TopBP1 contains a transcriptional activation domain suppressed by two adjacent BRCT domains

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TopBP1 has eight BRCT [BRCA1 (breast-cancer susceptibility gene 1) C-terminus] domains and is involved in initiating DNA replication, and DNA damage checkpoint signalling and repair. Several BRCT-domain-containing proteins involved in mediating DNA repair have transcriptional regulatory domains, and as demonstrated for BRCA1 these regulatory domains are important in mediating the functions of these proteins. These transcriptional regulatory processes involve modification of chromatin, and recent evidence has clearly demonstrated that the ability to modify chromatin plays an important role in regulating DNA damage signalling and repair. Here we report the identification of a TopBP1 transcriptional activation domain that is rich in hydrophobic residues, interspersed with acidic amino acids, characteristics

that are typical of transcriptional activation domains identified previously. Two adjacent repressor domains encoded by BRCT2 and BRCT5 silence this activator and experiments suggest that these repressors actively recruit repressor complexes. Both the activator and BRCT2 repressor domains function in yeast. The present study identifies several chromatin modification domains encoded by TopBP1, and the implications of these findings are discussed in the context of the DNA damage response and the understanding of TopBP1 function.

Key words: BRCT domain, chromatin, DNA damage, DNA replication, TopBP1, transcription.

INTRODUCTION

TopBP1 was first identified as an interacting partner for topoisomerase II β and a major feature of TopBP1 is that it encodes eight BRCT [BRCA1 (breast-cancer susceptibility gene 1) C-terminus] domains [1]. These domains were first identified in the C-terminal region of BRCA1 and are commonly found in proteins involved in regulating the response of the cell to DNA damage [2]. BRCT domains are hydrophobic and are involved in interacting with other proteins and phosphorylated peptides as well as interacting with single- and double-stranded DNA [3,4]. Therefore, following DNA damage, BRCT domains can alter their interacting partners or their affinity for existing partners thus playing a major role in mediating the DNA damage response. Although the precise role of TopBP1 in DNA damage signalling and repair is not known there is evidence demonstrating that it is involved in these processes. TopBP1 is essential for the activation of Chk1 (checkpoint kinase 1), the DNA damage response signalling kinase [5,6], and co-localizes with BRCA1 to sites adjacent to PCNA (proliferating-cell nuclear antigen) following DNA damage [7]. TopBP1 also interacts with the DNA damage response protein Rad9 and is therefore implicated in loading repair factors to sites of DNA damage [8], as well as being a substrate for ATM (ataxia telangiectasia mutated) and probably ATR (ATM- and Rad3-related) [9]. Recent findings have also demonstrated that TopBP1 is involved in mediating genome integrity throughout a normal S-phase [10]. TopBP1 has also been implicated in the recombination process during meiosis [11,12]. Further evidence implying a role for TopBP1 in mediating the DNA damage response and repair processes comes

from studies of TopBP1 homologues (for a review see [13]). The yeast homologues of TopBP1 are Cut5 in *Schizosaccharomyces pombe* and Dpb11 in *Saccharomyces cerevisiae*, and both of these proteins are involved in regulating DNA damage signalling checkpoints following damage [14–16]. Also, mutations in Mus101, the *Drosophila* homologue of TopBP1, result in defects in DNA replication and repair as well as in chromosome segregation [17]. Cut5 and Dpb11 also have a role in genome segregation following mitosis [16,18], while *Caenorhabditis elegans* TopBP1 is required for protection against DNA double-strand breaks [19].

As well as DNA damage and repair processes, TopBP1 is involved in other areas of nucleic acid metabolism. The *Xenopus* homologue of TopBP1, named either Xcut5 or Xmus101, is essential for the initiation of DNA replication in *Xenopus* nuclear extracts where it plays a role in loading the initiation complex on to DNA [20,21]. Yeast and *Drosophila* homologues are also implicated in DNA replication [14–17]. Injection of anti-TopBP1 antibodies into isolated mammalian nuclei results in failure to synthesize DNA, and knockdown of TopBP1 results in reduced cellular growth [7,9], further implicating TopBP1 in DNA replication. Mammalian TopBP1 also interacts with the HPV16 (human papillomavirus 16) transcription/replication factor E2 [22,23]; E2 is the viral origin recognition complex, which binds to the viral origin of replication and recruits the viral helicase E1, which, therefore, suggests that TopBP1 plays a role in the initiation of viral replication.

TopBP1 has also been proposed as a transcriptional regulator. When overexpressed, TopBP1 co-activates transcription with the HPV16 E2 protein when E2 is bound to target promoters

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; BRCA1, breast-cancer susceptibility gene 1; BRCT, BRCA1 C-terminus; Brg1, Brahma-related gene 1; BRM1, Brahma 1; Chk1, checkpoint kinase 1; DBD, DNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; HDAC, histone deacetylase; HEK-293T, human embryonic kidney-293 cells expressing the large T-antigen of simian virus 40; HPV16, human papillomavirus 16; ONPG, *O*-nitrophenyl- β -D-galactopyranoside; PCNA, proliferating-cell nuclear antigen; SC, synthetic complement; SV40, simian virus 40; TCA, trichloroacetic acid; TSA, trichostatin A.

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[23]. Other studies have implicated TopBP1 as a transcriptional repressor; TopBP1 can interact with the chromatin modification complex proteins Brg1 [BRM (Brahma)-related gene 1]/BRM1 and repress the transcriptional and apoptotic function of E2F1 [24]. This forms a feedback loop as E2F1 positively regulates the *TopBP1* promoter [25]. Also, in complex with Miz1, TopBP1 represses the *c-Myc* promoter, a repression that is removed following UV irradiation, suggesting that TopBP1 may not only regulate the DNA damage response directly, but may also regulate gene transcription directly following DNA damage [26]. TopBP1 also represses the *c-abl* promoter and again there is a feedback loop in this repression as phosphorylation of TopBP1 by c-Abl blocks the repression of the *c-abl* promoter [27].

Although TopBP1 is involved in several nucleic acid metabolism processes, the domains responsible for mediating these properties are not clearly defined. Obviously BRCT domains will be involved in mediating many, if not all, of TopBP1 functions via interactions with other proteins. In recent years it has been established that chromatin modification is an essential part of the DNA damage response, which allows access for repair proteins. While some BRCT domains regulate chromatin structure as evident by transcriptional regulation, others do not [28] and it is not known which, if any, of the BRCT domains of TopBP1 regulate chromatin structure. Here we report that TopBP1 has a transcriptional-activation domain, located partially in BRCT4. This activation domain is suppressed by two surrounding TopBP1 sequences, one incorporating BRCT2 and another incorporating BRCT5. We also demonstrate that the activation domain and the BRCT2 repressor domain function in yeast, providing a model system for the analysis of these domains. We discuss the significance of these findings in the light of the known TopBP1 functions and propose that these domains may play an important role not only in transcriptional regulation but also in the DNA damage response.

MATERIALS AND METHODS

Cell culture

HEK-293T cells [human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40)] were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) foetal calf serum and 1 % (v/v) penicillin/streptomycin mixture (Invitrogen) at 37 °C in a 5 % CO₂/95 % air atmosphere.

Transcription assays

Transcription assays were performed as described in [29] using 2×10^5 cells per 60 mm-diameter tissue culture dish coated with poly(L-lysine). The assays shown are representative of at least three independent experiments carried out in duplicate. The luciferase activity was normalized to the protein concentration present in each extract. pG5luc luciferase reporter (Promega) was used in all transcription assays. pGL3control, which contains the SV40 promoter and enhancer driving the expression of the luciferase gene, was always included in the individual experiments to confirm similar levels of transfection efficiency between experiments. For TSA (trichostatin A) treatment, the cells were treated with 300 ng/ml TSA in DMEM containing 10 % (v/v) foetal calf serum for 10 h prior to harvesting.

Western blotting

Cell lysates were prepared with 150 µl of lysis buffer [0.5 % Nonidet P40, 50 mM Tris/HCl, pH 7.8, 150 mM NaCl with 1 CompleteTM protease inhibitor cocktail tablet (Roche Molecular

Biochemicals) dissolved in 10 ml lysis buffer]. Samples containing 20 µg of protein were separated by SDS/PAGE (4–12 % gels) (Invitrogen) and blotted on to a nitrocellulose membrane. The membrane was placed in blocking solution [PBS containing 0.1 % Tween 20 and 5 % (w/v) skimmed milk powder], and then incubated with anti-Gal4 antibody (a gift from Dr Stefan Roberts, School of Biological Sciences, University of Manchester, Manchester, U.K.) diluted in blocking solution for 1 h. The membrane was washed with PBS containing 0.1 % Tween 20 then probed with horseradish-peroxidase-conjugated anti-sheep IgG diluted in blocking solution for 1 h. The proteins were detected using ECL[®]-Plus (Amersham Biosciences) and the membrane was exposed to film. Proteins from AH109 yeast were harvested using a TCA (trichloroacetic acid) method. Briefly, an A₆₀₀ 2–3 of cell culture was centrifuged at 13 000 g for 1 min; the pellet was then re-suspended in 1 ml 0.3 M NaOH, and incubated at room temperature (23 °C) for 10 min with agitation. The mixture was then incubated on ice for 10 min before the addition of 150 µl TCA and incubated on ice for a further 10 min. The suspension was then centrifuged at 14 000 rev./min for 10 min. The supernatant was removed and the pellet was resuspended in SDS/PAGE loading buffer (Invitrogen). The protein was then treated as above for the detection of Gal4 fusion proteins.

Cloning Gal4 fusion constructs

The Gal4 fusion proteins were cloned into pBIND (Promega). The TopBP1 fragments were amplified by PCR using the pTopBP1 plasmid [9] as a template and specific nucleotide primers corresponding to amino acid number. The 5'-primers contained a BamHI restriction enzyme site and the 3'-primers a KpnI site. PCR products were purified using Qiagen QIAquick PCR clean-up kits, digested with KpnI and BamHI, and the fragments were ligated in-frame into the similarly digested pBIND vector. For the yeast experiments, TopBP1 fragments were cloned into the pGBKT7 expression vector (Clontech), using the above method, except that EcoRI and BamHI restriction sites were used.

Cloning of Gal4–VP16 and VP16–TopBP1 constructs

The Gal4–VP16 fusion protein was generated by amplifying the VP16 activation domain (amino acids 1188–1325) from the expression vector pACT (Promega), using primers containing BamHI and KpnI restriction enzyme sites. Following a double digest with BamHI and KpnI, the fragment was ligated into pBIND. pBIND-VP16-TopBP1 (amino acids 2–258) was created using a PCR-stitch technique as follows. Two first-round PCRs were performed using pTopBP1 and pACT as templates, with the following primer pairs: (1) Top 2, 5'-CGCGGATCCGTCACACCAGCGATGTG-3' and Top 258 Del, 5'-GTTTCGGGGGGGCCGTCGATAGATTTTCAAGTGTAGG-3'; and (2) VP16 del, 5'-CCTACACTTGAAAATCTATCGACGGCCCCCCCCGACC-3' and VP16, 5'-CGGGGTACCTCCCGGACCCGGGGGAAATC-3'. The products of these reactions were pooled and amplified in a second round using primers Top 2 and VP16. This fragment was then digested with BamHI and KpnI and cloned into pBIND.

Nutritional selection and transformation of AH109 cells

pGBKT7 vector containing TopBP1 sequences were transformed separately into yeast *Saccharomyces cerevisiae* AH109 (*MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1_{UAS}-GAL1^{TATA}-HIS3*, *GAL2_{UAS}-GAL2^{TATA}-ADE2*, *ura3::MEL1_{UAS}-MEL1^{TATA}-lacZ*) by heat-shock and maintained on SC (synthetic complement) medium lacking tryptophan at 30 °C. Experiments to determine whether the activation domain of TopBP1 functions in yeast were carried out by replica plating

Figure 1 Assaying for transcriptional regulatory domains encoded by TopBP1

(a) Fusion proteins between the TopBP1 sequences shown and the Gal4 DBD were constructed in order to assay for transcriptional-control domains of intact TopBP1 and the regions shown. To the right-hand side of the Figure is the predicted size of the fusion proteins in kDa (kD). Boxes 1 to 8 represent the BRCT domains. (b) The plasmids encoding the proposed fusion proteins described in (a) were transfected into HEK-293T cells. Protein extracts were prepared from these cells and Western blots using an anti-Gal4 antibody were performed. A representative blot is shown. The numbers to the left-hand side of the blot represent the marker proteins sizes in kDa. (c) The ability of these proteins to activate transcription from a Gal4 DNA-binding site-containing promoter, pG5luc, was assayed in HEK-293T cells. The results are expressed relative to the Gal4 DBD-expressing plasmid pBIND.

on SC medium lacking adenine, histidine and tryptophan; growth on this medium demonstrates transcriptional activation of the *HIS3* and *ADE2* genes by the Gal4 fusion protein.

Liquid β -galactosidase assay

To perform the liquid β -galactosidase assay, colonies from a master SC – Trp plate were selected and diluted in 100 μ l of double-distilled water. An aliquot (20 μ l) was used to inoculate 1.5 ml of liquid SC–Trp and incubated at 30°C until a D_{600} of 0.8 was reached. Cells were pelleted at 13000 rev.min for 3 min and washed with 1.5 ml of Z-buffer (sodium phosphate buffer, pH 7.0, containing 10 mM KCl and 1 mM $MgSO_4$) before being centrifuged again. The pellet was then resuspended in 300 μ l of Z-buffer. To permeabilize the yeast cells, Eppendorf tubes containing yeast pellets with Z-buffer were frozen in liquid nitrogen and thawed at room temperature. Next, 700 μ l of Z-buffer containing 50 mM 2-mercaptoethanol plus 150 μ l of ONPG (*O*-nitrophenyl- β -D-galactopyranoside) buffer (4 mg/ml ONPG in Z-buffer) was added and the mixture was kept at 30°C until a yellow colour developed. To stop the reaction, 400 μ l of 1 M sodium carbonate was added. Reaction tubes were centrifuged at 13000 rev./min for 10 min and the

absorbance of the supernatant was measured at 420, 550 and 600 nm. β -Galactosidase expression levels were calculated using the following equation: Units = $(1000 \times A_{420} - 1.75 \times A_{550}) / [\text{time (min)} \times \text{volume of culture (ml)} \times A_{600}]$.

RESULTS

TopBP1 encodes a transcriptional-activation domain silenced by an adjacent repressor domain

In order to identify TopBP1 chromatin modification domains, a series of fusion proteins between TopBP1 sequences and the DBD (DNA-binding domain) of the yeast transcription factor Gal4 were constructed (Figure 1a). The plasmids encoding these fusions were sequenced and correct protein expression was confirmed by transfecting the plasmids into HEK-293T cells and preparing protein extracts. The protein extracts were probed by Western blotting using an antibody against the Gal4 DBD [30] (Figure 1b). It is clear that all of the fusion proteins are expressed, although the Gal4–TopBP1^{586–1435} was poorly expressed compared with the others. Figure 1(a) lists the predicted molecular masses of the fusion proteins (right-hand side) and in Figure 1(b) it is clear that the observed molecular masses are not always as expected,

although they are close to the predicted value. This is common with many proteins. All proteins tested were checked for expression at least twice with identical results. As these were transient transfections, no internal 'control' protein was tested, although identical amounts of cellular extracts were added to each lane. The loading on to the SDS/PAGE gel and subsequent transfer on to the membrane was checked by Ponceau S staining. The reproducible equivalent expression of most of the proteins confirms that they have similar stabilities and therefore the subsequent results described are not due to differences in protein stability between the fusion proteins. The ability of the fusion proteins to regulate transcription from a luciferase reporter containing Gal4 DNA-binding sites (pG5luc) was determined, and the results of these experiments are shown in Figure 1(c). This Figure, and all other Figures describing transcription assays, shows the means $\pm$ S.E.M. of at least three independent experiments carried out in duplicate. None of the fusion proteins activated transcription and most repressed relative to the Gal4 DBD alone (pBIND). The fusion Gal4–TopBP1^{586–1435} was a significantly better repressor of transcription compared with the other fusion proteins. This repression domain will be described in more detail in Figure 6. The other point of note from Figure 1(c) is that, although the Gal4–TopBP1^{2–258} sequence acts as a repressor, addition of amino acids 259–591, resulting in Gal4–TopBP1^{2–591}, removes some of this repressor function. This suggested that a transcriptional activation domain might reside between amino acids 259 and 591, which is repressed by sequences encoded between the residues at positions 2 and 258. In order to investigate this further, more deletion mutants of TopBP1 were prepared and these are described in Figure 2(a). The transcriptional activity and expression of the fusion proteins are shown in Figures 2(b) and 2(c) respectively. It is clear that removal of amino acids 113–181 revealed a transcriptional-activation domain between the residues at positions 181 and 591. This demonstrates that amino acids 113–181 encode a transcriptional-repressor domain that is able to suppress the transcriptional-activation domain. The residues at positions 113–181 map directly to the BRCT2 domain of TopBP1.

The repressor domain between amino acids 2 and 258 works on other activators and is independent of HDACs (histone deacetylases)

The results described in Figures 1 and 2 demonstrate that amino acids 2–258 of TopBP1 act as a suppressor of the TopBP1 transcriptional-activation domain. This repression could be due to either an intramolecular interaction where the residues between positions 2 and 258 physically mask the transcriptional-activation domain, or due to recruitment of a repressor complex that acts dominantly over the activation domain. To resolve which of these alternatives is likely to occur, the TopBP1 repressor sequence, 2–258, was expressed in-frame with the transcriptional activation domain of VP16 as a fusion protein with the Gal4 DBD (Figure 3a). The transcriptional activity and the expression of the proteins are shown in Figures 3(b) and 3(c) respectively. It is clear in Figure 3(b) that the insertion of amino acids 2–258 from TopBP1 adjacent to the VP16 activation domain suppresses the ability of VP16 to activate transcription. The VP16 activation domain is one order of magnitude stronger than that identified in TopBP1, but the repressor domain of TopBP1 can suppress over 90 % of the transcriptional-activation function of VP16. These results suggest strongly that the repressor identified in TopBP1 recruits proteins that are able to suppress adjacent transcriptional activation domains. This direct recruitment of a repressor complex is also supported by the observation in Figure 1 that amino acids 2–253 of TopBP1 can act as a repressor by

Figure 2 TopBP1 encodes a transcriptional-activation domain

(a) Further deletion mutants of TopBP1 were prepared as shown and to the right-hand side of the Figure is the predicted size of the fusion proteins in kDa (kD). (b) The ability of these proteins to activate transcription from a Gal4 DNA-binding site-containing promoter, pG5luc, was assayed in HEK-293T cells. The results are expressed relative to the Gal4 DBD-expressing plasmid pBIND. (c) The plasmids encoding the proposed fusion proteins described in (a) were transfected into HEK-293T cells. Protein extracts were prepared from these cells and Western blots using an anti-Gal4 antibody were performed. A representative blot is shown. The numbers to the left-hand side of the blot represent the marker protein sizes in kDa.

themselves. To investigate the mechanism involved in mediating the repressor function we treated cells with the HDAC inhibitor TSA prior to harvesting the cells and the results of these experiments are shown in Figure 3(d). TSA treatment did not alleviate repression of the TopBP1 activation domain by amino acids 2–258, suggesting that repression by this region operates in an HDAC independent manner. Of note, in this experiment, was the increase in transcription mediated by the Gal4 DBD by itself (pBIND) following TSA treatment, suggesting that there are cryptic activation domains within this region that are repressed by adjacent repressor domains interacting with HDACs.

Figure 3 The TopBP1 repressor domain is able to repress the strong transcriptional activation domain of VP16

(a) The diagram is a representation of the plasmids encoding Gal4 DBD fusion proteins with the VP16 activation domain with the TopBP1 repressor domain or without. (b) The ability of these proteins to activate transcription from a Gal4 DNA-binding site-containing promoter, pG5luc, was assayed in HEK-293T cells. The results are expressed relative to the Gal4 DBD-expressing plasmid pBIND. (c) The plasmids encoding the proposed fusion proteins described in (a) were transfected into HEK-293T cells. Protein extracts were prepared from these cells and Western blots using an anti-Gal4 antibody were carried out. A representative blot is shown. The numbers to the left-hand side of the blot represent the marker proteins sizes in kDa. (d) Similar

The TopBP1 transactivation domain is rich in hydrophobic amino acids

The results shown in Figure 2 demonstrate that TopBP1 encodes a transcriptional-activation domain between amino acids 253 and 591. To map the activation domain further, deletion constructs were made and assayed as described in Figure 4. These deletion mutants revealed that an essential part of the activation domain of TopBP1 is encoded between amino acids 460 and 500. Deletion of amino acids 460–470 removes two-thirds of the transcriptional activity, while the remaining activity is lost following deletion of amino acids 470–500. The Gal4–TopBP1^{500–591} fusion protein is relatively poorly expressed. However, over a range of plasmid concentrations from 10 ng to 1 μg, the Gal4–TopBP1^{460–591} fusion protein was a strong transcriptional activator, whereas the Gal4–TopBP1^{500–591} fusion protein failed to activate transcription (results not shown). This strongly suggests that the Gal4–TopBP1^{500–591} fusion protein is not a transcriptional activator. This modular nature of the TopBP1 transcriptional domain is typical of others where two or more domains can contribute to the overall transcriptional-activation potential. The predicted start residue for BRCT4 is at position 466 and therefore this activation at least partially overlaps with the N-terminal portion of BRCT4. In Figure 4(d) an alignment is shown between the identified human transcriptional-activation domain and the corresponding sequence from a variety of species. There is clear conservation in the nature of this region, as it has a high percentage of hydrophobic amino acids interspersed with acidic residues. Such sequences are typical of other transcriptional-activation domains.

The TopBP1 activation domain and adjacent repressor domain both function in yeast

To determine the functional conservation of the identified repressor and activator domains and develop a model system for their study, the ability of these domains to function in yeast was studied. As described in Figure 5(a), TopBP1 amino acids 2–591, 2–258 and 253–591 were cloned into pGBKT7, resulting in yeast expression vectors encoding Gal4 fusions of these TopBP1 sequences. These plasmids were introduced into the yeast strain AH109 and selected on SC – Trp medium. To investigate whether the activation and repressor domains function in yeast, several clones expressing these proteins were tested for their ability to grow on SC – Trp – His – Ade medium. Both the *HIS3* and *ADE2* genes were under the control of Gal4 DNA-binding sequences. An example of the results obtained in these experiments is shown in Figure 5(b). All of the yeast cells grew on the SC – Trp medium confirming the presence of the pGBKT7 plasmids; however, only Gal4–TopBP1^{253–591} allowed growth on the triple-dropout medium. This demonstrates that the activator identified in mammalian cells also functions in yeast cells, and that the repressor domain identified between amino acids 2 and 258 also functions in yeast cells. These results were confirmed using a liquid β -galactosidase assay as shown in Figure 5(c). Interestingly, the β -galactosidase assays also suggested that the repressor domains can actively repress the yeast promoter under investigation, as the activity of Gal4–TopBP1^{2–591} and Gal4–TopBP1^{2–258} was lower than that obtained with Gal4 DBD alone, with Gal4–TopBP1^{2–258} being the best repressor as shown in

experiments to those described in (b) were carried out with the samples indicated pre-treated with TSA prior to cell harvest. In this figure the results are shown as relative light units (RLU) per μg of protein used in the luciferase assay.

Figure 4 The TopBP1 activation domain maps to a region between amino acids 460 and 500, incorporating the N-terminal part of BRCT4

(a) A schematic representation of Gal4 DBD fusion proteins investigated for their ability to activate transcription. (b) The ability of these proteins to activate transcription from a Gal4 DNA-binding site containing promoter, pG5luc, was assayed in HEK-293T cells. The results are expressed relative to the Gal4 DBD-expressing plasmid pBIND. (c) The plasmids encoding the proposed fusion proteins described in (a) were transfected into HEK-293T cells. Protein extracts were prepared from these cells and Western blots using an anti-Gal4 antibody were performed. A representative blot is shown. The numbers to the left-hand side of the blot represent the marker proteins sizes in kDa. (d) An alignment of the human TopBP1 transcriptional-activation domain between amino acids 460 and 500 with that of other species. Of note are the clusters of hydrophobic residues. The closed circles (●) above the sequence indicates hydrophobic residues, the asterisk (*) highlights identical residues, the colon (:) conserved substitutions and the period (.) semi-conserved substitutions.

mammalian cells (see Figure 1). Expression of all of the fusion proteins was detected by Western blotting (Figure 5d), confirming that the activator and repressor domains are expressed in yeast. Of note is the molecular mass of the Gal4–TopBP1^{2–258} fusion protein, which appears higher than the predicted molecular mass. An asterisk (*) marks the predicted weight of the protein and indeed a band can be detected at this point, although the slower mobility protein is predominant (Figure 5d). There is no clear explanation for this, although it is certain that this fusion can function as a repressor as shown in Figure 5(c).

A second repressor domain resides on the C-terminal side of the activation domain

From Figure 1 it is clear that Gal4–TopBP1^{586–1435} represses transcription of pG5luc. However, as this fusion protein was expressed poorly, its ability to repress transcription over a range of concentrations was tested. As shown in Figure 6(a) it is clear that, over a 20-fold range in plasmid concentration, this fusion protein acts as an efficient repressor and, if anything, the repression increases following increased amounts of transfected plasmid.

Figure 5 The TopBP1 transcriptional-activation and -repressor domains function in yeast

(a) A schematic representation of the Gal4 DBD fusion proteins tested in yeast. (b) The ability of yeast expressing the proteins shown in (a) to activate transcription in yeast was monitored by plating cells out on SC — Trp medium, where they should all grow due to the presence of the plasmid, and SC — Trp — His — Ade medium, where growth should only occur following transcriptional-activation by the Gal4—TopBP1 fusion proteins. Growth was only seen on the triple-dropout medium with the Gal4—TopBP1^{253–591} expressing yeast. (c) The ability of the fusion proteins to activate transcription in yeast was tested directly by monitoring levels of β -galactosidase activity. The promoter controlling the expression of this protein is controlled by Gal4 DNA-binding sites. The results are expressed as attenuation units following the β -galactosidase assay. (d) Expression of the fusion proteins in yeast was confirmed by Western blotting using an anti-Gal4 antibody; the numbers to the left-hand side of the blot represent the marker proteins sizes in kDa. The Gal4—TopBP1^{2–258} fusion protein major band has a higher molecular mass than predicted. The predicted size is marked by a * where a protein can be detected.

Further deletion studies had determined that the repressor region around residue 586 resides in the region up to amino acid 675 (results not shown). Therefore a plasmid encoding a Gal4 fusion protein incorporating amino acids 460–675 was constructed as shown in Figure 6(b). The transactivation property and expression of this fusion protein is shown in Figures 6(c) and 6(d) respectively. Figure 6(c) demonstrates that incorporation of amino acids up to residue 675 represses the TopBP1 transcriptional-activation domain. This confirms the region between residues 586 and 675 as an additional repressor domain that can control the function of the TopBP1 transcriptional-activation domain. This region contains BRCT5.

DISCUSSION

TopBP1 has eight BRCT domains, and, as well as a role in DNA damage signalling and repair, it can act as both a transcriptional

co-activator [23] and as a transcriptional co-repressor [24]. It has also been proposed as a replication initiation factor, as it is required for this process in *Xenopus* extracts [20,21]. Recent studies have demonstrated that the modification of chromatin is a process required for the repair of DNA. For example, in mammalian cells, loading of Rad51 to DNA breaks is facilitated by localized chromatin unwinding mediated by the addition of acetyl groups to histones [31], while the transcriptional histone acetyltransferase cofactor TRRAP (transformation/transcription domain-associated protein) interacts with the MRN repair complex to facilitate DNA double-strand break repair [32]. The present report describes, for the first time, domains of TopBP1 that are able to regulate transcription when tethered to a promoter via Gal4 DNA-binding sites.

Figure 7 summarizes our results by highlighting the transcriptional regulatory regions that were identified. A transcriptional-activation domain between amino acids 460 and 591, which

Figure 6 An additional repression domain between amino acids 586 and 635

(a) To confirm that the Gal4–TopBP1^{586–1435} fusion protein was a repressor, an experiment with a titration of plasmid concentrations was carried out and the results show the ability of this protein to activate transcription from the Gal4 DNA-binding site-containing promoter pG5luc in HEK-293T cells. The results are expressed relative to pG5luc values. (b) To determine whether this region was able to repress the TopBP1 transcriptional-activation domain, an additional Gal4 fusion plasmid was constructed as shown. (c) The ability of the encoded fusion protein to activate transcription from a Gal4 DNA-binding site containing promoter, pG5luc, was assayed in HEK-293T cells. The results are expressed relative to the Gal4 DBD-expressing plasmid pBIND. (d) The plasmids encoding the proposed fusion protein described in (b) were transfected into HEK-293T cells. Protein extracts were prepared from these cells and Western blots using an anti-Gal4 antibody were carried out. A representative blot is shown. The numbers to the left-hand side of the blot represent the marker proteins size in kDa.

Figure 7 A schematic summary of the results presented in this paper

The solid box maps the transcriptional activation domain described, while the broken boxes describe two repressor domains, each of which are capable of repressing the transcriptional-activation domain.

incorporates the BRCT4 domain of TopBP1 was identified. Amino acids 460–500 were essential for this activity and this region is rich in hydrophobic amino acids interspersed with acidic residues (Figure 4d), typical of identified transcriptional-activation domains [33]. Adjacent to the transcriptional-activation domain, we identified a repressor domain encoded by BRCT 2 that is able to repress the TopBP1 transcriptional-activation domain. As shown in Figure 3, the TopBP1 repressor domain was also able to reduce transcriptional activation by VP16 by around 90% and suggests that the repressor domain directly recruits a repressor complex, which is supported by the observation in both mammalian and yeast cells (Figures 1 and 5) that the repressor

domain by itself is able to repress transcription from an adjacent promoter. In addition to this repressor domain, another exists on the C-terminal side of the activation domain, which requires amino acids 586–675, as shown in Figure 6(c). The juxtaposition of the two repressor domains surrounding the activator suggests a complex interaction between competing chromatin modification complexes to mediate TopBP1 function.

The clear role of TopBP1 in regulating the DNA damage response does not preclude a possible role for TopBP1 in regulating transcription directly. It has been proposed as a transcriptional co-activator with HPV16 E2 [23] and as a transcriptional repressor of E2F1 [24]. It can also repress transcription of

the *c-myc* and *c-abl* promoters [26,27]. It is also possible that chromatin modification may play a role in the regulation of DNA replication by TopBP1. The proposed role for TopBP1 in DNA replication is to interact with the origin recognition complex and assist in recruitment of factors onto the pre-initiation complex [20,21]. Such a role may also involve modification of chromatin surrounding the origin of replication to assist in the initiation of replication and/or the formation of the pre-initiation complex. Previous studies supporting such a link between DNA replication and chromatin modification have demonstrated that PCNA can interact with histone deacetylases [34] and that the ACF1 (ATP-utilizing chromatin assembly and remodelling factor 1)–ISWI chromatin-remodelling complex is required for DNA replication [35].

The ability of the TopBP1 activation domain to function in yeast is in common with other transcription activation domains. Of note is the BRCA1 activation domain; as well as being of a similar content to that of TopBP1, in that it is rich in hydrophobic amino acids interspersed with acidic residues, it also functions in yeast [28]. The similarities between TopBP1 and BRCA1 are many and are worth discussing in the context of the potential functional roles for TopBP1 and its chromatin modification domains. Both TopBP1 and BRCA1 are essential for an intact G₂/M checkpoint; abrogation of either compromises the checkpoint, while the lack of both completely abolishes it [36]. TopBP1 and BRCA1 co-localize following DNA damage of cells and both proteins are substrates for the ATM/ATR kinases that are essential for mediating the DNA damage response [7,9]. Indeed, recent results demonstrate that TopBP1 expression is essential for the activation of ATR and also therefore Chk1 [37]. TopBP1 and BRCA1 interact with ubiquitin ligase complexes EDD and BARD1 respectively, which are expressed aberrantly in breast cancer [38,39]. Both proteins also interact with the HPV E2 protein and are proposed to act as transcriptional co-activators for this protein [23,40]. There is also evidence to suggest strongly that TopBP1 and BRCA1 are involved in DNA double-strand-break repair, as homologous recombination is compromised in cells lacking BRCA1 [41], whereas TopBP1 is implicated in recombination during meiosis [11]. When BRCA1 is mutated in breast cancer, the predominant mutations that have been identified result in loss of the transcriptional-activation function from this protein, demonstrating that this property is essential for the normal functioning of the BRCA1 protein and may therefore play a role in mediating DNA double-strand-break repair [42]. Of additional note is the transcriptional-repressor domain encoded by the BRCT2 domain of TopBP1, which represses the function of the adjacent transcriptional-activation domain. Although not well characterized, it is clear that BRCA1 also encodes a similar repressor domain. Full-length BRCA1 is unable to activate transcription, whereas truncated versions containing the BRCT domains incorporating the transcriptional-activation domain are able to activate transcription [43]. The repression of the BRCA1 activation domain resulted in the prevention of chromatin modification by this domain.

The present study, used an established system (Gal4 DBD fusion proteins) to identify three domains of TopBP1 that regulate transcription: an activator and two repressors that map, at least in part, to BRCT domains (Figure 7). Due to the known functions of TopBP1, and its similarity with BRCA1, it is unlikely that these domains are an artefact of the system used, and that they play an important role in mediating TopBP1 function. We also propose a role for TopBP1 in the generation of breast carcinomas and this is supported by our recent observation that TopBP1 is aberrantly expressed in a significant number of such cancers [44].

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