

# Structure and function of the Rad9-binding region of the DNA-damage checkpoint adaptor TopBP1

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## ABSTRACT

**TopBP1 is a scaffold protein that coordinates activation of the DNA-damage-checkpoint response by coupling binding of the 9-1-1 checkpoint clamp at sites of ssDNA, to activation of the ATR-ATRIP checkpoint kinase complex. We have now determined the crystal structure of the N-terminal region of human TopBP1, revealing an unexpected triple-BRCT domain structure. The arrangement of the BRCT domains differs significantly from previously described tandem BRCT domain structures, and presents two distinct sites for binding phosphopeptides in the second and third BRCT domains. We show that the site in the second but not third BRCT domain in the N-terminus of TopBP1, provides specific interaction with a phosphorylated motif at pSer387 in Rad9, which can be generated by CK2.**

## INTRODUCTION

Activation of the Chk1 arm of the DNA-damage checkpoint response is triggered by assembly of a number of multiprotein complexes at segments of single-stranded DNA resulting from replication fork stalling or from resection of a double-strand break (1). Multiple copies of replication protein A, coat the single-strand DNA, and facilitate recruitment of ATR via interaction with its constitutive binding partner ATRIP (2). The Rad9-Rad1-Hus1 DNA-damage checkpoint clamp is loaded at the junction of single- and double-stranded DNA by a specialized form of the RFC clamp loader, incorporating Rad17 in place of Rfc1 (3,4). While there may be some direct interaction, at least in yeast (5), coupling of these two major complexes into a functional system is performed by TopBP1, which binds

simultaneously to the phosphorylated C-terminal tail of Rad9 (6–8), and to the ATR-ATRIP complex, upregulating its kinase activity (9,10).

TopBP1 was originally identified as a binding partner of DNA topoisomerase II in yeast 2-hybrid experiments (11), and in addition to its involvement in the DNA-damage checkpoint, it has been variously found to interact with transcription factors such as Miz-1, HPV16E2 and E2F1 (12–14), the protein kinase c-Abl (15), the DNA-damage sensor PARP-1 (16), replication protein Cdc45 (17), p53 (18) and the Nbs1 subunit of the MRN complex (19). Recently, TopBP1 has also been implicated in recruitment of DNA Pol- $\alpha$ , suggesting a role in the restart of stalled replication forks (20), although whether this involves a direct interaction is not clear.

Structurally, TopBP1 contains multiple copies of the BRCT domain, first identified in *Schizosaccharomyces pombe* Rad4 (21,22) and subsequently in the C-terminal region of the breast-cancer-associated DNA-damage mediator protein BRCA1 (23,24). Indeed *S. pombe* Rad4 (aka Cut5) is the orthologue of metazoan TopBP1. In *Xenopus*, binding of TopBP1 to the phosphorylated C-terminal tail of Rad9 has been shown to be dependent on the first-tandem pair of BRCT domains (BRCT1:2) (25). The TopBP1 orthologues Rad4 in *S. pombe* and Dpb11 in *Saccharomyces cerevisiae* also interact in a phosphorylation-dependent manner with the tail of Rad9 and Ddc1, respectively, but in these organisms it is the second-tandem pair of BRCT domains, BRCT3:4 that mediate this interaction (26–28). The first pair of BRCT domains on Rad4 is instead involved in interaction with a phosphorylated site in the N-terminus of Crb2, the *S. pombe* homologue of mammalian 53BP1 (29,30). Interaction of metazoan TopBP1 with ATR-ATRIP is mediated by a specific C-terminal domain of unknown structure, mapping between the sixth- and seventh-BRCT domains in the protein sequence (10), which interacts with conserved domains in ATR and ATRIP (9).

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A functionally comparable ATR-activating domain has also been identified in the budding yeast equivalent of TopBP1, Dpb11, at the C-terminus beyond the fourth BRCT domain (31,32).

Of the remaining BRCT domains in TopBP1, BRCT6 is implicated in binding to E2F1 (12) and PARP-1 (16), while BRCT domains 7 and 8 mediate interaction with the helicase BACH1/FANCI (33). Although the basis of these interactions remains to be described, structural analysis of TopBP1-BRCT6 (34) suggests that it does not involve a phospho-peptide interaction of the type commonly mediated by tandem pairs of BRCT domains (35–37). A clear role for the tandem-pair BRCT4:5 have not yet emerged.

To try and gain some insight into the specific roles of the individual domains of TopBP1 in mediating specific protein interactions, we have now determined the crystal structure of an N-terminal segment of human TopBP1, from the N-terminus to beyond BRCT2 and analysed its interactions with the C-terminal tail of Rad9 *in vitro*.

## MATERIALS AND METHODS

### Cloning, expression and purification

*Human TopBP1(1–290)*. DNA-encoding amino acids 1–290 of human TopBP1 was amplified by PCR from IMAGE CLONE 8991925 (Geneservice, Cambridge, UK), then sub-cloned into both an in-house modified pOPIN vector (38) and pGEX-6P-1 (GE Healthcare, Chalfont St Giles, UK) encoding N-terminal, 3C-protease cleavable, octa-histidine (His<sub>8</sub>) or Glutathione S-Transferase (GST) affinity tags, respectively.

His<sub>8</sub>- or GST-tagged TopBP1(1–290) was transformed into *Escherichia coli* expression strain Rosetta2(DE3) pLysS (Merck, KGaA, Darmstadt, Germany). A single transformed colony was used to inoculate a 250-ml flask containing 100 ml of Luria–Bertani broth (LB) supplemented with carbenicillin (100 µg/ml) and chloramphenicol (34 µg/ml). The inoculated culture was grown overnight at 37°C, at 220 rpm, in an orbital shaking incubator.

The following day, 20 ml of the overnight culture was used to inoculate a 2-l flask containing 1 l of LB, supplemented with antibiotics as before. Cultures were grown at 37°C, 220 rpm, until the optical density at 600 nm reached 0.6–0.8. They were then removed from the incubator, and rapidly cooled on ice for 30 min. Recombinant protein expression was induced by the addition of 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cultures incubated for a further 16 h, at 220 rpm, at a reduced temperature of 16°C. Cells were then harvested by centrifugation, and the resulting pellet stored at –80°C until required.

The cell pellet arising from 6 l of culture was resuspended in 100 ml of 25 mM HEPES pH 7.5, 1 M NaCl, 5% v/v glycerol, 0.1% v/v Tween 20, supplemented with protease inhibitors (Roche, Burgess Hill, UK), then lysed by sonication (18 × 5 s bursts, on ice, at 50% amplitude, Jencons Ultrasonic Processor). Cell debris and

precipitated material was removed by high-speed centrifugation at 48 834g for 60 min.

The supernatant arising from this step was applied to a batch/gravity column containing 10 ml of either Talon (TaKaRa Bio, Saint-Germain-en-Laye, France) or Glutathione Sepharose 4 Fast Flow (GE Healthcare) resin, depending on the encoded affinity tag.

The column containing the cell extract and resin was rotated/rolled at 4°C for a period of 1 h to facilitate protein binding, and then allowed to pack under gravity flow.

For the His-tagged protein, the column was washed with 250 ml of 25 mM HEPES pH 7.5, 1 M NaCl, 5% v/v glycerol, 5 mM imidazole. Bound protein was then eluted with the application of 25 mM HEPES pH 7.5, 1 M NaCl, 5% v/v glycerol, 400 mM imidazole. Rhinovirus 3C-protease (PreScission, GE Healthcare) was added to the eluate, and incubated overnight at 4°C to cleave the affinity tag.

For the GST-tagged protein, the column was instead washed with 250 ml of 25 mM HEPES pH 7.5, 1 M NaCl, 5% v/v glycerol. Rhinovirus 3C-protease was then added directly to the resin/buffer slurry and incubated overnight, with rolling, at 4°C to cleave the affinity tag. The released protein was collected by repacking the column under gravity flow, and then collecting the eluate.

In both cases, the eluted TopBP1 (1–290) protein was concentrated to a final volume of 5 ml (Vivaspin 20, 10 kD MWCO, Sartorius Stedim, Epsom, UK) then applied to a HiLoad Superdex 200 16/60 size exclusion chromatography column (GE Healthcare) pre-equilibrated in 25 mM HEPES pH 7.5, 1 M NaCl, 5 mM EDTA, 10 mM DTT and 5% v/v glycerol (for crystallographic studies), or 25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 mM DTT and 5% v/v glycerol (for peptide-binding studies).

*Human Rad9Tail*. DNA encoding the C-terminus of human Rad9 (amino acids 264–391) was amplified by PCR from a vector encoding full-length human Rad9 kindly provided by Dr Andrew Doré (ICR; The Institute of Cancer Research, UK) then sub-cloned into the expression vector pGEX-6P-1 (GE Healthcare).

Expression and purification of GST-Rad9Tail was essentially as that described for TopBP1(1–290) except that the cell pellet resulting from 4 l of cell culture was used to produce the initial cell lysate, only 5 ml of Glutathione Sepharose 4 FF resin was used in the affinity capture step, and the protein was eluted from the column using 25 mM HEPES pH 7.5, 1 M NaCl, 10 mM DTT, 5% v/v glycerol, 5 mM EDTA and 40 mM reduced glutathione and not cleaved with Rhinovirus 3C-protease.

*Selenomethionine-labelled TopBP1(1–290)*. The TopBP1(1–290) expression plasmid was co-transformed along with the pRARE plasmid (Merck) into the methionine auxotroph, *Escherichia coli* strain, B834(DE3) (Merck). Transformed colonies were selected on LB agar plates supplemented with antibiotics as before. From an overnight culture, 25 ml was used to inoculate a 2-l flask, containing 1 l of SelenoMet Medium Base plus Nutrient

Mix and SelenoMethionine solution (Molecular Dimensions, Newmarket, UK) supplemented with antibiotics. Cultures were incubated at 37°C, 220 rpm, in an orbital-shaking incubator, until they reached an optical density of  $\sim 0.7$  at 600 nm, when they were removed from the incubator and rapidly cooled on ice. Protein expression was induced with IPTG as before, and cultures incubated for a further 16 h, at 220 rpm and 16°C, before the cells were harvested by centrifugation.

Incorporation of the selenomethionine label was verified using mass spectrometry (Dr WJ Mawby, Department of Biochemistry, University of Bristol).

### Crystallization, data collection, phasing, model building and refinement

Selenomethionine-labelled TopBP1(1–290) was crystallized at 14°C using the hanging drop vapour diffusion method, by mixing equal volumes of the protein (10–15 mg/ml) with either 100 mM Tris–HCl pH 7.5, 400 mM MgCl<sub>2</sub>, 20–30% w/v polyethylene glycol 4000, 2–6% v/v glycerol (spacegroup P2<sub>1</sub>), or 100 mM Tris–HCl pH 6.8, 500 mM KI, 18–25% w/v PEG 3350, 2–8% v/v glycerol (spacegroup P2<sub>1</sub>3). Crystals were visible after 24 h, but generally took a week to reach their maximum size.

Cryoprotection, for data collection, was achieved by step-wise soaking in buffers containing increasing amounts of glycerol, to a final concentration of 30% v/v.

All diffraction data were collected at 100K on station ID14.4 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Data were processed and scaled using the software package Mosflm (39) and Scala (40).

Phases were calculated from a two-wavelength anomalous dispersion experiment with the P2<sub>1</sub>3 crystals, which comprised one molecule per asymmetric unit and a solvent content of 68%. Positions of eight Se atoms and five Iodide ions could be determined using SHELX (41) and refined using SHARP (42), yielding an interpretable electron density map at 2.8Å resolution.

Density modification and automated building was carried out by using the program RESOLVE (43) to produce an initial model, which was extended through a combination of manual building in Coot (44) and refinement with the PHENIX suite (45). This model was then used to solve the P2<sub>1</sub> data set by molecular replacement using PHASER (46), which contained four molecules per asymmetric unit, and a solvent content of 52%.

### Peptides

Biotinylated peptides were purchased from Pepceuticals Ltd, Nottingham, UK, or from the University of Bristol Peptide Synthesis Facility, Bristol UK.

Rad9Tail: pS387, Biotin-SPVLAED[pS]EGE

H2A.1: pS129, Biotin-GSG-YSGSRTGKP-[pS]-QEL

Fluorescein-labelled peptides were purchased from Peptide Protein Research Ltd, Fareham, UK.

Rad9, pS272: fluorescein-GGSDDTDSH[pS]QDLGSPE

Rad9, pT355: fluorescein-GGEPSTVPG[pT]PPPKKFR

Rad9, pS375: fluorescein-GGSILAPVR[pS]PQGSPV

Rad9, pS380: fluorescein-GGVRSPQGP[pS]PVLAEDS

Rad9, pS387: fluorescein-GGPPSPVLAED[pS]JEGEG

Crb2: pT215, fluorescein-GGSGQVET[pT]PTRLAT

Where [pS] or [pT] corresponds to phospho-serine or phospho-threonine, respectively.

### Co-precipitation (pull down) experiments

*GST-tagged proteins.* A volume of 100 µl of GST, GST-Rad9Tail WT or S387A mutant at a concentration of 31.5 µM, was mixed with 100 µl of Glutathione Sepharose 4 FF resin (GE Healthcare) pre-equilibrated in buffer A: 25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 mM DTT and 5% v/v glycerol, then mixed/incubated for a period of 3 h at 4°C to facilitate protein binding.

The resin was then washed three times, with successive applications of 1 ml aliquots of buffer B: 25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 mM DTT and 5% v/v glycerol supplemented with 0.1% v/v BSA (NEB, Hitchin, UK) and 0.1% v/v NP-40 (buffer B).

A volume of 100 µl of GST-CKII $\alpha$ , at 31.5 µM, in buffer B supplemented with 10 mM ATP and 30 mM MgCl<sub>2</sub> was added to the resin slurry, then mixed/incubated at 4°C for a period of 15 h. The resin was then washed with 1 ml of buffer B, and a further three times with 1 ml aliquots of buffer A.

Then, 100 µl of His-tagged TopBP1(1–290), at 94.5 µM in buffer B, was added to the beads and incubated for a further period of 3 h at 4°C. The beads were then washed four times, with 1 ml aliquots of buffer A. Samples of each co-precipitation were then analyzed by SDS–PAGE.

Dephosphorylation of immobilized GST-Rad9Tail WT, was performed after the CK2 $\alpha$  phosphorylation step, by the addition of 40 µg of  $\lambda$ -phosphatase and 30 U of calf intestinal alkaline phosphatase (CIP, NEB) in 100 µl of buffer B supplemented with 30 mM MnCl<sub>2</sub>, then incubated for 15 h at 4°C.

*Biotinylated peptides.* A volume of 100 µl of biotinylated Rad9Tail phospho-peptide at 140 µM, or H2A.1 phospho-peptide at 100 µM, in buffer A, was added to 100 µl of NeutrAvidin agarose beads (Thermo Fisher Scientific, Loughborough, UK), then incubated/mixed at 4°C for a period of 1 h, before being washed with three successive 1 ml aliquots of buffer A. A volume of 100 µl of His-tagged TopBP1(1–260), at 42 µM in buffer B, was then added to the resin slurry and incubated for a further period of 2 h at 4°C. Samples were analysed by SDS–PAGE, after washing the beads a further four times with buffer A.

Dephosphorylation and re-phosphorylation steps were carried out using  $\lambda$ -phosphatase/CIP and CKII $\alpha$  as before.

All samples were analysed by SDS–PAGE, on 4–12% NuPAGE Bis–Tris gels (Invitrogen, Paisley, UK) run in 1 $\times$  MES buffer (Invitrogen). Western blots, used a mouse anti-his primary (TaKaRa Bio, Cat No: 631212) and an HRP-conjugated anti-mouse (GE Healthcare, Cat No: NXA931) secondary antibody.



## Fluorescence polarization

Fluorescein-labelled peptides at a concentration of 10 nM, were incubated at 4°C, for a period of 30 min with increasing concentrations of WT or mutant TopBP1(1–290) in 25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM EDTA, 10 mM DTT, 5% v/v glycerol, 0.01% v/v NP40. The samples (typically a volume of 50 µl) were then transferred to a black 96-well polypropylene plate (VWR, Lutterworth, UK) for measurement of fluorescence polarization in a Victor V<sup>2</sup> Multilabel Reader (Perkin Elmer, Cambridge, UK). Two 0.1 s measurements were collected for each well with excitation/emission wavelengths of 485 and 535 nm, respectively, with either parallel or perpendicular polarizers in-line. Background fluorescence in wells containing only buffer was subtracted from all values obtained for the samples.

Polarization data were analysed using GraphPad Prism 5.0 by non-linear fitting with a one-site total binding model. The non-specific binding component was then subtracted from the data for presentation purposes. All data represent the mean of three separate experiments, and error bars represent one standard deviation.

## RESULTS

### Structure of TopBP1 N-terminus

An N-terminal construct of human TopBP1 (residues 1–290) was expressed in *E. coli* using a selenomethionine-supplemented medium, and two different crystal forms were obtained. The structure was determined by a two-wavelength anomalous diffraction experiment, and refined at a resolution of 2.6 Å ('Materials and Methods' section and Table 1).

As expected from the previous amino acid sequence analysis (28), the crystal structure of TopBP1(1–290) contained the predicted BRCT domains 1 and 2, formed by residues 106–198 and 199–289, respectively. More surprising was the presence of an additional undocumented BRCT domain, N-terminal to these, consisting of residues 1–105, although the possibility of this had previously been suggested (28). To maintain consistency with published nomenclature, the second- and third-BRCT domains in this structure will be referred to as BRCT1 and 2, while the unexpected BRCT domain at the extreme N-terminus is designated BRCT0 (Figure 1A and B).

Previous structural and bioinformatics analysis has identified two sub-classes of BRCT domains, those such as the C-terminal domain of XRCC1 (47) which occur as singletons, and those in proteins such as BRCA1, Crb2, 53BP1, Mdc1, etc., which are involved in phospho-peptide binding, and occur as tandem pairs, closely linked in sequence and in 3D structure. To our knowledge, the TopBP1(1–290) structure is the first example of a closely linked triple-BRCT, and is structurally distinct from the previously described singleton and tandem examples.

Examination of the TopBP1 BRCT0:1 or BRCT1:2 pairs, reveals a very different juxtaposition of the consecutive BRCT domains from each other, and from previously

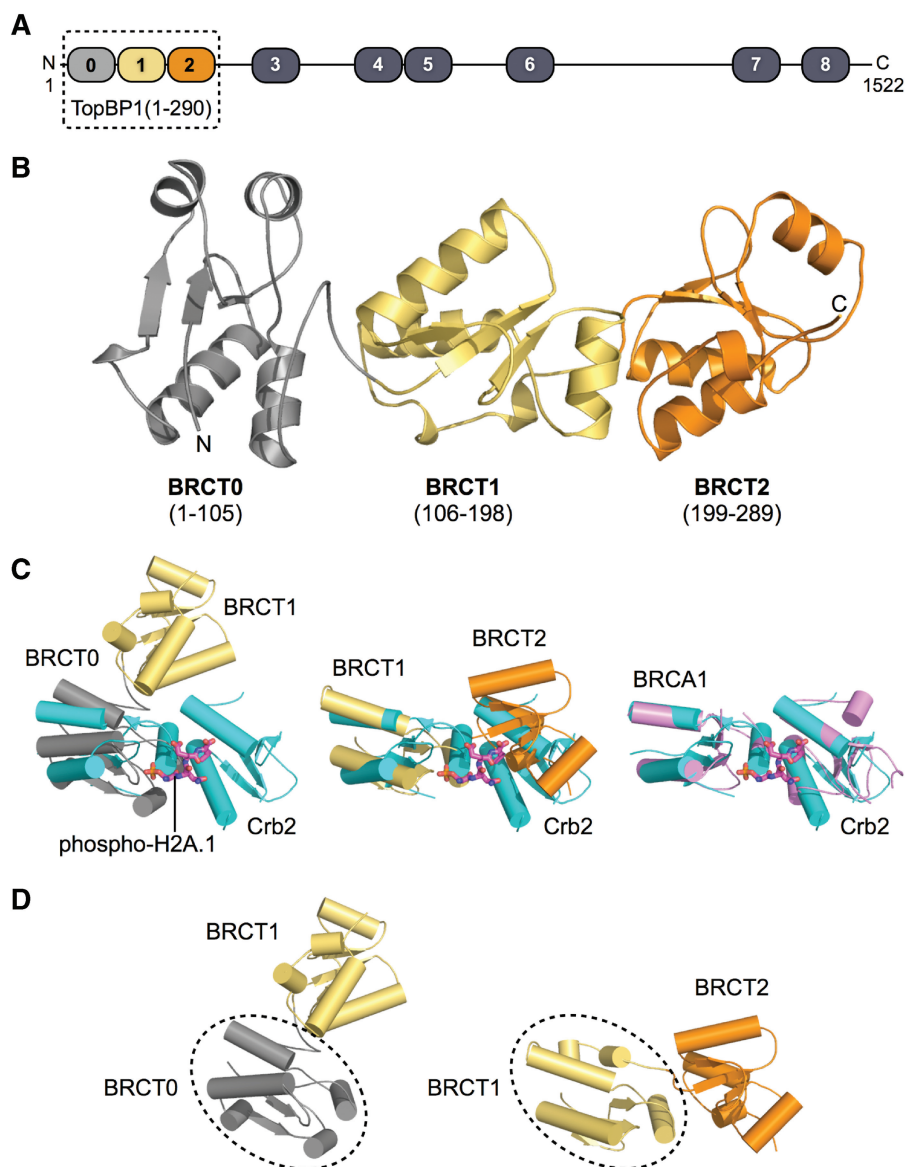
**Table 1.** Data collection, phasing and refinement statistics

Data collection		
Space group	P2 <sub>1</sub> 3	P12 <sub>1</sub> 1
Cell dimensions a, b, c (Å)	115.8, 115.8, 115.8	39.5, 290.3, 60.6
Cell angles $\alpha$ , $\beta$ , $\gamma$ (°)	90.0, 90.0, 90.0	90.0, 89.5, 90.0
Resolution (Å)	51.8–2.8 (2.95–2.8)*	55.9–2.6 (2.74–2.6)
$R_{\text{merge}}$	0.091 (0.50)	0.103 (0.38)
$1/\sigma I$	6.9 (1.5)	5.0 (2.0)
Completeness (%)	99.9 (99.9)	97.2 (97.2)
Redundancy	5.9 (6.1)	2.6 (2.6)
Wilson B (Å <sup>2</sup> )	75.2	56.2
Phasing power	Acentric	Centric
Phasing		
Se inflection anomalous	1.313 (0.300)	
Se peak anomalous	1.377 (0.310)	
Se peak isomorphous	0.123 (0.065)	0.097 (0.044)
FOM	0.3972	0.1495
Refinement		
Resolution (Å)	47.3–2.80	51.3–2.60
Reflections (work/free)	24687/1254	76051/3850
$R_{\text{work}}/R_{\text{free}}$	0.232/0.251	0.205/0.266
Number of atoms		
Protein	2280	9438
Ligands/ions	17	66
Solvent	73	343
B factors		
Protein	58.7	57.5
Ligands/ions	83.7	52.8
Solvent	37.1	43.2
R.m.s.d. values		
Bond lengths (Å)	0.003	0.005
Bond angles (°)	0.603	0.806
Ramachandran plot (%)		
Favoured	97.5	99.0
Allowed	2.5	1.0
Outliers	0.0	0.0

\*Highest resolution shell is shown in parenthesis.

documented 'conventional' tandem BRCT structures (Figure 1C and D). Despite the individual BRCT domains having a standard  $\beta 1$ - $\alpha 1$ - $\beta 2$ - $\beta 3$ - $\alpha 2$ - $\beta 4$ - $\alpha 3$  topology and each having its C-terminal  $\alpha 3$  helix connected to the N-terminal  $\beta 1$  strand of the subsequent BRCT, the central  $\beta$ -sheets in the BRCT domains are perpendicular to each other rather than parallel as in canonical tandem BRCT structures. In the TopBP1(1–290) structure this spatial arrangement is dictated by the very short nature of the linkers between consecutive BRCT domains: BRCT0  $\alpha 3$  helix is connected to BRCT1  $\beta 1$  strand through a 17-amino acid linker (91–108) and BRCT1  $\alpha 3$  helix is connected to BRCT2  $\beta 1$  strand through a 22-amino acid linker (181–203). This contrasts with all other known tandem BRCT structures where longer inter-domain linkers permit a more extended arrangement in which the  $\alpha 3$  helix of the upstream BRCT domain packs against the  $\alpha 1$  helix of the downstream BRCT domain (Supplementary Figure S1).

Overall, the three consecutive BRCT domains form a continuous molecular surface of roughly cylindrical shape, with significant burial of hydrophobic surface at the interfaces. Consistent with this, the overall arrangement of the three domains seems to be rigid, and is essentially identical



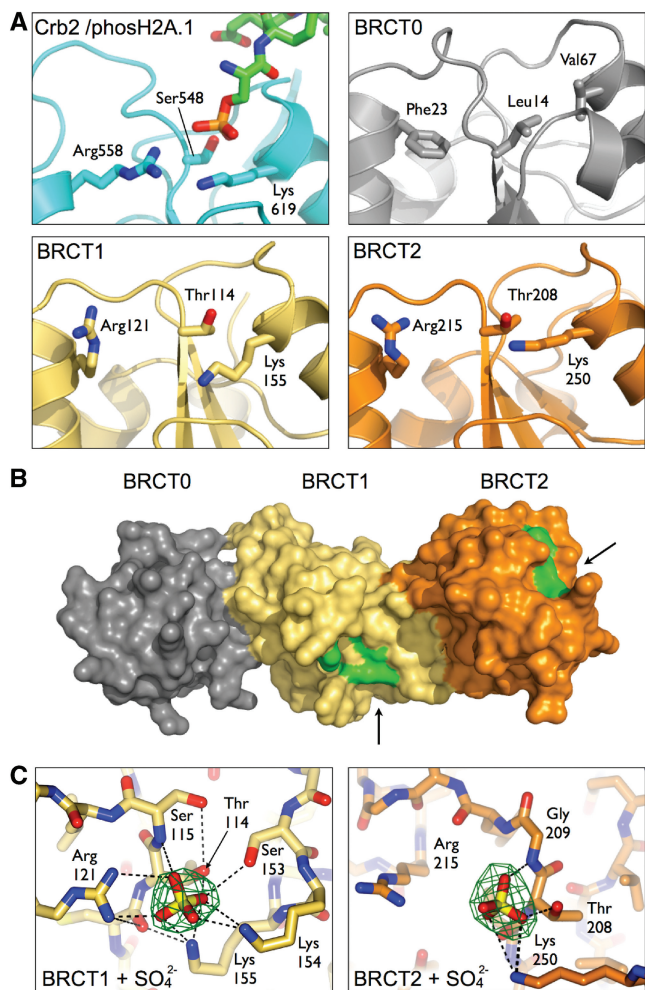
**Figure 1.** Structure of the N-terminus of TopBP1. (A) Schematic representation of human TopBP1, highlighting the position of each BRCT domain. The boxed region indicates the amino acid boundaries of the TopBP1(1–290) expression construct used in this study. (B) The X-ray crystal structure of TopBP1(1–290) reveals three consecutive BRCT domains, designated as BRCT0, BRCT1 and BRCT2 (coloured grey, yellow and orange, respectively). Amino acid boundaries of each domain are indicated in parentheses. This and all other molecular graphics figures were produced with MacPyMOL unless otherwise stated. (C) Superimposition of the first BRCT domain in the consecutive TopBP1 BRCT pairs BRCT0:1 and BRCT1:2 onto the first-BRCT domain in the canonical-tandem BRCT structure (cyan) of Crb2 in a phospho-H2A.1 peptide complex (PDB: 2VXC), reveals significant differences in juxtaposition of the two domains in both TopBP1 pairs (left, middle). In contrast (right), both BRCT domains in the canonical pair from BRCA1 (pink), align with their equivalents in Crb2. The bound phospho-peptide from the Crb2–pH2A.1 complex is shown in stick representation. In order to improve the clarity of the figure, some interconnecting loops have been omitted. (D) Comparison of the TopBP1 BRCT0:1 and BRCT1:2 pairs, based on superimposition of the outlined domain from each, but with the pairs separated for clarity, reveals distinct and idiosyncratic domain juxtapositions for each, and the absence of any repeating poly BRCT superstructure.

in the two different crystal forms (r.m.s.d of 0.91Å over 279 C $\alpha$  positions), which involve distinct crystal lattice contacts.

#### Phospho-peptide-binding sites

Previous studies have implicated TopBP1 BRCT domains 1 and 2 in interaction with the phosphorylated tail of Rad9 (25). Structures of tandem BRCT domains bound

to phosphopeptides (48–54), have identified a conserved cluster of charged and polar amino acids in the first-BRCT domain of a tandem pair, that provide generic interactions with the phosphorylated residue in a phosphopeptide ligand, while residues in the cleft formed by the junction of the two BRCT domains, furnish specificity for ligand residues C-terminal to the phosphorylated serine or threonine. Examination of the three BRCT domains in TopBP1(1–290) shows the presence of the



**Figure 2.** Phosphopeptide-binding sites in TopBP1 N-terminus. (A) Comparison of the phosphopeptide-binding site in a canonical tandem BRCT structure, Crb2, with the topologically equivalent sites in TopBP1 BRCT0, BRCT1 and BRCT2. The pattern of residues that provide the phosphate interaction in Crb2 and in other known phosphopeptide-binding BRCT domains, are present in BRCT1 and BRCT2 but not BRCT0 where the site is hydrophobic in nature. (B) Surface representation of TopBP1(1–290) structure coloured by domain as in Figure 1. The conserved putative phosphopeptide-binding patches conserved in BRCT1 and BRCT2 are highlighted in green. (C) Difference Fourier maps from crystals of TopBP1(1–290) grown in a buffer containing  $\text{MgSO}_4$ , show significant density ( $>2.5\sigma$ ) in the putative phosphopeptide-binding sites of BRCT1 and BRCT2, corresponding to bound sulphate. This confirms that both sites are competent for interacting with the phosphate of a phosphopeptide.

generic phospho-peptide recognition motif in BRCT domains 1 and 2, but not in BRCT0, where the topologically equivalent residues are hydrophobic (Figure 2A). The two putative phosphopeptide-binding patches on BRCT1 and 2 are disconnected from each other, and are arrayed  $\sim 90^\circ$  apart around the overall cylindrical axis of the TopBP1(1–290) structure. The interface between BRCT1 and 2 is quite different from the more conventional tandem BRCT structures as it does not provide the peptide-binding cleft. Instead, the phosphopeptide-binding patch on BRCT1 runs into a helical channel winding around the TopBP1(1–290) structure, and

connecting to the phosphopeptide-binding patch on BRCT2 (Figure 2B).

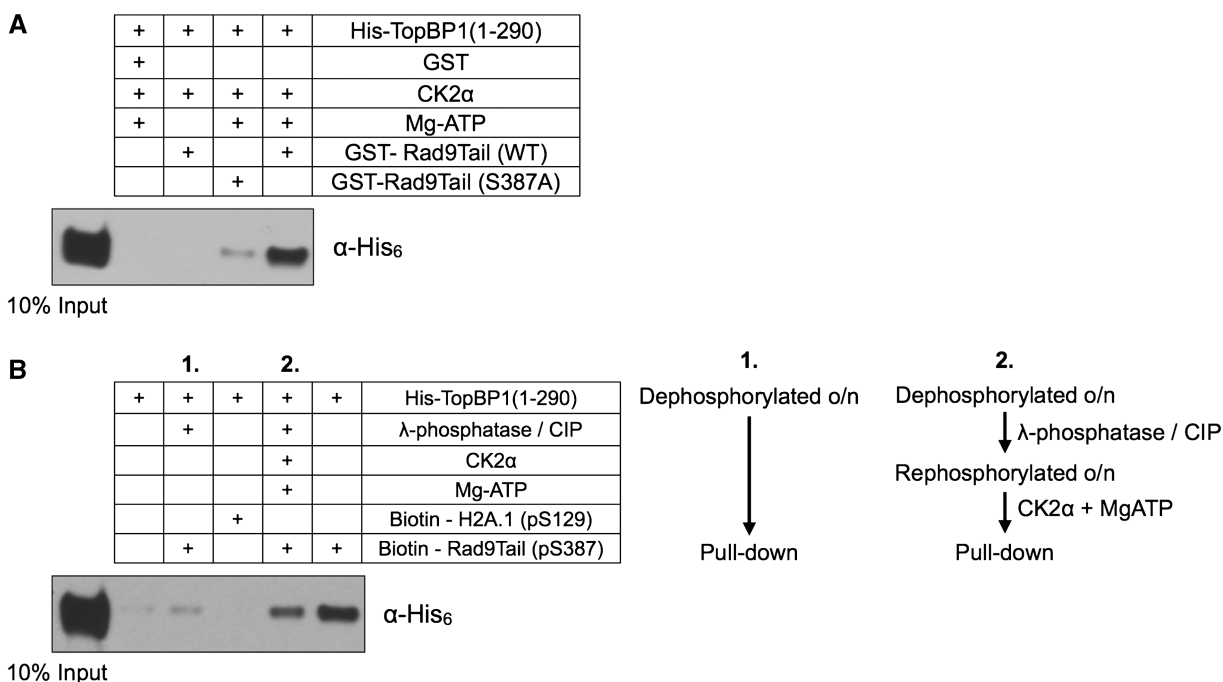
Phosphate-binding sites in proteins frequently show high affinity for sulphate ions, which are iso-structural with phosphate. Crystals of TopBP1(1–290) grown in buffers containing  $\text{MgSO}_4$  instead of the original 200 mM  $\text{MgCl}_2$  displayed strong electron density for sulphate ions bound to the putative phosphopeptide-binding sites in BRCT1 and BRCT2 (Figure 2C), where the key residues are conserved, but not in BRCT0, where they are not.

### Rad9 phosphorylation

The Rad9 C-terminal tail, extending beyond the structurally defined PCNA-like core (55–57), contains multiple sites of phosphorylation that are conserved to varying degrees from yeast to mammals (Supplementary Figure S2). A conserved site, Ser272 in human Rad9, occurs close to the N-terminus of the tail immediately following the PCNA-like core, and is believed to be phosphorylated by ATM/ATR on genotoxic stress (58,59). The opposite end of the Rad9 tail contains a second well-conserved site, Ser387 in humans, whose phosphorylation at least in metazoa, is constitutive and required for TopBP1 recruitment and consequent activation of ATR as part of checkpoint signalling (6,8,10,25). Multiple potential sites for phosphorylation by the cyclin-dependent kinase Cdc2 (7) can also be identified between the 272 and 387 sites, but the significance of these is not known.

The nature of the kinase responsible for providing the essential TopBP1-binding site at Rad9-pSer387, has not previously been determined, although bioinformatics analysis (60) suggested CK2, which would certainly be consistent with the constitutive nature of the phosphorylation. To determine whether CK2 could provide this essential phosphorylation, we expressed a GST-fusion with the C-terminus of Rad9 (GST-Rad9Tail; amino acids 264–391) in *E. coli*, incubated it with recombinant maize CK2 $\alpha$  and Mg-ATP as described earlier (61), and tested its ability to co-precipitate TopBP1(1–290) (Figure 3A). GST alone incubated with CK2 $\alpha$  and Mg-ATP, or GST-Rad9Tail incubated with CK2 $\alpha$  in the absence of Mg-ATP, failed to co-precipitate detectable TopBP1(1–290). GST-Rad9Tail incubated with CK2 $\alpha$  in the presence of Mg-ATP gave a robust co-precipitation of TopBP1(1–290), whereas a GST-Rad9Tail construct with an S387A mutation incubated in the same conditions failed to significantly co-precipitate TopBP1(1–290). To further test the specificity of the interaction, we repeated the experiment with a biotinylated-synthetic peptide consisting of the last 11 residues of human Rad9 with a phosphoserine at 387. Again we observed a robust co-precipitation of TopBP1(1–290) by neutravidin beads in the presence of the pS387 peptide or a dephosphorylated peptide that was incubated with CK2 $\alpha$  and Mg-ATP, but not with the dephosphorylated Rad9 peptide, or with an unrelated biotinylated phosphopeptide derived from the C-terminus of *S. pombe* histone H2A.1 (Figure 3B). Taken together these data confirm that the





**Figure 3.** Rad9-tail phosphorylation and TopBP1 binding. (A) Pull-down assay of binding of His<sub>6</sub>-TopBP1(1–290) to a GST-Rad9 tail construct. His<sub>6</sub>-TopBP1(1–290) was efficiently co-precipitated only when the GST-Rad9 tail construct was incubated with the protein kinase CK2 $\alpha$  in the presence of Mg-ATP, but not by GST alone, or a mutant in which the target phosphorylation site, Ser387 was changed to alanine. (B) Pull-down assay using a synthetic biotin-coupled peptide incorporating a phosphoserine at the equivalent of Ser387. His<sub>6</sub>-TopBP1(1–290) was efficiently co-precipitated by the biotin-pS387 peptide, but not by a different biotinylated-phosphopeptide, nor by the biotin-pS387 peptide treated with phosphatase. Incubation of phosphatase-treated peptide with CK2 $\alpha$  + MgATP restored binding.

C-terminal sequence of Rad9 including pSer387 is essential for interaction with TopBP1(1–290) and that the key phosphorylation is likely to be provided by CK2.

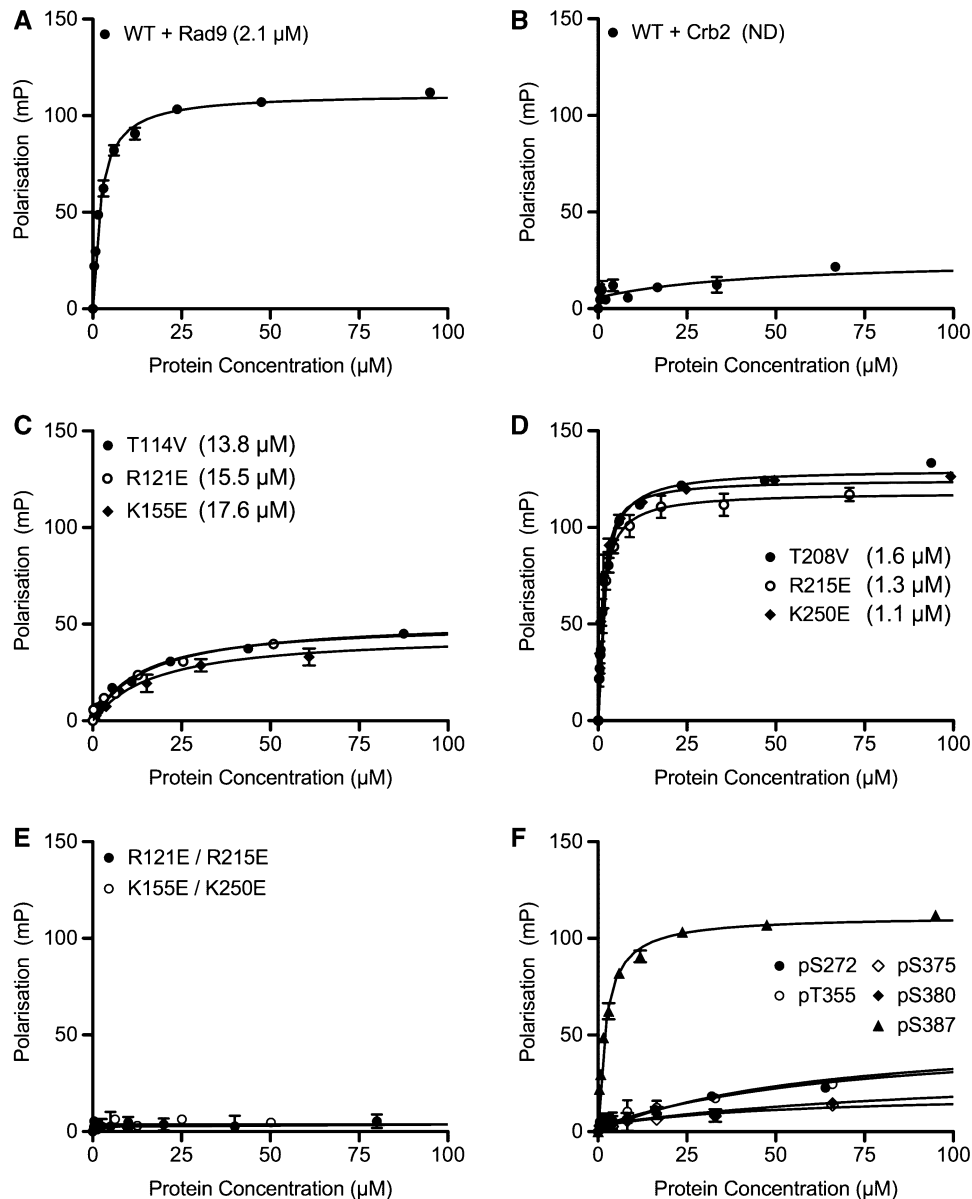
#### Identification of the Rad9 interaction site in TopBP1

Previous studies in *Xenopus*, have implicated the N-terminal region of TopBP1 as necessary for interaction with the C-terminally phosphorylated Rad9 tail (25). Within that region of TopBP1 the structural data presented here reveal three BRCT domains, two of which (BRCT1 and BRCT2) possess the characteristic motifs for phosphopeptide interaction, identified from studies of conventional tandem BRCT structures (48–54,62,63). While involvement of BRCT0 in mediating part of the interaction with Rad9 cannot be totally ruled out, the strong dependence of the interaction on phosphorylation suggests that one or both of BRCT1 and BRCT2 are likely to play the dominant role.

To test this, we generated a series of mutants, in which key residues in the phosphopeptide interaction motifs of BRCT1, BRCT2 or both, were changed to residues that would be likely to abrogate phosphopeptide interaction, based on analysis of other BRCT systems (49). We then determined the interaction of these with a fluorescently labelled synthetic phosphopeptide incorporating the last 13 residues of human Rad9 with the equivalent of Ser387 phosphorylated, using a fluorescence polarization (FP) assay (Figure 4A). Wild-type TopBP1(1–290) bound the Rad9 peptide with a  $K_d = 2.1 \mu\text{M}$ . The interaction was

specific to the Rad9 sequence, and no interaction was observed with a non-cognate fluorescent phosphopeptide derived from a segment of *S. pombe* Crb2, implicated in binding to the TopBP1 homologue, Rad4 (Figure 4B). Mutation of Thr114, Arg121 or Lys155 in the putative phosphopeptide-binding site in BRCT1, significantly decreased the strength of the interaction, but did not totally abolish it (Figure 4C). In contrast, mutation of Thr208, Arg215 and Lys250 in BRCT2, did not weaken the interaction, but actually caused a slight increase in affinity (Figure 4D). Double mutants, in which the putative binding sites in both BRCT domains were disrupted, showed no measurable interaction with the Rad9 phosphopeptide (Figure 4E).

The substantial loss of affinity on mutation of the BRCT1 site, clearly implicates it as the primary determinant of Rad9 C-terminal phosphopeptide binding in the TopBP1(1–290) construct. The weak-residual interaction observed when the BRCT1 site, but not the BRCT2 are mutated, could suggest that BRCT2 also contributes to the interaction and that the two sites cooperate. However the observation that binding is actually tighter when the BRCT2 site is disabled, contradicts this interpretation, and is more consistent with it displaying a weak non-specific binding activity for the Rad9 phosphopeptide that competes with specific binding to BRCT1 in this assay. Together with the observation of sulphate ion-binding this does strongly support the idea that BRCT2 possesses a competent phosphopeptide-binding site, but one whose specific target is something other



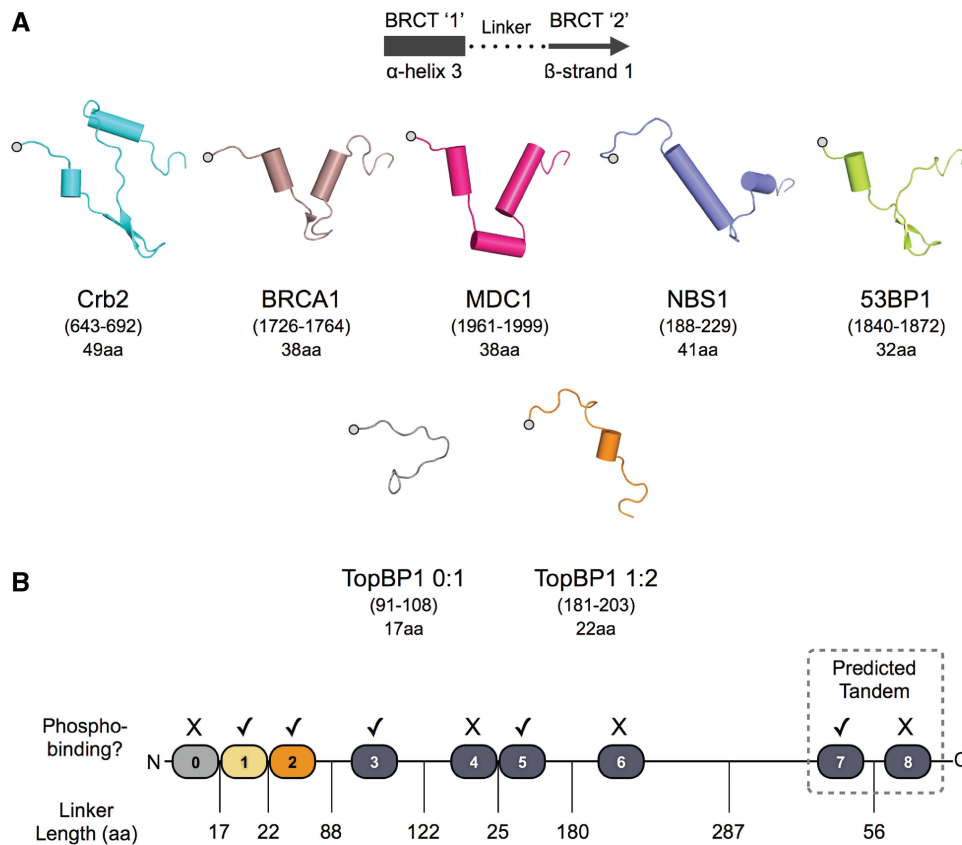
**Figure 4.** Specificity of phospho-peptide binding to TopBP1(1–290). (A) Binding affinity of TopBP1(1–290) for a fluorescein-labeled Rad9-derived peptide, incorporating pSer387, as determined by fluorescence polarization. The calculated  $K_d$  (shown in parenthesis) indicates a specific interaction. For calculation of  $K_d$  see ‘Materials and Methods’ section. (B) As (A) but with a labelled phosphopeptide derived from the *S. pombe* checkpoint mediator protein Crb2. No significant binding is observed, confirming the specificity of the Rad9 interaction. (C) As (A), but with TopBP1(1–290) constructs harbouring mutations in the putative phosphopeptide-binding site in BRCT1. The affinity of the interaction is substantially reduced, indicating a significant contribution to the interaction by BRCT1. (D) As (C), but with TopBP1(1–290) constructs harboring mutations in the putative phosphopeptide-binding site in BRCT2. The affinity of the interaction is comparable to that for wild-type TopBP1(1–290), indicating that there is no significant contribution to the interaction by BRCT2. (E) As (C), but with TopBP1(1–290) constructs with mutations in both putative phosphopeptide-binding sites. The weak-residual binding present in BRCT1 mutants is effectively abolished in the double mutants. (F) As (A), but with phosphopeptides corresponding to five different phosphorylation sites that have been mapped within the Rad9 tail. Only the peptide incorporating pSer387 shows significant affinity.

than the Rad9 C-terminal sequence incorporating pSer387.

Human Rad9 has a cluster of poorly conserved putative Cdc2 phosphorylation sites upstream of Ser387, at Thr355, Ser375 and Ser380 (7) (Supplementary Figure S2). The proximity of these to Ser387 in the linear sequence of the Rad9 tail, and the physical proximity of the BRCT1 and BRCT2 phosphopeptide-binding sites in

the TopBP1(1–290) structure suggested the possibility that BRCT2 might provide a binding site for one of these. To test this, we synthesized fluorescently labelled phosphopeptides incorporating pThr355, pSer375 or pSer380, and used these in FP assays as above. However, unlike the pSer387 peptide, neither of these phosphopeptides showed any significant ability to bind to TopBP1(1–290) specifically (Figure 4F). Finally, we





**Figure 5.** BRCT-domain classes in TopBP1 and other BRCT domain proteins. (A) Array of the linkers connecting consecutive BRCT domains in structurally characterized tandem BRCT proteins. The linker segments in the triple-BRCT structure of TopBP1(1–290) are substantially shorter, resulting in the novel non-canonical domain arrangement. (B) Assignment of BRCT domain classes in TopBP1. Domains 1, 2, 3, 5 and 7 show the characteristics of phosphopeptide binding, but only BRCT7:8 resemble the canonical tandem pair arrangement found in checkpoint mediators such as Crb2, BRCA1, etc.

considered the possibility that BRCT2 might provide a binding site for the relatively well-conserved pSer272 site at the N-terminal end of the Rad9 tail. Again, no significant affinity was observed for a fluorescently labelled pSer272 phosphopeptide.

## DISCUSSION

### BRCT domain architecture of TopBP1

BRCT domains are key sites of protein–protein interaction in the regulation and assembly of numerous complexes involved in replication and repair of DNA (64). From previous biochemical and structural studies they can be roughly partitioned into two functional classes—singleton BRCTs typically involved in mediating homo or hetero-dimerization with other BRCT domains (65,66), and tandem BRCTs where pairs of closely contiguous BRCT domains cooperate to provide sequence specific binding sites for (typically) phosphorylated peptide motifs on other proteins (35). At the sequence level, TopBP1 appears to be a composite of both types of BRCT, with the tandem pairs BRCT1:2, BRCT4:5 and BRCT7:8 conforming to the consensus for phosphopeptide binding, while BRCT domains 3 and 6

resemble non-phospho-binding singletons (34). The data presented here shows the architecture of TopBP1 to be far more complicated, with an additional BRCT domain at the N-terminus of the protein, contributing to a complex triple-BRCT structure that has no precedent in previously described systems.

In light of this new data, we have re-examined the amino acid sequence attributes of closely contiguous tandem BRCT pairs, and find that we can distinguish between the ‘canonical’ phosphopeptide-binding tandem pairs (e.g. Crb2, BRCA1, Mdc1, etc.) and the novel structural arrangements found in TopBP1, by consideration of the size of the peptide segment that links the individual BRCT domains. Thus, the canonical tandem BRCT structures, in which the individual domains of the pair have a similar orientation and are effectively related by a translation, have a segment of ~32–49 amino acid residues connecting the last  $\alpha$ -helix of the first-BRCT domain to the first  $\beta$ -strand of the second (Figure 5A). In the novel arrangements found in TopBP1, by contrast, these linker segments are substantially shorter, with 17 residues connecting BRCT domains 0 and 1, and 22 connecting BRCT domains 1 and 2. With the shorter linker, the juxtaposition of the consecutive BRCT domains is highly constrained and results in a screw relationship, with the putative

**Table 2.** Phospho-binding analysis of TopBP1 BRCT domains

	Phospho binding			
Crb2 (1)	Arg558	Ser548	Lys619	Yes
BRCT0	Phe68	Leu14	Val67	No
BRCT1	Arg121	Thr114	Lys155	Yes
BRCT2	Arg215	Thr208	Lys250	Yes
BRCT3	Leu374	Cys367	Glu407	No
BRCT4	Glu568	Leu561	Glu602	No
BRCT5	Lys661	Ser654	Lys704	Yes
BRCT6	Gln920	Val912	Glu957	No
BRCT7	Arg1280	Ser1273	Lys1317	Yes
BRCT8	Glu1408	His1402	Asn1452	No

Amino acids at the topologically equivalent positions associated with binding the phospho-group of phosphorylated peptides in a canonical phospho-binding BRCT domain (Crb-BRCT1), are conserved in TopBP1 BRCT domains 1, 2, 5 and 7.

functional sites on consecutive BRCT domains presenting on opposite sides of an overall cylindrical structure.

Based on this, we have re-examined the BRCT domain structure of the rest of TopBP1 itself, and find that BRCT4:5, with an estimated linker length of 25 amino acids more closely resembles the novel arrangement found in BRCT0:1 and BRCT1:2, than the canonical tandem arrangement. Significantly, BRCT4 lacks the cluster of conserved residues associated with phosphopeptide-binding-tandem BRCT structures. BRCT7:8 on the other hand, conforms more to a canonical tandem pair, with a predicted linker of ~56 amino acids, and conservation of phospho-binding residues in BRCT7 but not in BRCT8 (Figure 5B and Table 2).

### Phosphopeptide recognition by TopBP1

The data presented here identifies Rad9 Ser387 as a probable target of CK2, that when phosphorylated, interacts specifically with the N-terminal region of TopBP1 and thereby mediates the functionally essential coupling of TopBP1 and the 9–1–1 complex (25). The presence of three distinct BRCT domains within TopBP1(1–290) considerably complicates the previous expectation that BRCT1:2 would constitute a canonical phosphopeptide-binding tandem BRCT repeat of the type previously documented in BRCA1, Mdc1, 53BP1/Crb2, Nbs1, etc. (48–54,62,63), where the first BRCT provides the phosphate recognition, while the second defines the additional sequence specificity. Instead, TopBP1 BRCT1:2 defines a new class of tandem BRCT arrangement in which the two consecutive domains independently offer binding sites for phosphorylated Ser/Thr residues.

Our mutagenesis and interaction data strongly implicates BRCT1 as the primary binding site for the Rad9 pSer387 motif. BRCT2, while conserving all the residues usually involved in phosphopeptide binding, and displaying the characteristic affinity for sulphate, only has a low affinity for the pSer387 peptide. As in other BRCT systems, we have analysed (49), while interaction with the phosphorylated residue provides a significant component of affinity for the ligand peptide, there are other overriding specificity determinants. In the canonical

tandem-BRCTs, these are furnished by the second BRCT domain—the structural basis for this specificity in the non-canonical TopBP1 BRCT1:2 structure is still to be defined.

The tail of Rad9 provides a site for multiple phosphorylations in yeasts and in animals, although the location and genesis of these vary amongst different organisms. For example, the extreme C-terminal sites (Thr412/Ser423) in *S. pombe* Rad9 are modified by ATR (Rad3) and/or ATM (Tel1) in response to cell-cycle progression and/or DNA damage, whereas the equivalent C-terminal site (Ser387) in mammals is constitutively modified, probably by CK2 as we have shown here. In both cases however, these phosphorylations are essential for mediating the physical coupling of Rad9 to TopBP1(Rad4), that functionally connects the 9–1–1 and ATR (Rad3) complexes. Metazoan Rad9 does possess an ATR/ATM damage regulated phosphorylation site (Ser272), but at the N-terminal end of the tail segment close to the PCNA core, and several cell cycle-regulated CDK phosphorylation sites have also been mapped upstream of the TopBP1-binding pSer387. Despite the presence of multiple phosphorylation sites on Rad9, only phosphopeptides containing the pSer387 site shows the level of affinity for the TopBP1 BRCT0:1:2 segment, that is consistent with a biologically significant interaction, and while some small involvement of BRCT domains 0 and 2 in that interaction cannot be totally ruled out, the interaction with BRCT1 alone seems both necessary and sufficient. What protein–protein interactions are mediated by the other phosphorylation sites on Rad9, or by the BRCT0 and BRCT2 domains of TopBP1 remain to be defined. Experiments to address this are in progress.

During the preparation and review of this article, additional evidence supporting the role of CK2 in mediating the 9-1-1—TopBP1 interaction was published by Takeishi *et al.* (67); demonstrating that residues Ser341 and Ser387 are both *in vitro* targets of the kinase, are phosphorylated *in vivo*, and that HeLa cells over-expressing a phospho-deficient form of Rad9 display hypersensitivity to both UV and methyl methane sulphonate (MMS) treatment.

### ACCESSION NUMBERS

2xnh and 2xnk.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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