Chapter 3

Solution NMR Structure of the Second BRCT Domain from Human BRCA1

3.1 Identification of Stable BRCT Constructs

Proteolytic dissection of the BRCA1 BRCT region was performed with a set of six different (site-specific) proteases (trypsin, chymotrypsin, thermolysin, elastase, subtilisin, and V8 protease). The experiments were started with purified 1559-BRCT ($BRCA1^{[1559\text{-}1863]}$) and 1590-BRCT ($BRCA1^{[1590\text{-}1863]}$), both of which were expected to comprise the complete BRCT tandem repeat with an additional amino-terminal extension of approximately 90 and 60 un-structured residues, respectively. This approach yielded one exceptionally stable polypeptide with a molecular size very close to that of the predicted BRCT tandem repeat (25 kDa), thus providing the first clue for the physical existence of BRCT domains (Figure 8). To follow the time course of proteolysis and to trap transiently stable intermediates, the amount of protease was then adjusted for each protease such that proteolytic fragments accumulated for several hours without being further degraded. Strikingly the final results as visualized on the gels were virtually independent of the protease type, indicating that the suggested BRCT domains represented a compact fold with hardly any accessible protease cleavage sites in between. The most stable reaction product generated by trypsin cleavage was analyzed by N-terminal sequencing and mass spectrometry and further confirmed to correspond to the BRCA1 sequence patch ^{1646}VNKRM(…)PHSHY^{1863}, thus being almost identical to the predicted BRCT tandem repeat region. Notably, the nearby potential trypsin cleavage sites ^{1648}K↓R^{1649} and ^{1649}R↓M^{1650} were not affected. It was therefore concluded that the BRCT tandem repeat starts actually at or very near to Val1646. As to the carboxy-terminal domain boundary, limited proteolysis was much less informative due the lack of suitable protease recognition sites. In addition, the C-terminus was known to contribute significantly to the DNA damage-responsive cell cycle checkpoint activation. Therefore structural studies were performed on BRCT constructs without further C-terminal truncations.

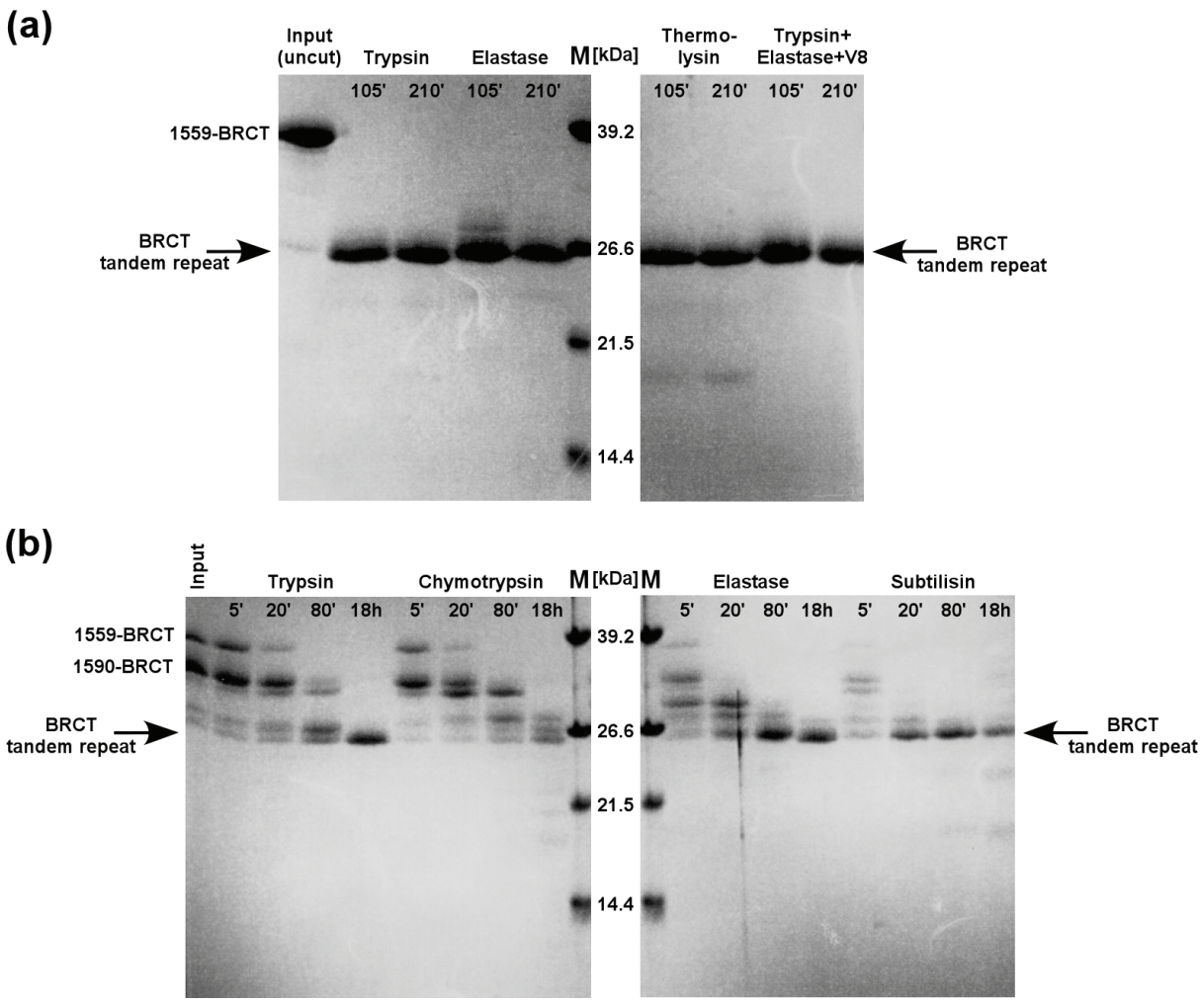

Figure 8: Proteolytic dissection of the BRCA1 BRCT region.
(a) Limited proteolysis of 1559-BRCT, using trypsin, elastase, thermolysin, and a mixture of trypsin, elastase and V8 protease. **(b)** Extended time course of the proteolytic dissection of combined 1559-BRCT and 1590-BRCT, using the proteases trypsin, chymotrypsin, elastase and subtilisin. Note that the most stable reaction products are all very similar in molecular size, corresponding to the BRCT tandem repeat. Protein samples were analyzed by 17.5% SDS-PAGE, Coomassie Brilliant Blue staining. M: protein molecular weight marker.

Interestingly, the double BRCT repeat was very resistant to proteolytic degradation without being further cleaved to yield the individual BRCT domains (BRCT-n and BRCT-c). Nevertheless the second BRCT domain of the tandem repeat (BRCT-c) was equally stable when recombinantly expressed with the GroEL/GroES chaperone system, thus indicating for the first time that some individual BRCT domains may fold independently even in the absence of an intramolecular BRCT tandem partner. The first BRCT domain (BRCT-n), however and most strikingly, was extremely sensitive toward proteases upon thrombin cleavage of the GST tag and failed to withstand protein purification without being degraded.

3.2 Crystallization Screening

Following the results from limited proteolysis which clearly marked the BRCT tandem repeat as the most stable fragment of the BRCA1 C-terminal region, 1646-BRCT (BRCA1$^{[1646-1863]}$) and the N-terminally truncated variant 1650-BRCT (BRCA1$^{[1650-1863]}$) were selected for sparse matrix crystallization screening. However, despite much effort using large amounts of protein for many hundreds of different screening conditions, all wild-type BRCT constructs including the tandem BRCT constructs and the isolated BRCT-c domain (1756-BRCT, BRCA1$^{[1756-1863]}$) failed to crystallize. As even small amounts of impurities may interfere with nucleation and subsequent crystal formation, protein purity might be considered the sticking point. However, the BRCT constructs were stable over the course of purification and yielded highly pure and homogeneous protein samples. It was therefore suggested that crystallization was severely hampered due to solvent-exposed hydrophobic amino acid residues and consequently reduced solubility. This observation was later confirmed by Glover et al. when reporting the crystal structure of the tandem BRCT repeat with a short C-terminal truncation [50].

3.3 NMR Structure Determination

As diffraction quality crystals of wild-type BRCT constructs were not obtained, NMR spectroscopy was used to analyze the dynamic behavior in solution and to derive structural information about the BRCT region. In a first step both the BRCT tandem repeat and the isolated BRCT-c domain were used in preliminary NMR experiments to assess the relaxation properties of NMR signals and the overall quality of the NMR data. The BRCT constructs showed well dispersed NMR signals, indicating that the samples were properly folded. The BRCT tandem repeat (220 residues), however, showed (i) consistently weaker and (ii) partially overlapping NMR signals, most likely due to an increased rotational correlation time and the larger number of amino acid residues, respectively.

Thus, the isolated BRCT-c domain representing the smallest stable subunit of the BRCT region was most promising in initial NMR screening experiments and consequently selected as target for the structure determination by NMR spectroscopy.

3.3.1 NMR Screening

^{1}H-NMR and ^{1}H-^{15}N HSQC experiments with BRCT-c were used as diagnostic tools to identify suitable buffer conditions for NMR data acquisition. Figure 9 shows a series of ^{1}H-NMR spectra with systematic variations of temperature, ionic strength (salt concentration) and pH. In the range of 10°C to 32°C the temperature had little effect in *short-term* experiments, such as 1D ^{1}H-NMR spectra, besides the observation that at 10°C the signal intensity was slightly decreased, most probably the result of reduced molecular motion and rotation. In *long-term* experiments, however, the signal-to-noise ratio was significantly reduced above 30°C due to sample aggregation and precipitation. This effect was even more pronounced at increased ionic strength (330 mM salt) and lower pH (6.0) (Figure 9e). NMR data acquisition with BRCT-c was consequently performed at 296 K (~23°C) in phosphate buffer pH 6.8 with very low ionic strength, containing only 5 mM KCl.

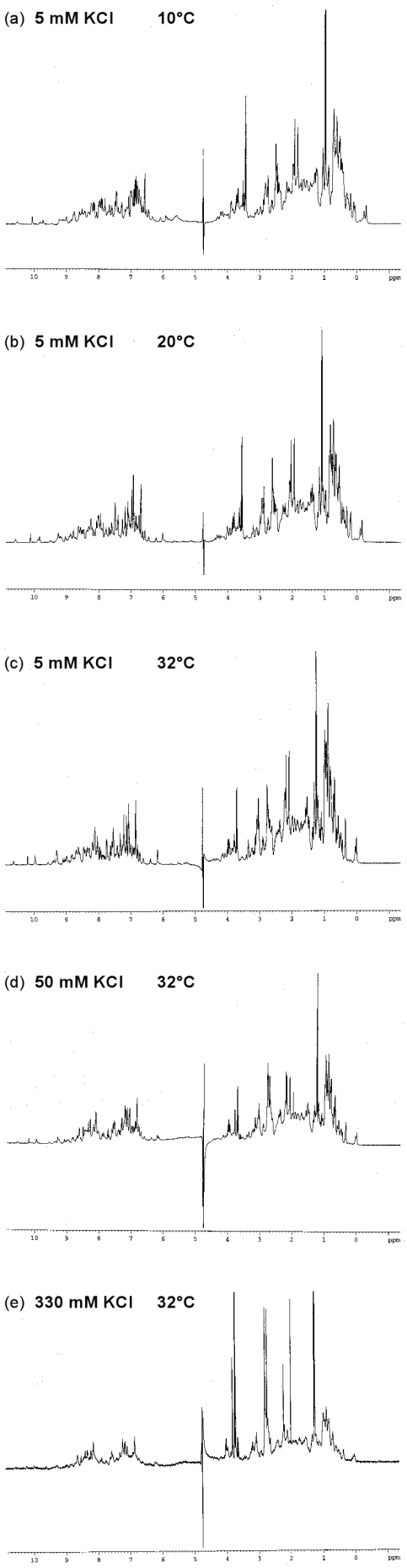

Figure 9: ^{1}H-NMR screening of the second BRCA1 BRCT domain (BRCT-c).

(I) Temperature dependency (a-c):
^{1}H-NMR spectra of BRCT-c in phosphate buffer (pH 7.0) containing 5 mM KCl, supplemented with 10 mM fresh fully deuterated DTT. Spectra were acquired at **(a)** 10°C, **(b)** 20°C, and **(c)** 32°C, using a 600 MHz spectrometer (DRX600, BRUKER BIOSPIN).
(II) Salt dependency (c-e):
^{1}H-NMR spectra of BRCT-c in phosphate buffer (pH 6.0*) containing **(c)** 5 mM KCl, **(d)** 50 mM KCl and **(e)** 330 mM KCl, each supplemented with 10 mM fresh fully deuterated DTT. Spectra were acquired at 32°C, using 600 MHz spectrometer (DRX600, BRUKER BIOSPIN). * (c) pH 7.0

3.3.2 NMR Assignment of the Second BRCA1 BRCT Domain (BRCT-c)

Resonance assignments and by that the chemical shift values for ^{1}H, ^{13}C and ^{15}N nuclei were obtained from analysis of 2D and 3D NMR spectra, using ^{15}N-labeled and ^{13}C,^{15}N double-labeled BRCT-c samples (1.3 mM and 0.7 mM protein concentration, respectively). Almost all (non-proline) backbone $^{13}C\alpha$, ^{15}N and ^{1}H resonances, as well as 101 of 110 ^{13}CO resonances were assigned. In addition, more than 90% of the aliphatic and aromatic side chain ^{13}C and ^{1}H resonances were assigned. Asparagine and glutamine side chain amide ^{15}N and ^{1}H resonances were also partially assigned, as were the side chain $^{15}N\varepsilon/^{1}H\varepsilon$ nuclei of Arg1835 (BRCA1 numbering). The resonance assignments for ^{1}H, ^{13}C and ^{15}N nuclei of BRCT-c have been published and are available from the Biological Magnetic Resonance Bank (BMRB) (http://www.bmrb.wisc.edu/) under the accession code BMRB-6114 [42]. ^{1}H-^{15}N correlations of BRCT-c are shown in the assigned 2D ^{1}H-^{15}N-HSQC spectrum (Figure 10).

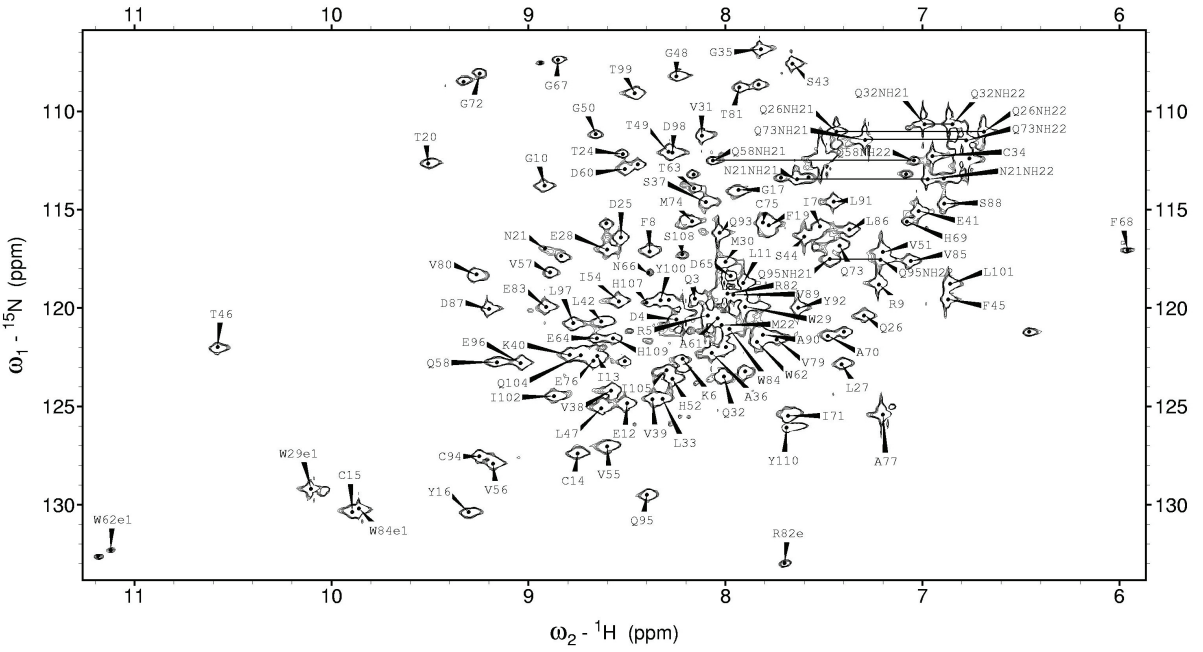

Figure 10: Assigned ^{1}H-^{15}N-HSQC spectrum of BRCT-c.
The 2D HSQC spectrum was acquired at 296 K on a 750 MHz spectrometer (DMX750, BRUKER BIOSPIN). Isolated BRCT-c was dissolved in a buffer containing 10 mM KH_2PO_4 and 10 mM K_2HPO_4 (pH 6.8), 5 mM KCl, 10 mM deuterated DTT, and 7% D_2O (v/v) [42].

3.4 Solution NMR Structure of BRCT-c

The structure of BRCT-c was calculated with a total number of 1387 NOE restraints derived from 2D and 3D NOESY spectra, including 747 long-range NOEs, 108 dihedral angle restraints and 33 hydrogen bond restraints. The restraints used for NMR structure calculations and the structural statistics for the final BRCT-c ensemble are listed in [43]. The NMR structure of BRCT-c (15 lowest energy structures) is well restrained and has a mean backbone RMSD of 0.311 Å considering the well-structured regions (essentially β-strands and α-helices). The three loop regions and a number of amino acid residues close to the N- and C-terminus were less well defined due to the intrinsic flexibility and consequently the low number of experimental restraints. The BRCT-c domain has a central four-stranded all-parallel β-sheet with two neighboring extended β-strands (β1 and β3) flanked by two short β-strands (β2 and β4). The β-sheet contains many conserved hydrophobic (mostly aliphatic) amino acid residues forming the hydrophobic core of the BRCT fold (Figure 11). The central β-sheet is surrounded by three rather short α-helices which are anchored to the core by additional conserved hydrophobic amino acid residues.

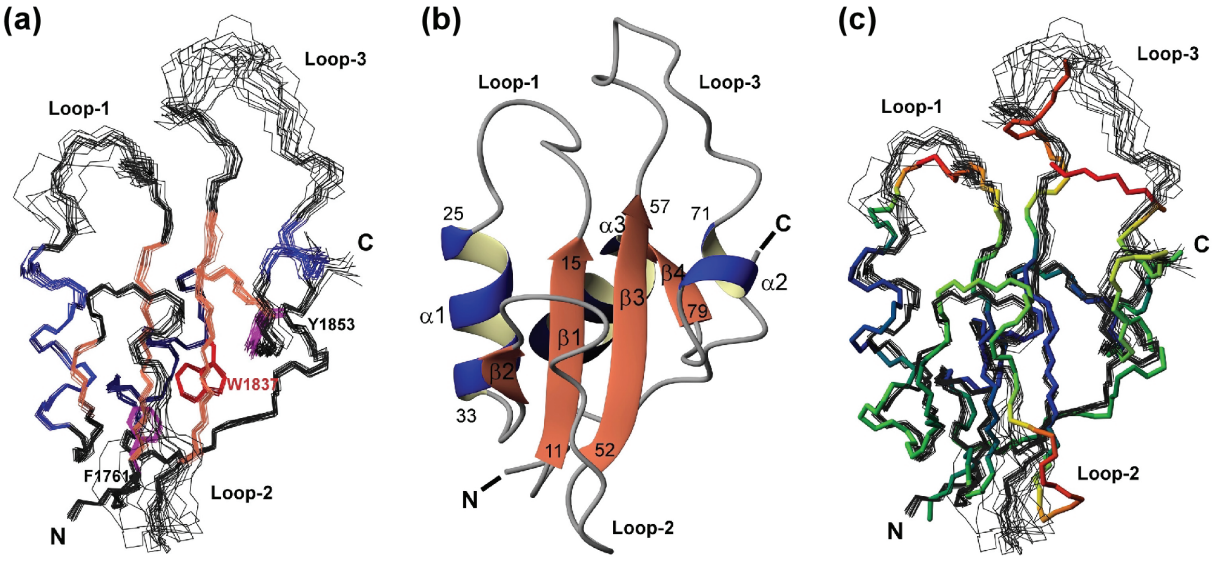

Figure 11: Solution NMR structure of the second BRCA1 BRCT domain (BRCT-c).
(a) NMR ensemble of BRCA1 BRCT-c (residues 1759-1855). Superposition of the 15 lowest energy structures considering the backbone atoms (N, C^{α}, and C'). β-strands (β1-β4) and α-helices (α1-α3) are colored brown and blue, respectively. Loop regions are additionally labeled. The side chains of the three most conserved aromatic residues, Phe1761, Tyr1853 and Trp1837, are shown in magenta (Phe/Tyr) and red (Trp). Amino- and carboxy-terminus are marked N and C, respectively. **(b)** Ribbon diagram of the structure closest to the mean structure of the NMR ensemble. Numbering is as in the BRCT-c expression construct. **(c)** Superposition of the BRCT-c NMR ensemble with the crystal structure of the second BRCT domain as part of the BRCA1 BRCT tandem repeat [50]. Coloring according to the crystallographic *B* values: blue (≤ 30 $Å^2$), green (~ 40 $Å^2$), yellow (~ 50 $Å^2$), and red (≥ 60 $Å^2$).

3.4.1 Conserved Amino Acid Residues

Most conserved residues within the BRCT domain family have aliphatic or aromatic side chains and apparently stabilize the hydrophobic core. Trp1837 from helix α3 is by far the most conserved residue; it is almost completely excluded from the solvent and deeply buried in the hydrophobic core, making direct contact to other conserved residues, such as Leu1746 and Ile1766 (both from β-strand β1), Val1805 and Val 1810 (both from β3), Val 1833 (from β4) and Val1784 (from α1). In addition Trp1837 interacts with Phe1761 and Tyr1853, two other highly conserved aromatic residues. However, although situated very close to Trp1837, Tyr1853 is only partially embedded into the hydrophobic core. In addition to the numerous conserved aliphatic and aromatic residues, some conserved glycine (Gly) and alanine (Ala) residues at specific positions already identified by sequence analysis are particularly important for the folding and/or the stability of BRCT domains. In fact, one of the most conserved residues is Gly1788 which is located in the turn connecting helix α1 and β-strand β2, and the structure of BRCT-c reveals that only glycine with its lacking side chain fits into the limited space in this area. The same applies to Ala1789 that follows Gly1788 in sequence, and also to both Gly1763 and Gly1770. Other amino acid residues with larger side chains at these positions are therefore expected to interfere with protein folding and/or stability.

In 2001, Glover at al. reported the successful crystallization and structure determination of the BRCT tandem repeat from BRCA1, using a BRCT variant with a short C-terminal truncation [50]. The comparison of the solution NMR structure of BRCT-c with the crystal structure of the second BRCT domain from the BRCT tandem repeat shows subtle differences at the interface between the two BRCT repeats (BRCT-n and BRCT-c) and in the loop regions (Figure 11c). The interface of the tandem BRCT repeats is predominantly hydrophobic and involves residues from α1, α2-n (helix α2 from BRCT-n) and α3. In the absence of the first BRCT domain (BRCT-n), interface residues of BRCT-c become solvent-exposed and change conformations, thereby affecting the orientation of α1 and α3 with respect to each other. Most striking is the reorientation Met1775 near the end of loop 1. In the presence of BRCT-n, the side chain of Met1775 extends into the BRCT-n–BRCT-c interface and contacts Met1783, Leu1839 (α3), and Leu1701 (α2-n), thereby forming part of the binding pocket for the interaction with phosphorylated peptide motifs, whereas in the isolated BRCT-c domain Met1775 loses contact with all other interface residues and becomes solvent exposed.

3.4.2 Association Behavior

To investigate the self-association behavior of isolated BRCT-c, analytical UCF was applied. As a first approach sedimentation velocity (SV) experiments were performed to reveal concentration-dependent heterogeneity in the sedimentation behavior of BRCT-c. Analysis of the data indicated only very weak association. To obtain absolute values for the apparent molecular mass in solution, sedimentation equilibrium (SE) experiments were performed. The fit on equilibrium gradients converged to a molecular mass of 16.5 (± 0.1) kDa, excluding the possibilities of either a pure monomer or pure dimer. However, deconvoluting the data using a monomer-dimer self-association model resulted in a molecular mass of 12.2 (± 0.8) kDa for the monomeric species (Figure 12). This is in very good agreement with the theoretical value of 12.5 kDa for ^{15}N-labeled BRCT-c. The dissociation constant (K_D) of 3.7 mM suggests a fairly weak self-association in solution which is well consistent with the observation that isolated BRCT-c shows a tendency toward unspecific self-association in high-ionic strength buffer at protein concentrations in the millimolar range. In agreement with the results from the ultracentrifugation experiments, both the BRCT tandem repeat and the isolated BRCT-c domain eluted as monomers from the SEC column during protein purification.

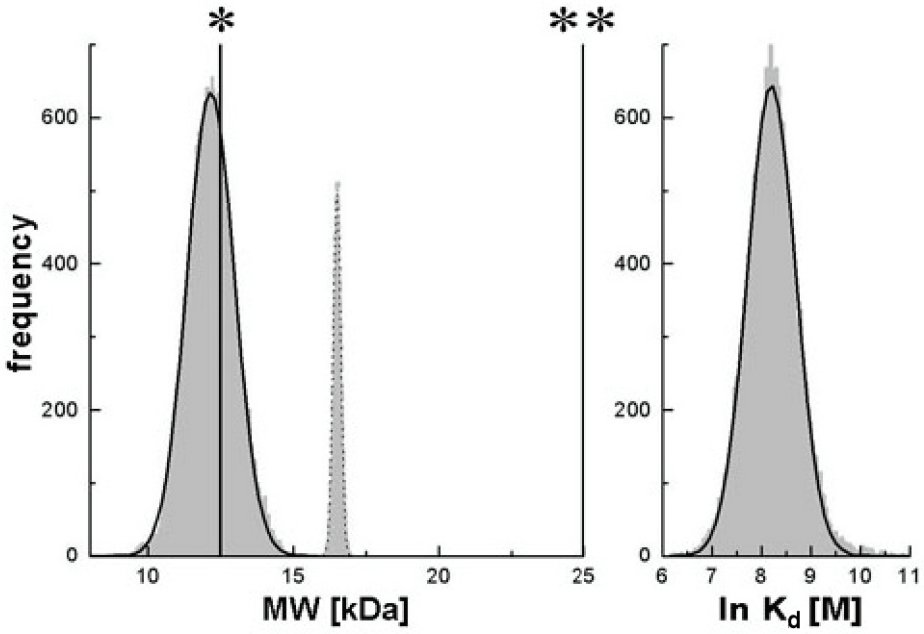

Figure 12: Analytical Ultracentrifugation (UCF).
Monte Carlo distributions of the parameters for the molecular mass (left) and the dissociation constant (right). The best fit to a Gaussian distribution of the data is shown for the monomer-dimer model and the single-ideal species (solid line and dotted line, respectively). One and two asterisks indicate the theoretical molecular mass of the ^{15}N-labeled BRCT-c monomer and dimer, respectively.

3.5 Discussion

3.5.1 Functional Implications of Disease-Related Mutations

BRCA1 variants with mutations in structurally conserved regions of BRCT-c, such as the central four-stranded β-sheet, are correlated with an increased risk for the development of breast and ovarian cancer. However, only a small number of key amino acid residues appear to be essential for the stabilization of the BRCT fold. These include Phe1761, Trp1837 and Tyr1853, the aliphatic residues Leu1780 and Val1838 which anchor helix α1 to the central β-sheet, Val1838 and Val1842 from helix α3 which contact helix α1 through hydrophobic interactions with Leu1780 and Met1783, respectively, the small residues Gly1788 and Ala1789 forming the sharp turn that connects helix α1 and β-strand β2, and a number of additional, mostly hydrophobic residues from the extended central β-strands (β1 and β3). Consequently, Ile1766Ser, Met1783Thr, Gly1788Val, Val1804Asp, Val1809Phe, Tyr1853Cys, and other disease-related mutations are expected to destabilize the BRCT fold and are very likely to be functionally deleterious. One of the most striking cancer-predisposing mutations in BRCA1 is the nonsense mutation Tyr1853stop which truncates the protein by 11 amino acid residues. The highly conserved tyrosine is part of a hydrophobic cluster comprising Tyr1853, Leu1854 and Ile1855 which anchor the C-terminal tail of BRCA1 to the core of the second BRCT domain. C-terminal truncations of more than eight amino acid residues render the BRCT tandem repeat sensitive to proteolytic degradation [51] and severely interfere with the cellular functions of BRCA1, pointing at an important role in stabilization of the BRCT fold by hydrophobic interactions with the utmost conserved Trp1837 and other core residues. However, despite the fact that the C-terminal hydrophobic cluster, including Tyr1853, is exceedingly important for the tumor suppressor function of BRCA1, this sequence motif is *not* strictly conserved within the BRCT domain family. BRCT domains with C-terminal truncations are found in BARD1 and some DNA-modifying enzymes which completely lack this hydrophobic cluster, thus indicating that the function of Tyr1853, Leu1854 and Ile1855 in BRCA1 might not be restricted to fold stabilization alone. Instead they might additionally confer BRCA1-specific function such as recognition and binding of molecular targets.

Using the combined information from mutagenesis studies and the different structures of BRCT domains, it may be possible to improve the assessments of cancer risks associated with previously uncharacterized mutations. In addition, the method of NMR spectroscopy provides resonance assignments which may be monitored in CSP mapping experiments to identify new binding partners and the specific residues mediating these interactions.

3.5.2 Intra- and Intermolecular BRCT-BRCT Interactions

Since the discovery of BRCT domains there was controversy about the molecular and cellular functions. Particularly, the finding that BRCT domains may occur in double or multiple repeats opened the possibility that pairs of BRCT domains may physically interact if they are close together in sequence, thus forming basically an intramolecular dimer. Single BRCT domains with no sequential BRCT counterpart, however, could obviously not be involved in *intra*molecular BRCT-BRCT interactions as in the case of BRCT tandem repeats [50]. Yet they were suggested to play a role in *inter*molecular dimer formation. As such, the crystal structure of the second single BRCT domain from XRCC1 (X-ray cross complementation group 1 protein), a protein implicated in DNA repair, showed a BRCT *homodimer* in the asymmetric unit [52]. The authors suggested that the XRCC1 BRCT-BRCT interaction as observed in the crystal might be representative of the reported *heterodimer*ic interaction between XRCC1$^{[BRCT-II]}$ and the single BRCT domain of DNA ligase IIIα (LigIIIα$^{[BRCT]}$) [53], thereby ascribing BRCT domains a general role in protein-protein interactions.

However, the mode of *inter*molecular homo- and heterodimeric BRCT-BRCT assembly still remains a matter of debate. The BRCT-BRCT interaction site in XRCC1$^{[BRCT-II]}$ has been located to helix α1 and the preceding unstructured region, both for the XRCC1$^{[BRCT-II]}$ self-association and heterodimer formation with LigIIIα$^{[BRCT]}$. In contrast, another study reported that a 20-residue peptide derived from the second β-strand (β2) of XRCC1$^{[BRCT-II]}$ bound to LigIIIα$^{[BRCT]}$ with high affinity [54]. This peptide encompassed basically β2 and some additional loop residues but not helix α1, thus suggesting that the BRCT-BRCT interaction between XRCC1$^{[BRCT-II]}$ and DNA ligase IIIα is different from the XRCC1$^{[BRCT-II]}$ self-association. In addition the BRCT domain from DNA ligase IIIα is reported to form dimers in solution [55], whereas the first BRCT domain from XRCC1 (XRCC1$^{[BRCT-I]}$) interacts with the BRCT domain from poly(ADP-ribose) polymerase 1 (PARP1, pADPRT1) [56,57]. The BRCT-BRCT interactions have been recently analyzed with biophysical methods [58].

To date there is a persistent lack of high-resolution structures which might reveal details of heteromolecular BRCT-BRCT interactions. In addition, the functional role of single BRCT domains *in vivo* beyond the suggested assembly of BRCT proteins in response to DNA damage remains largely elusive, raising questions as to potential non-homologous binding targets. Noteworthy, *single* BRCT domains apparently do not bind pPep motifs as it has been demonstrated for *double* BRCT repeats. However, *heterodimer*ic BRCT interactions may in fact result in the assembly of intermolecular BRCT tandem repeats with defined functions. This view is discussed in Chapter 3.5.6 ("Perspective Views: It Takes Two to Tango").

3.5.3 Binding Partners of the BRCT Repeats

Despite numerous publications reporting the molecular interaction between the BRCT repeats of BRCA1 and putative binding partners, phosphorylated peptides (pPeps) represent the first and only class of binding targets to date which have been clearly shown by biochemical and structural studies to form stable complexes with the BRCA1 BRCT repeats. The suggested interaction with the tumor suppressor protein p53, formerly brought about by sequence homology between the BRCT regions of BRCA1 and the p53-binding protein 53BP1, relates to binding studies based on co-precipitation experiments [48,49] but was never approved by structural biology methods. Notably, the binding studies identified different but contradicting p53 interaction sites, and a close look at the results revealed that the control experiments were in fact mutually exclusive.

NMR titration experiments performed with the second BRCT domain of BRCA1 (BRCT-c) and the DNA-binding domain of p53 ($p53^{[94-292]}$) did not show any significant chemical shift perturbations (CSPs) and by that did not indicate a physical interaction between the two domains. These results, however, are consistent with reports showing the molecular interactions between the BRCT tandem repeats of 53BP1 and the DNA-binding domain of p53 to be restricted exclusively to the first BRCT domain and the inter-repeat linker [59,60].

3.5.4 Transcriptional Activation

Soon after the identification of BRCA1 as important gatekeeper and/or caretaker protein, the BRCT repeats were found to possess an intrinsic transcriptional activation function. However, despite much effort to reveal the molecular mechanisms of transcriptional activation by the BRCT repeat region, this issue remains largely a black spot. Nevertheless, as the transcriptional activation function of BRCT was found to be highly correlated with an early onset of breast and ovarian cancer in affected patients, a Gal4-based reporter-gene assay for unclassified mutations in the BRCT region of the *BRCA1* gene has been developed: Wild-type BRCA1 display full activity whereas disease-related mutations in the BRCT repeat region reduce or even abolish the transcriptional activation property completely [34-36].

Mutagenesis studies revealed that the transcriptional activation of the BRCT region is partially preserved in the isolated second BRCT domain (BRCT-c) upon removal of the first BRCT domain (BRCT-n) by means of molecular cloning techniques. Truncations of more than eight residues at the C-terminus of the BRCT repeats completely abolish the activity, as do a number of cancer-associated single point mutations in BRCT-n and/or BRCT-c, thus

highlighting the importance of an intact BRCT-c domain. Particularly surprising, mutations in BRCT-n abolish the transcriptional activation function completely even in the presence of an intact BRCT-c domain. In addition, the cancer-related mutation Met1775Arg severely interferes with the transcriptional activation function of the BRCT repeats [34-36]. In case of the BRCT double repeat, activation of transcription is completely abolished upon mutation of Met1775, whereas in case of the single BRCT-c domain this function is reduced but not entirely lost when Met1775 is mutated. This suggests that BRCT-n may possibly modulate the transcriptional activation properties of BRCT-c by means of allosteric cross-talk between the two BRCT domains. In a simple model this might be accomplished by conformational changes and structural rearrangements by direct interactions at the BRCT interface. In fact, comparison of the BRCT-c NMR structure with the crystal structure of the BRCT tandem repeats [50] reveals that helices α1 and α3 of BRCT-c slightly reorient in the absence of the intra-molecular tandem partner BRCT-n. In case of the complete tandem arrangement, helices α1 and α3 from BRCT-c interact with helix α2-n from the first BRCT domain (BRCT-n) to form a compact interface between the two BRCT repeats.

To test the hypothesis of structural and functional cross-talk between the tandem BRCT domains, NMR titration experiments were envisioned using both the isolated BRCT-n and ^{15}N-labeled BRCT-c domains as binding partners. The idea behind was that a stable BRCT tandem repeat might potentially form upon the addition of BRCT-n, even without the short inter-repeat linker. For this purpose, unlabelled BRCT-n was recombinantly expressed in *E. coli*, using the co-expression systems already successfully applied to BRCT-c and other BRCT constructs. However, despite much effort, it was not possible to isolate BRCT-n in a soluble form. This was rather surprising, given the fold conservation of homologous BRCT domains and the precise knowledge about the domain boundaries. The data strongly suggest that recombinantly expressed BRCT-n failed to fold properly even in excess amounts of the GroEL/GroES chaperonins. This might point at a coupled interaction between the tandem BRCT domains during protein folding, such that BRCT-c might possibly function as a folding scaffold for BRCT-n *in vivo*. In fact, by that BRCT-c might take the role of an intramolecular chaperone.

3.5.5 Molecular and Cellular Functions of the BRCT Repeats

The molecular basis of the highly diverse BRCA1 functions had been a matter of debate for years, when there were several reports published in 2003 which revealed that BRCT tandem repeats bind specifically to phosphorylated peptide motifs [61,62]. The latter accumulate in response to DSBs upon γ-irradiation and subsequent activation of signaling kinases (ATM, ATR, see also Figures 2 and 5). The BRCT tandem repeat of BRCA1 was found to bind preferentially to phosphorylated peptides. The consensus sequence pSer-X-X-F is present in BACH1, a target protein of BRCA1 which is constitutively activated by phosphorylation. In *vivo*, BACH1 is phosphorylated by ATM upon γ-irradiation and subsequently interacts with the BRCT region of BRCA1, thereby activating the G_2-M checkpoint. The molecular basis for phosphopeptide recognition and binding was revealed by a number of independent reports describing the crystal structures of the BRCA1 BRCT repeats in complex with phosphorylated target peptides which were found to be bound at the BRCT-BRCT interface [63-66]. These findings were very well consistent with previous mutagenesis studies and cancer associated mutations, suggesting that the phosphopeptide interaction is functionally linked to the tumor suppressor function of BRCA1. As recently reported, BRCA1 not only regulates *transcription* of checkpoint genes in response to DNA damage but also modulates *translation* by interaction with the poly(A)-binding protein 1 (PABP1) [67]. The cellular and molecular functions of BRCA1 and the BRCT tandem repeats are summarized in the publication describing this part of the thesis [43] and were recently reviewed in [38].

More than 10 years after the discovery of the first gene related to breast cancer, the riddles associated with the molecular mechanisms of BRCA1-mediated cell cycle regulation and tumor suppression slowly unravel: The emerging picture attributes a central role to BRCA1 as component of the BASC genome surveillance complex and as signal transducer in the cellular DNA damage response. BRCA1 appears to mediate DNA damage signals to key components of the cell cycle regulation network. This is apparently accomplished by (i) the direct interaction of the tandem BRCT domains with phosphorylated BACH1 and other target effector and signal transducer proteins, and (ii) the transcriptional activation function of the BRCT region by stimulating the expression of MDM2, the CDK inhibitor $p21^{WAF1/CIP1}$ and other cell cycle checkpoint proteins. This view holds a rational explanation for both the observed embryonic lethality and increased risk of cancer development upon the functional knockout of BRCA1 (Figure 7). However, BRCA1 might be additionally involved in signal transduction pathways and molecular interactions which still need to be discovered.

3.5.6 Perspective Views: It Takes Two to Tango

The tandem BRCT repeats of BRCA1 were found to bind phosphorylated peptide motifs that are present in protein targets upon activation in response to DNA damage and/or cell cycle signaling events. In general, the phosphopeptide binding epitope apparently resides at the interface between the two BRCT repeats. This raises the question as to the binding properties of *single* BRCT domains. Since most BRCT proteins identified so far are involved in signaling pathways initiated by the cellular DNA damage response, it is reasonable to suggest that also single BRCT domains may serve as adaptor modules to recruit DNA repair proteins to sites of damaged DNA or to assemble protein complexes for downstream signal transduction. If the latter happens to occur by phosphorylation events, it is tempting to speculate that two single BRCT domains may assemble *in trans*, thus forming a *hetero*-dimeric tandem repeat that might be capable of binding pPep motifs by virtue of its newly formed BRCT-BRCT interface. By that, BRCT modules may function basically as building blocks that can be assembled and combined to constitute functional intra- or intermolecular BRCT repeats. This offers a playground for further structural and functional studies on other members of the BRCT domain family.