Structure of BRCA1-BRCT/Abraxas Complex Reveals Phosphorylation-Dependent BRCT Dimerization at DNA Damage Sites

Graphical Abstract

Highlights
- Abraxas C terminus S404 is phosphorylated in response to ionizing radiation
- Phosphorylated S404 promotes stable BRCA1 BRCT/Abraxas dimer formation
- Abraxas S404 is critical for BRCA1 accumulation at DNA damage sites
- BRCA1 germline mutations disrupt BRCA1-BRCT/Abraxas dimer formation

In Brief
Abraxas interacts with BRCA1 through a phosphor-SPxF motif. Wu et al. show that a nearby serine 404 residue is phosphorylated in response to ionizing radiation in an ATM-dependent manner. S404 phosphorylation promotes BRCA1-BRCT/Abraxas dimer formation and is crucial for BRCA1 accumulation at DNA damage sites.

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Structure of BRCA1-BRCT/Abraxas Complex Reveals Phosphorylation-Dependent BRCT Dimerization at DNA Damage Sites

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SUMMARY

BRCA1 accumulation at DNA damage sites is an important step for its function in the DNA damage response and in DNA repair. BRCA1-BRCT domains bind to proteins containing the phosphorylated serine-proline-x-phenylalanine (pSPxF) motif including Abraxas, Bach1/FancJ, and CtIP. In this study, we demonstrate that ionizing radiation (IR)-induced ATM-dependent phosphorylation of serine 404 (S404) next to the pSPxF motif. Crystal structures of BRCT/Abraxas show that phosphorylation of S404 is important for extensive interactions through the N-terminal sequence outside the pSPxF motif and leads to formation of a stable dimer. Mutation of S404 leads to deficiency in BRCA1 accumulation at DNA damage sites and cellular sensitivity to IR. In addition, two germline mutations of BRCA1 are found to disrupt the dimer interface and dimer formation. Thus, we demonstrate a mechanism involving IR-induced phosphorylation and dimerization of the BRCT/Abraxas complex for regulating Abraxas-mediated recruitment of BRCA1 in response to IR.

INTRODUCTION

Patients with hereditary breast and ovarian cancer (HBOC) have high germline gene-mutation rates on chromosome 17q21 tumor suppressor gene BRCA1 (breast cancer susceptibility genes 1) (Futreal et al., 1994; Hall et al., 1990; Miki et al., 1994). BRCA1 stabilizes genomic integrity by interacting with various DNA damage response (DDR) sensors, mediators, and effecter proteins, thereby coordinating recognition of the DNA damage sites, cell-cycle checkpoint, DNA repair, transcription, and apoptosis/senescence. BRCA1, a large protein of 1,863 amino acids, contains an N-terminal RING domain and two C-terminal tandem BRCT domains. BRCT domains can recognize phosphorylated proteins with a phosphorylated serine-proline-x-phenylalanine (pSPxF) motif (Manke et al., 2003; Rodriguez et al., 2003; Yu et al., 2003) including Abraxas (Kim et al., 2007a; Liu et al., 2007; Wang et al., 2007), Bach1/FancJ (Cantor et al., 2001; Yu et al., 2003), and CtIP (Wong et al., 1998; Yu et al., 1998). The phosphopeptide-binding ability of BRCA1 BRCT is essential for BRCA1’s tumor suppression function (Shakya et al., 2011), where many breast and ovarian cancer related mutations occur (Clapperton et al., 2004; Couch and Weber, 1996; Friedman et al., 1994; Shattuck-Eidens et al., 1995; Shiozaki et al., 2004; Williams et al., 2004).

The two BRCA1-BRCT domains (BRCT1 and BRCT2) each contain about 100 residues and associate in a head-to-tail manner (Williams et al., 2001). Structural analysis of BRCA1-BRCT domains with pSPxF-containing phosphopeptides of Bach1 (Clapperton et al., 2004; Shiozaki et al., 2004), CtIP (Varma et al., 2005), synthetic optimized phosphopetide (Williams et al., 2004), or other binding proteins (Campbell et al., 2010; Liu and Ladias, 2013; Shen and Tong, 2008) have revealed that phosphorylated serine and phenylalanine in the pSPxF motif bind in a cleft formed at the junction of two BRCT domains in a “two-anchor” mode and the structural integrity of both binding sites is essential for peptide recognition (Glover et al., 2004; Leung and Glover, 2011; Wu et al., 2015). However, little information exists regarding the importance of the sequence surrounding the pSPxF motif. Nor is it known how the specificity is determined for BRCT binding to different pSPxF motif-containing proteins.

Abraxas mediates the interaction of BRCA1 to other components of the BRCA1-A complex, which include BRCC36, NBrA1/MERIT40, BRE, and Rap80. Abraxas, a 409-residue polypeptide, contains a non-catalytic Mrp1, Pad1 N-terminal (MPN)
domain at its N terminus, followed by a coiled-coil (CC) region, an unstructured region, and a BRCA1-binding pSPTF motif at the C terminus. While the N-terminal region including the MPN domain binds to Rap80, BRE, and NBA1/MERIT40, the CC domain is required for interaction with BRCC36 (Hu et al., 2011; Kim et al., 2007a; Wang and Elledge, 2007; Wang et al., 2009). Although the structures for BRCA1 BRCT in complex with other phosphopeptides have been solved previously (Castillo et al., 2014), the structure for BRCT/Abrazas has remained unknown.

Abrazas and the BRCA1-A complex recruit BRCA1 to DNA double-strand-break sites (DSBs) in an ATM-dependent ubiquitin-mediated signaling pathway involving E2 conjugase Ubc13, E3 ligases RNF8/RNF168, and Rap80 binding to ubiquitin lys63-linked polyubiquitin conjugates (Doil et al., 2009; Harper and Elledge, 2007; Hu et al., 2012; Huen and Chen, 2008; Kim et al., 2007b; Kolas et al., 2007; Lee and Paull, 2004; Mailand et al., 2007; Sato et al., 2009; Uziel et al., 2003; Wang, 2012; Wang and Elledge, 2007; Wu et al., 2009). Abrazas-deficient mice exhibit decreased survival and increased tumor incidence (Castillo et al., 2014). The interaction of Abrazas with BRCA1 has been shown critical for the function of Abrazas in DNA repair of DSBs and maintenance of genomic stability. Mutation of the serine residue in the pSPxF motif leads to defective DNA repair and chromosomal aberration. The importance of the Abrazas-BRCA1 interaction in tumor suppression is also suggested by identification of an Abrazas mutation in tumor in the phenylalanine residue of the pSPxF motif (F409C) (Castillo et al., 2014). Thus, structural and functional analysis of Abrazas and BRCA1 interaction is necessary to facilitate the understanding of Abrazas-mediated BRCA1 signaling in tumor suppression.

In this study, we have solved the crystal structures of BRCT with Abrazas phosphorylated peptides and revealed an ionizing radiation (IR)-induced, ATM-dependent Abrazas phosphorylation mechanism, which promotes dimerization of BRCT/Abrazas complex at the DNA damage sites. The IR-induced phosphorylation of Abrazas and the subsequent stabilization of BRCA1-BRCT dimerization are likely to be an important mechanism for accumulation of BRCA1 to DNA-damaged chromatin and BRCA1 mediated tumor suppression.

RESULTS

Abrazas Is Double Phosphorylated at S404 and S406 Residues in Response to IR

Analysis of the C-terminal sequence of Abrazas reveals an additional serine (S404) located close to the pSPxF motif (Figure 1A). Double-phosphorylated Abrazas peptide containing phosphorylated S406 and S404 (GFGEYpS406RpS406PTF) has been identified to bind to BRCA1-BRCT domains in response to IR (Wang et al., 2007). We decided to investigate whether the S404 residue is important and whether it is phosphorylated upon IR. Previously, we have generated S406 phospho-specific antibody and showed that phosphorylation of S406 (pS406) occurs independently of IR (Wang et al., 2007). In view of the fact that S406 is nearby and is phosphorylated in the presence and absence of IR, we generated antibodies specifically recognizing double phosphorylated S404 and S406 (pS404pS406). The pS404pS406 specific antibody recognized Abrazas in parental, but not Abrazas knockout 293T cells and the intensity of the Abrazas band increased significantly when cells were treated with IR, indicative of IR-induced phosphorylation (Figure 1B). Mutation of either S404 (S404A), S406 (S406A), or double mutation (S404A and S406A) abolished the recognition of Abrazas by the pS404pS406 antibody (Figure 1C). Upon IR treatment, double phosphorylation of Abrazas S404 and S406 residues increased immediately (within 10 min), peaked at 1 hr, and gradually decreased to nearly basal levels at late time points (Figure 1D). Furthermore, double phosphorylation occurs in a dose-dependent manner in response to IR (Figure 1E). Since phosphorylation of S406 is not changed upon IR treatment, phosphorylation of S404 is likely to be IR-induced. As ATM plays a central role in the IR-induced signaling pathway that recruits Abrazas and the BRCA1-A complex (Harper and Elledge, 2007), we investigated whether ATM regulates the phosphorylation. Indeed, the IR-induced S404 phosphorylation is ATM dependent, treatment of an ATM inhibitor KU55933 completely abolished the IR-induced phosphorylation (Figure 1F). Other DDR kinases including ATR, DNA-PK, Chk1, or Chk2, however, did not appear to have a major effect on the IR-induced phosphorylation recognized by the pS404pS406 antibody (Figures 1G and S1).

Crystal Structures of BRCA1 BRCT Domains in Complex with Single and Double-Phosphorylated Abrazas Peptides

Since S404 is separated by just one residue from S406, which is part of the pSPxF motif, we hypothesized that an additional mechanism exploiting phosphorylation of S404 might regulate Abrazas interaction with BRCA1. In order to test this, we used structural information to allow comparison of the interactions between BRCT and Abrazas in single and double-phosphorylated states. We solved crystal structures of BRCA1-BRCT in complex with both single (1p) and double phosphorylated (2p) synthesized Abrazas peptides (Ab). The BRCT-Ab1p (GFGEYRpS406PTF) complex was solved at 3.5 Å resolution with no clear main-chain electron density for the N-terminal GFE motif of the peptide, indicating that this region is flexible. BRCT-Ab2p_short without the GFGE residues (YpSRpSPTF) complex was then crystalized and solved at 2.5 Å resolution. The structure of BRCT domains in complex with the 2p Ab (Ab2p: GFGEYRpS406PTF) was solved at 3.5 Å resolution. However, the BRCT-Ab2p_short (YpSRpSPTF) crystal did not diffract to a high resolution. We therefore used BRCT-Ab1p_short and BRCT-Ab2p structures (Figures 2A and S2A) for analysis. Statistics of these two structures are shown in Table 1. As in structures solved previously (Campbell et al., 2010; Clapperton et al., 2004; Liu and Ladias, 2013; Shen and Tong, 2008; Shiozaki et al., 2004; Varma et al., 2005; Williams et al., 2001, 2004), two BRCT domains of BRCA1 (BRCT1 and BRCT2) associate in a head-to-tail manner in both structures. In each domain, a four-stranded parallel β sheet is surrounded by three α helices with α1 and α3 on one side of the β sheet and α2 on the other side. Helices α2 (from BRCT1), α′1, and α′3 (from BRCT2) form
the hydrophobic interface, and the two domains are further linked by extra helix xL (Figures 2A and 2E). The pSPTF motif from Abraxas binds to the BRCT domains in a similar two-anchor mode using pS406 and F409. Residues P407 and T408 do not make major interactions with the BRCT domains. The phosphate group of Abraxas S406 interacts with the side chains of BRCT K1702 and S1655, as well as the main chain of G1656 (Figure 2B). The side chain of F409 in Abraxas inserts into the BRCT hydrophobic pocket created by L1701, F1704, N1774, M1775, and L1839 (Figure 2C). As F409 is the terminal residue for Abraxas, an extra salt bridge is present between the main chain carboxyl group of F409 with the BRCT domain residue R1699 (Figure 2D).

A notable difference between BRCT-Ab2p and BRCT-Ab1p_short structure is the conformation of the Y403S404R405 region (Figures 2E–2G). Extra electron density corresponding to the phosphate group of pS404 and the side chain of Y403 is observed only in BRCT-Ab2p. Unlike pS406, the pS404 phosphate group is oriented away from the BRCT domains into the solvent region, thus avoiding contact with G1656, L1657, and T1658 (Figure 2G). In BRCT-Ab2p, the Y403 side chain is positioned to interact through a hydrophobic interaction with BRCT P1659 at the N terminus of BRCT1_a1. The extra interaction could explain the increased proximity of a1 toward the N terminus of the Abraxas phosphopeptide in BRCT-Ab2p compared to BRCT-Ab1p_short (Figure S3A). Superimposition of all available BRCA1 BRCT related crystal structures also showed that a1 movement toward the phosphopeptide is most prominent in BRCT-Ab2p (Figure S2B). It indicates that one of the roles of pS404 is to fix the side chain of Y403, which is conserved in higher organisms (Figure 2I), such that a trans peptide bond can form and collision is avoided. Superimposition of the BRCT/Abraxas structures with the BRCT/Bach1 (PDB: 1T29) (Shiozaki et al., 2004) and BRCT/CtIP structures (PDB: 1Y98) (Varma et al., 2005) shows similar pSPxF-motif binding. However, compared to Bach1 and CtIP, the N-terminal sequence of Abraxas in both BRCT-Ab1p and BRCT-Ab2p structures exits
on the opposite side, close to the α1 of BRCT1 domain (Figure 2H). Interestingly, a similar side chain arrangement was also seen in BRCA1 BRCT bound with “optimized peptide” (GAAYDiSpQVFAKKK) (PDB: 1T2V) (Williams et al., 2004) (Figure S3C), in which the tyrosine residue (Y) at α3 position (phosphorylated serine in pSxxF motif as 0 position) and negatively charged residues glutamic acid (E) or aspartic acid (D) at α2 position were shown more favored for interaction with BRCA1 BRCT domains (Manke et al., 2003; Rodriguez et al., 2003).

**Dimerization of BRCT-Abrazas Complex in Crystal Structures**

There are eight copies of BRCT-Ab2p in the asymmetric unit (ASU), and each BRCT-Ab2p appears to dimerize through the same interface either within the ASU or between ASUs (Figure S2B). The dimerization of the 1:1 BRCT-Ab2p complex results in a 2:2 BRCT/Abrazas complex dimer. Dimerization involves α1 and β2 of the BRCT1 domain and the Ab2p (Figures 3A–3D) burying about 1,880 Å² area. In the dimer interface, two of the α1 helices from each BRCT-Ab2p complex form isologous interactions burying a hydrophobic patch formed by F1662, M1663, and Y1666 with aromatic side chains stacking on each other (Figure 3B). Interestingly, BRCA1 germline mutations of F1662 (F1662S) and M1663 (M1663K) have been identified in germline cancer patients as recorded in the Breast Cancer Information Core database (Szabo et al., 2000). Extensive hydrogen bonds also form between the two equivalent antiparallel β2 strands (residues T1,675–L1,679) (Figure 3D). The two-fold symmetry axis within the BRCA1/Abrazas dimer lies perpendicular to the two β strands. The cross interaction between the two BRCT/Abrazas complexes is also mediated by the ionic interaction between Abrazas and BRCT α1 of the opposite BRCT/Abrazas complex. The negative surface patch, generated by the phosphate group of pS404 and side chain of E402 at the N terminus of Ab2p peptide, leads to cross interaction with...
BRCT-Ab1p and BRCT-Ab2p also show similar results (Figure S4). Small angle X-ray scattering (SAXS) experiments of the BRCT-Ab1p complex are shown to exist mostly as 1:1 complexes, consistent with the finding that higher protein concentration is required for formation of the dimer interface: (1) the N-terminal hydrophobic region of BRCT 2a-1 and Ab2p-1; (2) extensive hydrogen bonds formed between the phosphate group of pS404 and the side chain of E402; and (3) the N-terminal region of Ab2p including the phosphoepitope containing only phosphorylated S404 (Figure S3A). Under the same condition, the BRCT-Bach1 and BRCT-Ctip form complexes similar to that of BRCT-Ab1p (Figure 3F). Standard protein markers were also run and the positions of their elution peaks are indicated in Figure 3F. The size of BRCT-Ab2p is roughly double that of BRCT-Ab1p according to the protein markers.

We tested whether a higher concentration of BRCT-Ab1p leads to dimer formation as we observed in crystals. Indeed, the BRCT-Ab1p peak shifts left to the BRCT-Ab2p peak position once the concentration is increased (Figure S3E), indicating the tendency of BRCT-Ab1p to form higher order complexes at high protein concentrations as observed in the crystal structure. It is likely that at high concentrations, the BRCT-Ab1p complex is packed in a conformation that is not stable at low protein concentrations without a contribution from phosphorylated S404. Therefore stable dimerization of two BRCT/Abraxas complexes is unique for BRCT-Ab2p.

To confirm dimer formation, we also measured the exact molecular weight of peak fractions eluted from gel filtration using nanoelectrospray mass spectrometry analysis under native conditions (Figures 3G and 3H). BRCT-Ab1p is shown to exist predominantly as a 1:1 complex with a small portion forming a 2:2 dimer. In contrast, the majority of BRCT-Ab2p is detected as 2:2 complexes, indicating a much more stable dimer. BRCT/Bach1 and BRCT/Ctip are detected only as 1:1 complexes, consistent with the finding that higher protein concentration facilitates dimer formation (Figure S3E), the proportion of BRCT-Ab1p forming dimer increases significantly when the protein concentration is increased from 15 m to 25 m (Figure S3F). Small angle X-ray scattering (SAXS) experiments of the BRCT-Ab1p and BRCT-Ab2p also show similar results (Figure S4). Thus, our results indicate that only double-phosphorylated Abraxas C-terminal peptide induces stable dimerization of BRCT/Abraxas complexes in vitro.

**Mutagenesis Studies of BRCT-Ab2p Dimer Interface**

Reveal the Importance of S404 Phosphorylation and Residues of BRCA1 Germline Mutations for Stable BRCT/Abraxas Dimer Formation

In order to test the dimer interface, we have generated various mutants for both BRCT domains and Abraxas (summarized in Figure 4C with peptide sequence indicated) based on the crystal structure. As shown in a simplified graph of the dimer interface (Figure 4A), three regions of interactions appear to contribute to formation of the dimer interface: (1) the N-terminal hydrophobic region of BRCT x1-x1; (2) extensive hydrogen bonds formed by (2)-x2; and (3) the N-terminal region of Ab2p including the phosphorylated S404 interaction with BRCT x1. The interacting residues in x1-x1 and Ab2p-x1 are shown in Figure 4B.
By size exclusion chromatography, we first tested the importance of S404 phosphorylation. Mutations of Abraxas S404 to proline or aspartic acid were generated. While BRCT-Ab1p (S404P) leads to 1:1 complex formation, BRCT-Ab1p (S404D) can maintain the 2:2 complex dimer as BRCT-Ab2p (Figure 4D). This confirms that S404 phosphorylation is essential for dimerization. A previous report using optimized peptide containing aspartic acid in the equivalent position to Abraxas S404 was reported to not form a dimer in solution (Williams et al., 2004). We reasoned that the difference between the optimized peptide and Ab2p is in the N-terminal region (G_{168}F_{1690}G_{1690}E_{1690}) of Ab2p. We demonstrated that BRCT-Ab2p_short without GEGE still forms a dimer indicating that GEGE is not absolutely required for dimerization when Y_{168} and pS_{168} are present. However, when we analyzed BRCT-Ab2p in solution, we found that while F400D did not affect dimer formation, E402R, as well as BRCT K1671E or R1670E, partially destabilizes dimer formation. Double mutation of BRCT(K1671E)-Ab2p(E402R) further destabilized the complex, leading to elution at a position close to that of 1:1 stoichiometry (Figure 4E). These results indicate that although GEGE is not absolutely required for the dimer formation, it contributes to the dimer stabilization when it is present.

We also tested the importance of the BRCT P1659 interaction with Abraxas Y403 to the stability of the BRCT-Ab2p dimer. In the presence of the N-terminal region (GFGE) of Ab2p, mutation of Abraxas Y403A did not destabilize the dimer complex formation in either BRCT-Ab2p(Y403A) or BRCT(P1659G)-Ab2p(Y403A). But when the N-terminal region of Abraxas was absent, the dimer complex of BRCT-Ab2p(Y403A_short) became unstable. This destabilization was further enhanced in BRCT(P1659G)-Abraxas(Y403A_short) complex, which was eluted near the 1:1 complex (Figure 4F). Together, these results are consistent with the crystal structure analysis showing that Ab2p promotes dimer formation in two different ways: (1) phosphorylated S404 fixes the side chain of Y403, which generates additional interaction with BRCT K1671 and (2) the phosphorylated group of pS404 and E402 form a negative surface region that leads to cross interaction with BRCT K1671.

We then evaluated the contribution of the hydrophobic interactions between the two helices (z1-z1) and the extensive hydrogen bonds between the two antiparallel β strands (β2-β2) in the two protomers that comprise the dimer (Figure 4G). Our results indicate that the z1-z1 interaction contributes more significantly toward the stabilization of the dimer interface than the β2-β2 interaction. The BRCT mutant N1678A, which disrupted the β2-β2 interaction (Figure 3D), reduced hydrogen bonds between the side chain of N1678 and the nearby residue T1675, but did not destabilize BRCT-Ab2p dimer formation. In contrast, F1662S and M1663K, two BRCA1 germline mutations identified in cancer patients (Szabo et al., 2000), led to complete disruption of the dimer formation as the elution peaks of these two mutants in complex with Ab2p moved to the 1:1 complex position. BRCT Y1666A mutant did not result in complete disruption of the dimer and the peak in the elution profile is located between that for the 2:2 and 1:1 complexes. These results support our observation from the crystal structure that F1662S and M1663K have a much more significant effect on disruption of the dimer interface because these two residues are located at the point of the isologous dimer interface, while Y1666A is further away.

**Figure 3. 2p Ab Induces Dimerization of BRCT-Ab2p Complex**

(A) Crystal structure of BRCT-Ab2p complex dimer viewed from three different directions with a two-fold axis. The dimer interface is within the dashed circles and zoomed in (B)-(D).

(B) Dimer interface between two BRCT z1 helices.

(C) Interaction between BRCT z1 helix and Ab2p.

(D) Dimer interface between two BRCT β2 strands. The polar interactions between labeled residues are shown in black dashed lines. The key residues are indicated in the image.

(E) Surface representation of BRCA1-1p_short (yellow) and BRCT-Ab2p dimer (blue and pink). The Abraxas pS406 binding region is indicated in the dashed circle.

(F) Gel filtration BRCT in complex with Ab1p, Ab2p, Bach1, and CtIP phosphopeptides at a concentration of 40 μM (1 mg/mL). The regions for dimer complex (2:2 complex) and monomer complex (1:1 complex) are highlighted in yellow and gray shades. The elution positions for void and protein markers aprotinin (Mr = 6,500 Da), ribonuclease A (Mr = 13,700 Da), carbonic anhydrase (Mr = 29,000 Da), Ovalbumin (Mr = 44,000 Da), and Conalbumin (Mr = 75,000 Da) are indicated.

(G) The molecular weight of BRCT and its complexes with phosphopeptide, measured using native mass spectrometry.

(H) The native mass spectra of BRCT-Ab1p, BRCT-Ab2p, BRCT-Bach1, and BRCT-CtIP complexes tested at 15 μM (see also Figures S3 and S4).
We tested whether BRCA1 forms dimers in vivo and whether the stable dimer formation is dependent on Abraxas. We co-expressed differentially Myc- or FLAG-tagged BRCA1 full-length protein in control (Ctrl) cells or Abraxas knockout (KO) cells. In the co-immunoprecipitation experiment with lysates prepared from cells treated with IR, immunoprecipitated FLAG-BRCA1 interacts with Myc-tagged BRCA1, indicating that BRCA1 indeed dimerizes in vivo. The dimerization was decreased in Abraxas KO cells, indicating the dependency of dimerization on Abraxas (Figure 6A). Similarly, a construct containing only the BRCA1-BRCT domains also dimerizes when co-expressed in cells and the dimerization is decreased in Abraxas KO cells (Figure 6B).

We then tested whether the germline mutations F1662S and M1663K interfere with BRCA1 dimerization in vivo. We compared the interaction of a Myc-tagged full-length BRCA1 and a HA-tagged wild-type BRCT fragment with that of the F1662S or M1663K mutant of BRCA1 and a mutant BRCA1 BRCT fragment with three residues localized in the dimer interface mutated (F1662S/M1663K/R1670E). Both the Myc-immunoprecipitation (Figure 6C) and reciprocal HA-immunoprecipitation (Figure 6D) experiments showed that the
interaction/dimerization of BRCA1 and BRCT was decreased with mutation of the critical residues at the dimer interface F1662S or M1663K. Thus, BRCA1 germline mutations interfere with stable dimer formation in vivo.

To understand dimerization of the BRCT/Abraxas complex in vivo, we examined whether Abraxas forms a dimer in which the phosphorylated C-termini of Abraxas in complex with BRCT could be in close vicinity for dimerization. We expressed both GFP-tagged Abraxas and HA-FLAG-tagged Abraxas in cells and tested whether the differentially tagged Abraxas molecules interact with each other using the immunoprecipitation assay. We found that wild-type Abraxas, as well as the S404A...
Figure 6. Abraxas Promotes BRCA1 BRCT Dimerization In Vivo

(A) Abraxas-dependent BRCA1 dimerization in vivo. The differentially Myc- and FLAG-tagged BRCA1 full-length constructs were transiently transfected into parental 293T (Ctrl) or Abraxas KO cells. The lysates from cells treated with 10 Gy IR followed by 1 hr incubation at 37°C were used for FLAG-immunoprecipitation. The intensity of individual bands was quantified by densitometric analysis using NIH ImageJ software. The normalized value (IPed_mycBRCA1/Input_mycBRCA1) was shown in the bar graph.

(B) Abraxas-dependent BRCA1-BRCT domains dimerization in vivo. The differentially Myc- and HA-tagged BRCA1-BRCT domains constructs were transiently transfected into parental 293T (Ctrl) or Abraxas KO cells. The lysates from cells treated with 10 Gy IR followed by 1 hr incubation at 37°C were used for HA-immunoprecipitation. The band intensity was quantified with NIH imageJ software. The normalized value (IPed_mycBRCT/Input_mycBRCT) was shown in the bar graph. (legend continued on next page)
and S406A mutant, interact with differentially tagged counterpart, indicating that Abraxas dimerizes/oligomerizes in vivo independently of its binding to BRCA1 (Figure 5E). We then investigated what region of Abraxas mediates the dimerization by examining various deletion mutants of Abraxas. Deletion of the CC domain abolished the self-interaction of either wild-type or mutant Abraxas (Figures 6F and S6). Thus, in vivo, Abraxas dimerizes/oligomerizes through the CC domain.

**DISCUSSION**

BRCA1 accumulation to DNA damage sites is a crucial step for BRCA1’s function in DNA damage repair, and BRCT domains of BRCA1 are important for the tumor suppressor function of BRCA1. IR-induced ubiquitination at DNA damage sites generates docking sites for the recruitment of the Abraxas/BRCA1-A complex and accumulation of BRCA1 at sites of damage. Our data provide evidence for an IR-induced, ATM-dependent mechanism specific to Abraxas-mediated recruitment of BRCA1. In such a model (Figure 7), IR-induced phosphorylation of S404 next to the pSPxF induces stable dimer formation of the BRCA1 BRCT/Abraxas complex.

The crystal structural analysis of BRCT in complex with Abraxas phosphorylated peptides revealed that, although both Ab1p and Ab2p bind to BRCT domains through the same pSPxF motif, the phosphorylation of S404 in Ab2p induces stable dimerization of the BRCT/Abraxas complex. The dimer interface locates to the BRCT1 of BRCA1 tandem BRCT domains. As expected from previous reports, BRCT1 also provides the interaction site for pS406 of the pSPxF motif, while the side chain of F of the motif inserts into a hydrophobic pocket created mainly by BRCT2 domain. The dimer surface formed between two BRCT1 domains does not directly influence the interaction between the pSPxF motif and the BRCT domains. Although the hydrophobic z1 interface observed in the BRCT-Ab2p dimer was buried in a similar manner to that of the BRCT-Ab1p or other BRCA1 BRCT related crystal structures (Wu et al., 2015), this interface alone is not strong enough to form a stable dimer in solution as we observed for BRCT domain only, BRCT-Ab1p, BRCT-Bach1, or BRCT-CtIP. In contrast, under the same condition, the stable-dimer state of BRCT-Ab2p is triggered by the phosphorylation of S404. A phosphorylation-mimetic point mutant S404D stabilizes BRCT/Abraxas dimer formation in solution in a similar way to phosphorylated S404, further supporting the conclusion that phosphorylation of S404 promotes dimer formation. The impact of phosphorylation of Abraxas S404 is the following: (1) the highly charged phosphate group of pS404 faces away from BRCT domains, resulting in stabilization of the interaction of the side chain of Y403 with BRCT P1659 located at the N terminus of z1. This interaction causes the shift of z1 closer to the N terminus of the phosphopeptide; and (2) the negatively charged side chains of pS404 and E402 provide extra ionic interaction sites with K1671 of BRCT. All together, this leads to a stable BRCT-Ab2p complex dimer formation. Owing to the symmetric pairing shape, we describe the BRCT-Ab2p dimerization interaction as a “pair-hugging” interaction mode, in which the Abraxas phosphopeptide acts as an arm wrapping around the other BRCT domain therefore stabilizing the interaction (Figure 7).

The IR-induced phosphorylation of Abraxas S404 and the subsequent stable BRCA1-BRCT dimerization are likely to comprise an important mechanism for cellular response to IR since mutation of S404 leads to decreased BRCA1 accumulation to DNA damaged chromatin and increased cellular sensitivity to IR. IR-induced phosphorylation of Abraxas S404 may facilitate the accumulation of BRCA1 at DNA damage sites by stabilizing the BRCA1 protein dimerization, forming more stable higher order complexes at sites of damage. In addition, S404 phosphorylation may further facilitate the interaction of Abraxas and BRCA1 by reducing the dissociation of pS406, so prolonging the Abraxas interaction with BRCT domains or limiting the accessibility of pS406 by other proteins such as phosphatase.

Alternatively, induced dimerization of BRCA1 BRCT by phosphorylation of S404 of Abraxas could increase the local concentration of BRCA1 at damaged chromatin, which is likely critical for efficient DNA damage signaling and repair.

Many tumor-derived truncation and missense mutations have been identified in the BRCA1 BRCT domains. While some of these mutations have been shown to either destabilize the protein fold of the BRCT domains or disrupt the binding surface to pSPxF-containing phosphopeptides (Cantar et al., 2001; Clapperton et al., 2004; Coquelle et al., 2011; Manke et al., 2003; Shiozaki et al., 2004; Williams and Glover, 2003; Williams et al., 2004; Yu et al., 2003), resulting in cancer predisposition, the function of a large number of BRCT mutations is still unknown (Easton et al., 2007). Our analyses reveal that germline mutations F1662S and M1663K disrupt the ability of BRCT to dimerize, in vitro and in vivo, providing a structural explanation for the possible role of these mutations in inactivating BRCA1 tumor suppressor function. Future study is needed to further characterize the effect of these mutations in the function of BRCA1 in DNA repair and damage signaling.

How is the dimerization of BRCT-Abraxas achieved in vivo? Since the phosphorylation of S406 is not IR-dependent, Abraxas binds to BRCA1 through the pSPxF motif even in the absence of DNA damage (Wang et al., 2007). The dimerization of Abraxas
through the CC domain could potentially position the two BRCT tandem domains that interact at the C terminus of Abraxas into close vicinity, leading to an unstable dimer of the BRCT-Abraxas complex in the absence of DNA damage. In response to IR, IR-induced phosphorylation of S404 leads to an increase of affinity between the phosphate group and the residues at the dimer surface, which consequently results in a much more stable dimer complex of BRCA1 BRCT/Abraxas. Since the CC domain of Abraxas also appears to dimerize with the CC domain of BRCC36 (Wang and Elledge, 2007), it is likely that, in the BRCA1-A complex, Abraxas and BRCC36 form an oligomeric bundle through the CC domain present on each of the Abraxas and BRCC36 molecules. Future structural analysis of the BRCA1-A complex is needed to further understand the
multimerization of Abraxas and BRCC36 of the BRCA1-A complex. Nevertheless, IR-induced phosphorylation at Abraxas S404 appears to function as a regulatory switch, which leads to stable dimerization of two nearby BRCT domains.

The phosphorylation-induced BRCT dimerization is observed only in the BRCT/Abraxas complex. We demonstrate that, in addition to the pSpxF-binding motif, IR-induced phosphorylation of a nearby S404 residue can further regulate the interaction of BRCT and Abraxas. Thus, amino acid sequences outside the pSpxF motif may confer specificity in regulation of the BRCT binding to phosphorylated proteins. Indeed, in addition to phosphorylated S404, the N-terminal region of Ab (GFGE\textsuperscript{402}V\textsuperscript{403}R\textsuperscript{405}pS\textsuperscript{406}R\textsuperscript{407}PSPVF) also contributes to stable BRCT/Abraxas dimer formation. The E402 residue cooperates with the phosphorylated S404 in stabilizing the dimer formation; the side chain of Y403 is fixed to interact with BRCT P1659 when S404 is phosphorylated. Thus, the unique sequence feature outside of the pSpxF motif ensures that stable dimer formation occurs only with BRCT/Abraxas, but not with other BRCT complexes. Since Abraxas-BRCT1 interaction has been shown critical for DNA repair and maintenance of genomic stability, the additional regulatory mechanism uncovered in this study in regulating the interaction of Abraxas and BRCT1 further highlights the importance of this interaction in BRCT1 signaling and tumor suppression.

In summary, our study reveals a phosphorylation-dependent mechanism in Abraxas-mediated recruitment and accumulation of BRCT1 at DNA damage sites, deepening our understanding of BRCT1 and Abraxas tumor suppressor function and related cellular signaling. Our study also provides structural insights that will assist the design of small molecules modulating BRCT1-Abraxas interaction in the future.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Culture, and Antibodies**

U2OS cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS). 293T cells were grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% FBS. Details of generation of Abraxas knockdown or KO cells are described in the **Supplemental Information**. Antibodies used are described in the **Supplemental Information**.

**Immunofluorescence**

Cells were fixed with 3.6% formaldehyde for 10 min, permeabilized with 0.5% Triton X-100 solution, and incubated with primary antibodies for 1 hr at 37 °C followed by appropriate Alexa 488-conjugated (green; Invitrogen) and Alexa 555-conjugated (red; Invitrogen) secondary antibodies. Additional information is included in the **Supplemental Information**.

**Cell Lysis and Immunoprecipitation**

Cell lysates were prepared from 293T cells or 293T Abr1-gene KO cells either untreated or treated with 10 Gy IR followed by incubation at 37 °C for 1 hr. FLAG immunoprecipitation (IP) was performed with lysates prepared from cells either treated or untreated with 10 Gy IR followed by 1 hr incubation at 37 °C. Western blots were carried out using indicated antibodies. 293T cells were incubated with kinase inhibitors for 2 hr at indicated concentrations. Cells were then either exposed to 4 Gy IR or untreated. After 1 hr incubation, cells were lysed and Abraxas pS404pS406 levels were determined by western blot. Additional information is included in the **Supplemental Information**.

**Clonogenic Survival Assay**

Stable U2OS cell lines were seeded at low density in 10 cm dishes and irradiated with 4 Gy ionizing irradiation using a \textsuperscript{137}Cs source. The cells were then cultured at 37 °C for 14 days to allow colonies to form. Colonies were stained with 2% methylene blue and 50% ethanol for 10 min.

**Chromatin Fractionation**

Abraxas knockdown cells complemented with vector, wild-type (WT), S404A, S406A, and double mutant (S404A and S406A) were irradiated at 10 Gy and incubated for 1 hr at 37 °C. Cells were then subjected for chromatin fractionation followed by detection with indicated antibodies. Details are described in the **Supplemental Information**.

**BRCT Construct, Purification, Crystallization, and Data Collection**

Construction and purification of BRCT1 BRCT and mutants are described in the **Supplemental Information**. Purified BRCT was mixed with Abs at a 1:3 molar ratio as has been previously reported (Shiozaki et al., 2004) and incubated at 4 °C for 30 min. Final protein concentration was 30 mg/ml. Crystallization was set up using the hanging-drop vapor diffusion method with drops containing 1 μl of protein sample and 1 μl of crystallization solution. Crystals appeared after 3–4 days. BRCT-Ab2p was crystallized in 0.1 M HEPES (pH 7.0), 60 mM ammonium sulfate, and 5% (w/v) PEG 4000. BRCT-Ab1p short was crystallized in 1 M lithium chloride, 0.1 Tris (pH 8.0), and 20% (w/v) PEG 6000. X-ray diffraction data were processed by XDS (Kabsch, 2010) and Scala (Winn et al., 2011). The phases for the structure factors were obtained through molecular replacement using Phaser module in Phenix 1.8.4-1496 (Adams et al., 2010). More detail of protein crystal and structure determination can be found in **Supplemental Information**.

**Size Exclusion Chromatography for BRCT with Abraxas, Bach1, and CtIP Phosphopeptides**

BRCT protein and Abraxas, Bach1, and CtIP peptides were mixed in a 1:3 molar ratio to a final concentration of 1 mg/ml (about 40 μM) in 500 μl loops. Gel filtration was performed in Buffer A using Superdex 75 10/300 column (GE Healthcare life) with a flow rate of 0.5 ml/min. For studying the high protein concentration effects on BRCT-Abraxas complex formation, a final concentration of 10 mg/ml was used. Protein markers (GE Gel Filtration LMW Calibration Kit) were run following the kit protocol.

**Native Mass Spectrometry**

Samples were diluted to 15 or 75 μM protein concentration in 300 mM ammonium acetate (pH 7.6) and further buffer exchanged into 300 mM ammonium acetate using Bio-Spin 6 (Bio-Rad) column. The desalted samples were loaded into the in-house prepared gold-coated glass capillaries (Hernández and Robinson, 2007). Nano-electrospray mass spectrometric analyses were performed under native conditions on a hybrid quadrupole time-of-flight mass spectrometer previously modified for high mass transmission (Sobott et al., 2002). Typically the following instrumental conditions were used: capillary voltage 1.3 kV, sample cone 200 V, and collision cell energy 5 V.

**ACCESSION NUMBERS**

The accession numbers for the BRCT-Ab1p_short and BRCT-Ab2p data reported in this paper are PDB: 4V2G, 4V18.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.12.017.

**AUTHOR CONTRIBUTIONS**

Q.W., B.W., and T.L.B. initiated this project. Q.W. performed most of the in vitro work and solved structures. A.P., D.S., and B.W. designed the in vivo experiments. D.S. generated Abraxas KO cells and analyzed Abraxas double...
phosphorylation in response to IR and in the presence of kinase inhibitors, as well as Abraxas dimerization in vivo. A.P. generated Abraxas knockdown cells, examined the Abraxas mutants in rescuing the defects of Abraxas-deficient cells, and Abraxas-dependent BRCA1 dimerization in vivo. S.M. and C.V.R. performed and analyzed the native mass spectrometry experiment. T.O. conducted SAXS experiment. E.L.B. made and purified the BRCT P1659G mutant. T.K.F. and B.X. generated constructs expressing Myc-tagged BRCA1 F1662S and M1663K mutants. Q.W., A.P., B.W., and T.L.B. wrote the paper together with comments from other co-authors.

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