison between BARD1 and BRCA1 at the peptide-binding site. The phosphopeptide, BARD1-BRCT and BRCA1-BRCT are shown in yellow, pink and green, respectively. Hydrogen bonds are shown in red dashes. **b. Sequence alignment of BARD1 orthologs from human, chimpanzee, dog, mouse, rat and human BRCA1.** The residues that participate in hydrogen bonding with the BACH1 peptide are marked by red dots.

functional assays are used to evaluate several cancer related genes such as BRCA1, BRCA2, mismatch repair genes (Lynch syndrome) and CDKN2A (familial melanoma) (Parry & Peters 1996, Ou et al. 2007).

Classifying BRCA1 mutations

Genetic screening of BRCA1 intronic and exonic sequences by Myriad Genetics Inc. (Salt Lake City, USA) can establish presence or absence of alterations. Yet, the finding of a variant of uncertain significance (VUS) can complicate rather than improve the risk assessment process. BRCA1 VUS carriers and their at-risk family members often delay making decisions about their cancer risk management and are not able to take full advantage of prevention and therapeutic strategies offered to deleterious mutation carriers.

Because the BRCA1 protein is involved in multiple cellular pathways, the exact biochemical functions are yet to be fully defined. Studies have focused on assays limited to specific domains of N-terminal RING and C-terminal BRCT domains.

N-terminal RING domain mutants

The BRCA1/BARD1 heterodimer formed via their RING domains displays E3 ubiquitin ligase activity (Baer & Ludwig 2002, Brzovic et al. 2003). The BRCA1 RING domain cancer-associated variants have been shown to correlate with a loss of ubiquitin ligase activity as well as the protection from radiation hypersentivitiy (Ruffner et al. 2001). Based on

these observations, a functional assay was developed measuring the interaction between not only BRCA1 and BARD1 but also BRCA1 and UbcH5a (E2 ubiquitin conjugating enzyme) (Morris et al. 2006). Deleterious variants failed to interact with neither BARD1 nor UbcH5a and lost ubiquitin ligase activity (Morris et al. 2006), thereby establishing the potential utility of this BARD1 binding/ubiquitin ligase activity assay for classification of the BRCA1 RING domain variants. However, this assay is domain-specific and may not be used to interrogate variants in other regions of the protein.

C-terminal BRCT domain mutants

1. Transcription activation assay

When expressed as a fusion protein to a heterologous DNA binding domain, the carboxy-terminal region of BRCA1 functions as a transactivation domain (Monteiro, August & Hanafusa 1996, Monteiro 2000). Cancer-predisposing nonsense, frameshift and missense mutations in this region of BRCA1 showed to impair this transcriptional activity (Hayes et al. 2000). This lead to the development of transcription activation assay, which tested the integrity of the BRCT domain. The assay correctly classified 24 out of 24 known deleterious and neutral BRCA1 variants (Carvalho et al. 2007). Though it is an excellent method for evaluating effects of the mutants on BRCA1 function, it is limited to the C-terminus of BRCA1.

2. Protease sensitivity assay

The protease-based assay we used to test the stability of the BRCT domains senses the protein destabilizing effects of both missense and truncation mutations. The simplicity of this assay is advantageous for large-scale studies, however subtle deleterious changes in surface residues may be incorrectly identified in this assay.

However, this would be a general assay to assess single nucleotide polymorphisms (SNPs) in any structured domain. Easton et al. studied 1,433 distinct sequence variants in BRCA1 and BRCA2 and found that the percentages of deleterious missense variants in unstructured and structured regions are 0.1% and 6.5%, respectively (Easton et al. 2007). The findings suggest that if a SNP is found in an unstructured region, a chance of the mutation being a deleterious one is extremely low. On the other hand, if a SNP is found in a structured region, an assay such as the protease-based assay can clarify further the effect of the mutation.

3. Phosphopeptide binding assay

Our *in vitro* functional assay can evaluate biological effects of the BRCT mutations on its ability to interact with BACH1. Our assays are fully evaluated for the ability to discriminate between a series of well-defined positive and negative controls such as M1775R, A1708E and M1652I. Because BRCA1 is multifunctional, no single assay may be able to account for the functional effects of all variants. In the case of our functional assays, the variants that induce aberrant splicing or mRNA

stability will be overlooked. Unfortunately, our approach is limited to the BRCT domain and cannot be applied to the entire protein.

Confounding factors in the interpretation of functional assays

The ultimate goal of the use of functional tests is to be used clinically for the individual to make informed decisions. In principle, only when a classification is made with a high level of certainly, it is clinically useful. However, many of the variants are rare in the general population and the affected families would be denied the available information that may never achieve the “gold standard” (Couch et al. 2008). It is plausible that patients and clinicians choose to make decisions based on reasonable estimates of cancer-risk from incomplete or less-than-optimal data rather than using no data at all (Plon et al. 2008). Then the results of our assays are useful, in a qualitative manner, for clinical classification of the BRCA1 variants. However, there are few issues with using the functional assay data. The results should be distributed to the biomedical community through locus-specific databases (Greenblatt et al. 2008). Moreover, the clinicians should receive specific training in the interpretation of results to take full advantage of the information. Most importantly, the individuals must be aware that the results of functional assays may include flaws before they make clinical decisions.

Detecting novel genetic alterations within the breast cancer patients

Standard PCR-based screening methods (eg. Genetic screening method by Myriad Genetics Inc.) are highly sensitive and routinely used

for detecting BRCA1 mutations. However, the large genomic rearrangements cannot be detected by the automated sequencing methods and therefore are commonly overlooked (Mazoyer 2005). The rearrangement of BRCA1/BRCA2 seem to account for a relatively small proportion of familial breast cancer cases (<10%) (Gutierrez-Enriquez et al. 2007, Armaou et al. 2007, Vasickova et al. 2007, Palanca Suela et al. 2008). However, the presence of large rearrangements found in patients, who are non-carriers of BRCA1/BRCA2 mutations, reinforces the need of studying large genomic rearrangements of BRCA1/BRCA2 in genetic counseling programs.

Conclusions

Significant advances in the understanding the DDR and BRCA1 function have been made in recent years. The molecular recognition processes that are responsible for targeting BRCA1 and its associated partners to DNA damage sites have been revealed. Several distinct macromolecular protein complexes containing BRCA1 have been identified and we now have a better understanding of the BRCA1 tumor suppressor network. However, many aspects of the tumor suppressor pathway, such as the biological function of the BARD1 BRCT domain and the BRCA1/BARD1 heterodimer, still remain enigmatic. Our future work should be directed towards elucidation of the molecular details of BRCA1 tumor suppressor network and how mutations disrupt the processes related to the genesis of malignancy.

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