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To cite this version:

Roni Hg Wright, Edward S Dornan, Mary M Donaldson, Iain M Morgan. TOPBP1 contains a transcriptional activation domain suppressed by two adjacent BRCT domains. Biochemical Journal, 2006, 400 (3), pp.573-582. 10.1042/BJ20060831 . hal-00478610

HAL Id: hal-00478610

https://hal.science/hal-00478610v1

Submitted on 30 Apr 2010

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Biochemical Journal Immediate Publication. Published on 20 Sep 2006 as manuscript BJ20060831

TOPBP1 CONTAINS A TRANSCRIPTIONAL ACTIVATION DOMAIN SUPPRESSED BY TWO ADJACENT BRCT DOMAINS

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Running title: TopBP1 contains a transcriptional activation domain

Key words: TopBP1, DNA damage, transcription, DNA replication, chromatin, modification

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SYNOPSIS

TopBP1 has eight BRCT [BRCA1 carboxyl 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 rich in hydrophobic residues interspersed with acidic amino acids, typical of previously identified transcriptional activation domains. This activator is silenced by two adjacent repressor domains encoded by BRCT2 and BRCT5 and experiments suggest that these repressors actively recruit repressor complexes. Both the activator and BRCT2 repressor domains function in yeast. This report 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.

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INTRODUCTION

TopBP1 was first identified as an interacting partner for Topoisomerase IIβ and a major feature of TopBP1 is that it encodes eight BRCT domains [1]. These domains were first identified in the carboxyl 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, the DNA damage response signalling kinase [5, 6], and co-localises with BRCA1 to sites adjacent to PCNA 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, and probably ATR [9]. Recent findings have also demonstrated that TopBP1 is involved in mediating genome integrity throughout a normal S phase [10]. Also, TopBP1 has 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 S. pombe and Dpb11 in S. cerevisiae and both of these proteins are involved in regulating DNA damage signalling checkpoints following damage [14, 15, 16]. Also, mutations in the Drosophila homologue of TopBP1, Mus101, results 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 [18, 16] while C. 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,

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21]. Yeast and Drosophila homologues are also implicated in DNA replication [17, 14, 15, 16]. Injection of TopBP1 antibodies into isolated mammalian nuclei results in failure to synthesise DNA, and knock down of TopBP1 results in reduced cellular growth [7, 9], further implicating TopBP1 in DNA replication. Mammalian TopBP1 also interacts with the human papillomavirus [HPV] 16 transcription/replication factor E2 [22, 23]; E2 is the viral origin recognition complex as it binds to the viral origin of replication and recruits the viral helicase E1 and therefore TopBP1 is proposed to play a role in the initiation of viral replication.

TopBP1 has also been proposed as a transcriptional regulator. When over expressed, TopBP1 co-activates transcription with the HPV16 E2 protein when E2 is bound to target promoters [23]. Other studies have implicated TopBP1 as a transcriptional repressor; TopBP1 can interact with the chromatin modification complex proteins Brg1/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 directly regulate the DNA damage response but may also directly regulate gene transcription 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 to allow access for repair proteins. While some BRCT domains regulate chromatin structure as evidenced by transcriptional regulation, others do not [28] and it is not known which, if any, of the TopBP1 BRCT domains regulate chromatin structure. Here we report that TopBP1 has a transcriptional activation domain, partially located in BRCT4. This activation domain is suppressed by two surrounding TopBP1 sequences, one incorporating BRCT2 with another incorporating BRCT5. We also demonstrate that the activation domain and the BRCT2 region repressor domain function in yeast providing a model system for the analysis of these domains. We discuss the significance of these findings in light of the known TopBP1 functions and propose

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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. Human embryonic kidney 293-T cells were maintained in Dulbecco modified Eagle medium supplemented with 10% foetal calf serum and 1% penicillin / streptomycin mix [Invitrogen] at 37°C , 5% CO_2 .

Transcription Assays. Transcription assays were performed as described in [29] using 2 x 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 normalised to the protein concentration present in each extract. pG5luc luciferase reporter (Promega) was used in all transcription assays. pGL3 control, 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 treatment the cells were treated with 300ng/ml Trichostatin A in DMEM 10% FCS, penicillin streptomycin, 10 hrs prior to harvesting.

Western Blotting. Cell lysates were prepared with 150µl lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.8, 150 mM NaCl with 1 protease inhibitor cocktail tablet (Roche Molecular Biochemicals) dissolved in 10 ml lysis buffer). Samples containing 20µg of protein were separated on a 4-12% SDS PAGE gel (Invitrogen) and blotted onto a nitrocellulose membrane. The membrane was blocked in PBS containing 0.1% Tween 20 and 5% skimmed milk powder then incubated with anti-GAL4 antibody (a kind gift from Dr Stefan Roberts, University of Manchester) diluted in blocking solution for 1hour. The membrane was washed with PBS containing 0.1% Tween 20 then probed with horseradish peroxidase-conjugated α-sheep IgG diluted in blocking solution for 1 hour. The chemiluminescence was developed using ECL-Plus (Amersham Biosciences) and the membrane was exposed to film. Proteins from AH109 yeast were harvested using a TCA method. Briefly, 2-3 OD cell culture was centrifuged at 14,000rpm for 1 minute; the pellet was then re-suspended in 1ml 0.3M NaOH, and incubated at room temperature for 10 minutes with agitation. The mixture

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was then incubated on ice for 10 minutes before adding 150µl TCA (w/w) and incubated on ice for a further 10 minutes. The suspension was then centrifuged at 14,000rpm for 10 minutes, the supernatant was removed and the pellet re-suspended in SDS-PAGE loading buffer (Invitrogen). The protein was then treated as above for the detection of Gal4-fusion proteins.

Cloning GAL-4 fusion constructs. The GAL4 fusion proteins (Figure 1a, 2a, 4a and 6b) were cloned into pBIND (Promega). The TopBP1 fragments were amplified by polymerase chain reaction using pTopBP1 plasmid [9] as a template and specific nucleotide primers corresponding to amino acid number. The 5' primers contained a BamH1 restriction enzyme site and the 3' primers a Kpn1 site. PCR products were purified using Qiagen QIAquick PCR clean up kits, and digested with Kpn1 and BamH1 and the fragments were ligated in frame into the pBIND vector similarly digested. For the yeast experiments, TopBP1 fragments were cloned into the pGBKT7 expression vector (Clontech), using the above method with the exception of an EcoR1/ BamH1 restriction digest.

Cloning GAL4/VP16, VP16/TopBP1 constructs. The GAL4/VP16 fusion protein (Figure 3a) 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 enzymes sights. Following a double digest with BamH1 and Kpn1 the fragment was ligated into pBIND. pBINDVP16-TopBP1 (amino acids 2 -258) was created using a PCR-stitch technique as follows. Two first-round PCR reactions were performed using pTopBP1, and pACT (Promega) as templates and primer pairs 1) Top 2; CGC GGA TCC GTC ACC ACC AGC GAT GTG, Top 258 Del; GTT CGG GGG GGC CGT CGA TAG ATT TTC AAG TGT AGG and 2) VP16 del; CCT ACA CTT GAA AAT CTA TCG ACG GCC CCC CCG ACC and VP16; CGG GGT ACC TCC CGG ACC CGG GGA ATC respectively. 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 BamH1 and Kpn1 and cloned into pBIND.

Nutritional selection and Transformation of AH109

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pGBKT7 vector containing TopBP1 sequences were transformed separately into yeast *Saccharomyces cerevisiae* AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2*::$GAL1_{UAS}$-$GAL1^{TATA}$-*HIS3*, $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2*, *ura3*::$MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*) by heat-shock and maintained on synthetic complement media lacking tryptophan and incubated at 30ºC. Experiments to determine whether the activation domain of TopBP1 functions in yeast were carried out by replica plating on synthetic complement media lacking adenine, histidine and tryptophan; growth on this media demonstrates transcriptional activation of HIS3 and ADE2 genes by the Gal4 fusion protein.

Liquid β galactosidase Assay

To perform the liquid β galactosidase assay, colonies from master Sc-trp plate were selected and diluted in 100µl ddH_20. 20µl was used to inoculate 1.5ml liquid Sc-trp and incubated at 30ºC until an A_{600} = 0.8 was reached. Cells were pelleted at 13,000rpm for 3 min and washed with 1.5ml Z- buffer [60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH7]. The pellet was then resuspended in 300µl of Z-buffer. To permeabilize the yeast cells, eppendorfs containing the yeast pellet with Z-buffer was frozen in liquid nitrogen and thawed at room temperature. Next, 700µl of Z-buffer containing 50mM β-mercaptoethanol plus 150µl of ONPG buffer [4mg/ml in Z buffer] was added and the mixture was kept at 30ºC until a yellow colour developed. To stop the reaction, 400µl of 1M sodium carbonate was added. Reaction tubes were centrifuged at 13,000rpm for 10 minutes and the absorbance of the supernatant was measured at 420nm, 550nm and 600nm. β-gal expression levels were calculated using the following equation: Units=1000x [OD_{420}]-1.75 x [OD_{550}]]/ Time [min] volume culture x [OD_{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 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

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confirmed by transfecting plasmids into 293T cells and preparing protein extracts. The protein extracts were probed by western blotting using an antibody against the Gal4 DNA binding domain [30] (Figure 1b). It is clear that all of the fusion proteins are expressed although the 586-1435 poorly so. Figure 1a lists the predicted molecular weight of the fusion proteins and in Figure 1b it is clear that the observed molecular weights 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 and equivalent transfer in western 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 1c. This figure, and all other figures describing transcription assays, show the average of at least three independent experiments carried out in duplicate with the standard error bars. None of the fusion proteins activated transcription and most repressed relative to the Gal4 DNA binding domain by itself (pBIND). The fusion 586-1435 was a significantly better repressor of transcription than the other fusion proteins. This repression domain will be described in more detail in Figure 6. The other point of note from Figure 1c is that although the 2-258 sequence acts as a repressor, addition of amino acids 259-591, resulting in 2-591, removes some of this repressor function. This suggested that a transcriptional activation domain may reside between amino acids 259 and 591 that is repressed by sequences encoded by 2-258. In order to investigate this further, more deletion mutants of TopBP1 were prepared and these are described in Figure 2a. The transcriptional activity and expression of the fusion proteins are shown in Figure 2b and 2c respectively. It is clear that removal of amino acids 113-181 revealed a transcriptional activation domain between 181 and 591. This demonstrates that amino acids 113-181 encode a transcriptional repressor domain that is able to suppress the transcriptional activation domain. 113-181 maps directly to BRCT2 of TopBP1.

The repressor domain between amino acids 2 and 258 works on other activators and is independent of HDACs. The results described in Figures 1 and 2

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demonstrate that amino acids 2 to 258 of TopBP1 act as a suppressor of the TopBP1 transcriptional activation domain. This repression could be due to either an intramolecular interaction where 2-258 physically masks the transcriptional activation domain, or due to recruitment of a repressor complex that is dominant 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 DNA binding domain (Figure 3a). The transcriptional activity and the expression of the proteins are shown in Figure 3b and 3c respectively. It is clear in Figure 3b that the insertion of amino acids 2-253 from TopBP1 adjacent to the VP16 activation domain suppresses the ability of VP16 to activate transcription. The VP16 activation domain is an 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 strongly suggest 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 2-253 can act as a repressor by itself. To investigate the mechanism involved in mediating the repressor function we treated cells with the histone deacetylase inhibitor (HDAC) trichostatin A (TSA) prior to harvesting of the cells and the results of these experiments are shown in Figure 3d. TSA treatment did not alleviate repression of the TopBP1 activation domain by amino acids 2 to 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 DNA binding domain 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.

The TopBP1 transactivation domain is rich in hydrophobic amino acids. Figure 2 demonstrates 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 500-591 fusion protein is relatively poorly expressed. However

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over a range of plasmid concentrations from 10ng to 1μg the 460-591 fusion was a strong activator while the 500-591 fusion never activated (not shown). This strongly suggests that the 500-591 fusion 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 466 and therefore this activation at least partially overlaps with the amino terminal portion of BRCT4. In Figure 4d 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 5a, 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. Both the HIS3 and ADE2 genes are under the control of Gal4 DNA binding sequences. An example of the results obtained in these experiments is shown in Figure 5b. All of the yeast cells grow on the Sc-Trp medium confirming the presence of the pGBKT7 plasmids; however only the TopBP1 sequence 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 2-258 also functions in yeast cells. These results were confirmed using a liquid β-gal assay as shown in Figure 5c. Interestingly, the β-gal assays also suggested that the repressor domains can actively repress the yeast promoter under investigation as the activity of 2-591 and 2-258 was lower than that obtained with Gal4 alone, with 2-258 being the best repressor as shown in mammalian cells (see Figure 1). Expression of all of the fusion proteins was detected using western blotting [Figure 5d] confirming that the activator and repressor domains

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are expressed and function in yeast. Of note is the molecular weight of the 2-258 fusion protein which appears more than the predicted weight. A * marks the predicted weight of the protein and indeed a band can be detected at this point, although the slower mobility protein is predominant. There is no clear explanation for this although it is certain that this fusion can function as a repressor as shown in Figure 5c.

A second repressor domain resides carboxyl terminus to the activation domain. In Figure 1 it is clear that TopBP1 586-1435 represses transcription of pG5luc. However, as this fusion protein was poorly expressed its ability to repress transcription over a range of concentrations was tested. As shown in Figure 6a 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 plasmid transfected. Further deletion studies had determined that the repressor region around 586 resides in the region up to amino acid 675 (not shown). Therefore a plasmid encoding a Gal4 fusion protein incorporating amino acids 460 to 675 was constructed as shown in Figure 6b. The transactivation property and expression of this fusion protein is shown in Figure 6c and 6d respectively. Figure 6c demonstrates that incorporation of amino acids up to 675 represses the TopBP1 transactivation domain. This confirms the region 586 to 675 as an additional repressor domain that can control the function of the TopBP1 transcriptional activation domain. This region contains BRCT5.

DISCUSSION

TopBP1 has 8 BRCT domains and as well as a role in DNA damage signalling and repair, it can act as 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 localised chromatin unwinding mediated by the addition of acetyl groups to histones [31] while the transcriptional histone acetyltransferase cofactor TRRAP interacts with the MRN repair complex to facilitate DNA double-strand break repair [32]. This 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.

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Figure 7 summarises our results by highlighting the transcriptional regulatory regions that were identified. A transcriptional activation domain between amino acids 460 and 591 which incorporates BRCT4 of TopBP1 was identified. Amino acids 460 to 500 were essential for this activity and this region is rich in hydrophobic amino acids interspersed with acidic residues [Figure 4d], typical of transcriptional activation domains identified [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 and this is supported by the observation in both mammalian and yeast cells [Figure 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 carboxyl terminal to the activation domain requiring amino acids 586 to 675 as shown in Figure 6c. 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 directly regulating transcription. It has been proposed as a transcriptional co-activator with HPV16 E2 [23] and as a transcriptional repressor of E2F1 [24]. It also can repress transcription of the c-myc and c-able gene 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-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

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similarities between TopBP1 and BRCA1 are many and are worth discussing in the context of the potential functional roles for TopBP1 and its encoded 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-localise 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]. Both TopBP1 and BRCA1 interact with ubiquitin ligase complexes aberrantly expressed in breast cancer; EDD and BARD1 respectively [38, 39]. Both proteins also interact with the human papillomavirus E2 protein and are proposed transcriptional co-activators for this protein [23, 40]. Evidence also strongly suggests that TopBP1 and BRCA1 are involved in DNA double strand break repair, as homologous recombination is compromised in cells lacking BRCA1 [41] while TopBP1 is implicated in recombination during meiosis [11]. When BRCA1 is mutated in breast cancer the predominant mutations 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 TopBP1 BRCT2 domain that represses the function of the adjacent transcriptional activation domain. Although not well characterised, it is clear that BRCA1 also encodes a similar repressor domain. Full length BRCA1 is unable to activate transcription while 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 studies reported here use an established system (Gal4 DNA binding domain 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

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observation that TopBP1 is aberrantly expressed in a significant number of such cancers (44).

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REFERENCES

1. Yamane, K., Kawabata, M., and Tsuruo, T. (1997) A DNA-topoisomerase-II-binding protein with eight repeating regions similar to DNA-repair enzymes and to a cell-cycle regulator. Eur. J. Biochem. **250**, 794-9

2. Huyton, T., Bates, P.A., Zhang, X., Sternberg, M.J., and Freemont, P.S. (2000) The BRCA1 C-terminal domain: structure and function. Mutat. Res. **460**, 319-32

3. Glover, J.N., Williams, R.S., and Lee, M.S. (2004) Interactions between BRCT repeats and phosphoproteins: tangled up in two. Trends Biochem. Sci. **29**, 579-85

4. Yamane, K.,, and Tsuruo, T. (1999) Conserved BRCT regions of TopBP1 and of the tumor suppressor BRCA1 bind strand breaks and termini of DNA. Oncogene **18**, 5194-203

5. Jurvansuu, J., Raj, K., Stasiak, A., and Beard, P. (2005) Viral transport of DNA damage that mimics a stalled replication fork. J. Virol. **79**, 569-80

6. Smits, V.A., Reaper, P.M., and Jackson, S.P. (2006) Rapid PIKK-Dependent Release of Chk1 from Chromatin Promotes the DNA-Damage Checkpoint Response. Curr. Biol. **16**, 150-9

7. Makiniemi, M., Hillukkala, T., Tuusa, J., Reini ,K., Vaara, M., Huang, D., Pospiech, H., Majuri, I., Westerlin,g T., Makela, T.P., and Syvaoja, J.E. (2001) BRCT domain-containing protein TopBP1 functions in DNA replication and damage response. J. Biol. Chem. **276**, 30399-406

8. Greer, D.A., Besley, B.D., Kennedy, K.B., and Davey, S. (2003) hRad9 rapidly binds DNA containing double-strand breaks and is required for damage-dependent topoisomerase II beta binding protein 1 focus formation. Cancer Res. **63**, 4829-35

9. Yamane, K., Wu, X., and Chen, J. (2002) A DNA damage-regulated BRCT-containing protein, TopBP1, is required for cell survival. Mol. Cell Biol. **22**, 555-66

10. Kim, J.E., McAvoy, S.A., Smith, D.I., and Chen, J. (2005) A DNA damage-regulated BRCT-containing protein, TopBP1, is required for cell survival. Mol. Cell. Biol. **25**, 10907-15

11. Barchi, M., Mahadevaiah, S., Di Giacomo, M., Baudat, F., de Rooij, D.G., Burgoyne, P.S. Jasin, M., and Keeney, S. (2005) Surveillance of different recombination defects in mouse spermatocytes yields distinct responses despite elimination at an identical developmental stage. Mol. Cell. Biol. **25**, 7203-15

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12. Perera, D., Perez-Hidalgo, L., Moens, P.B., Reini, K., Lakin, N., Syvaoja, J.E., San-Segundo, P.A., and Freire, R. (2004) TopBP1 and ATR colocalization at meiotic chromosomes: role of TopBP1/Cut5 in the meiotic recombination checkpoint. Mol. Biol. Cell **15**, 1568-1579
13. Garcia, V., Furuya, K., and Carr, A.M. (2005) Identification and functional analysis of TopBP1 and its homologs. DNA Repair [Amst]. **4**, 1227-39
14. Saka, Y., Esashi, F., Matsusaka ,T., Mochida, S., and Yanagida, M. (1997) Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1. Genes Dev. **11**, 3387-400
15. McFarlane, R.J., Carr, A.M., and Price, C. (1997) Characterisation of the Schizosaccharomyces pombe rad4/cut5 mutant phenotypes: dissection of DNA replication and G2 checkpoint control function. Mol. Gen. Genet. **255**, 332-40
16. Araki, H., Leem, S.H., Phongdara, A., and Sugino, A. (1995) Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc. Natl. Acad. Sci. **92**, 11791-5.
17. Yamamoto, R.R., Axton, J.M., Yamamoto, Y., Saunders, R.D., Glover, D.M., and Henderson, D.S. (2000) The Drosophila mus101 gene, which links DNA repair, replication and condensation of heterochromatin in mitosis, encodes a protein with seven BRCA1 C-terminus domains. Genetics **156**, 711-21
18. Saka, Y., Fantes, P., and Yanagida, M. (1994) Coupling of DNA replication and mitosis by fission yeast rad4/cut5. J. Cell Sci. Suppl. **18**, 57-61
19. van Haaften, G., Vastenhouw, N.L., Nollen, E.A., Plasterk, R.H., and Tijsterman, M. (2004) Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality. Proc. Natl. Acad. Sci. **101**, 12992-6
20. Van Hatten, R.A., Tutter, A.V., Holway, A.H., Khederian, A.M., Walter, J.C., and Michael, W.M. (2002) The Xenopus Xmus101 protein is required for the recruitment of Cdc45 to origins of DNA replication. J. Cell Biol. **159**, 541-7
21. Hashimoto, Y., and Takisawa, H. (2003) Xenopus Cut5 is essential for a CDK-dependent process in the initiation of DNA replication. EMBO J. **22**, 2526-35
22. Boner, W., and Morgan, I.M. (2002) Novel cellular interacting partners of the human papillomavirus 16 transcription/replication factor E2. Virus Res. **90**, 113-8

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23. Boner, W., Taylor, E.R., Tsirimonaki, E., Yamane, K., Campo, M.S., and Morgan, I.M. (2002) Functional interaction between the human papillomavirus 16 transcription/replication factor E2 and the DNA damage response protein TopBP1. J. Biol. Chem. **277**, 22297-303

24. Liu, K., Luo, Y., Lin, F.T., and Lin, W.C. (2004) TopBP1 recruits Brg1/Brm to repress E2F1-induced apoptosis, a novel pRb-independent and E2F1-specific control for cell survival. Genes Dev. **18**, 673-86

25. Yoshida, K., and Inoue, I. (2004) Expression of MCM10 and TopBP1 is regulated by cell proliferation and UV irradiation via the E2F transcription factor. Oncogene **23**, 6250-60

26. Herold, S., Wanzel, M., Beuger, V., Frohme, C., Beul, D., Hillukkala, T., Syvaoja, J., Saluz, H.P., Haenel, F., and Eilers, M. (2002) Negative regulation of the mammalian UV response by Myc through association with Miz-1. Mol. Cell. **10**, 509-21

27. Zeng, L., Hu, Y., and Li, B. (2005) Identification of TopBP1 as a c-Abl-interacting protein and a repressor for c-Abl expression. J. Biol. Chem. **280**, 29374-80

28. Miyake, T., Hu, Y.F., Yu, D.S., and Li, R. (2000) A functional comparison of BRCA1 C-terminal domains in transcription activation and chromatin remodeling. J. Biol. Chem. **275**, 40169-73

29. Taylor, E.R., Boner, W., Dornan, E.S., Corr, E.M., and Morgan, I.M. (2003) UVB irradiation reduces the half-life and transactivation potential of the human papillomavirus 16 E2 protein. *Oncogene* **22**, 4469-77

30. McKay, L.M., Carpenter, B., and Roberts, S.G. (1999)] Regulation of the Wilms' tumour suppressor protein transcriptional activation domain. Oncogene **18**, 6546-54

31. Murr, R., Loizou, J.I., Yang, Y.G., Cuenin, C., Li, H., Wang, Z.Q., and Herceg, Z. (2006) Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. Nat. Cell. Biol. **8**, 91-9

32. Robert, F., Hardy, S., Nagy, Z., Baldeyron, C., Murr, R., Dery, U., Masson, J.Y., Papadopoulo, D., Herceg, Z., and Tora, L. (2006) The transcriptional histone acetyltransferase cofactor TRRAP associates with the MRN repair complex and plays a role in DNA double-strand break repair. Mol. Cell Biol. **26**, 402-12

33. Erkine, A.M., and Gross, D.S. (2003) Dynamic chromatin alterations triggered by natural and synthetic activation domains. J. Biol. Chem. **278**, 7755-64

Copyright 2006 Biochemical Society

34. Milutinovic, S., Zhuang, Q., and Szyf, M. (2002) Proliferating cell nuclear antigen associates with histone deacetylase activity, integrating DNA replication and chromatin modification. J. Biol. Chem. **277**, 20974-8

35. Collins, N., Poot, R.A., Kukimoto, I., Garcia-Jimenez, C., Dellaire, G., and Varga-Weisz, P.D. (2002) An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. Nat. Genet. **32**, 627-32

36. Yamane, K., Chen, J., and Kinsella, T.J. (2003) Both DNA topoisomerase II-binding protein 1 and BRCA1 regulate the G2-M cell cycle checkpoint. Cancer Res. **63**, 3049-53

37. Kumagai, A., Lee, J., Yoo, H.Y., and Dunphy, W.G. (2006) TopBP1 activates the ATR-ATRIP complex. Cell **124**, 943-55

38. Honda, Y., Tojo, M., Matsuzaki, K., Anan, T., Matsumoto, M., Ando, M., Saya, H., and Nakao, M. (2002) Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response. J. Biol. Chem. **277**, 3599-605

39. Wu, L.C., Wang, Z.W., Tsan, J.T., Spillman, M.A., Phung, A., Xu, X.L., Yang, M.C, Hwang, L.Y., Bowcock, A.M., and Baer, R. (1996) Identification of a RING protein that can interact in vivo with the BRCA1 gene product. Nat. Genet. **14**, 430-40

40. Kim, J., Lee, D., Gwan Hwang, S., Hwang, E.S. and Choe, J. (2003) BRCA1 associates with human papillomavirus type 18 E2 and stimulates E2-dependent transcription. Biochem. Biophys. Res. Commun. **305**, 1008-16

41. Scully, R., Xie, A., and Nagaraju, G. (2004) Molecular functions of BRCA1 in the DNA damage response. Cancer Biol. Ther. **3**, 521-7

42. Hayes, F., Cayanan, C., Barilla, D., and Monteiro, A.N. (2000) Functional assay for BRCA1: mutagenesis of the COOH-terminal region reveals critical residues for transcription activation. Cancer Res. **60**, 2411-8

43. Ye, Q., Hu, Y.F., Zhong, H., Nye, A.C., Belmont, A.S., and Li, R. (2001) BRCA1-induced large-scale chromatin unfolding and allele-specific effects of cancer-predisposing mutations. J. Cell Biol. **155**, 911-21

44. Going, JJ., Nixon, C., Dornan, ES., Boner, W., Donaldson, MM., and Morgan, IM. (2006). Aberrant expression of TopBP1 in breast cancer. Histopathology, in press.

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ACKNOWLEDGEMENTS

RHGW is the recipient of a studentship from the Biotechnology and Biological Sciences Research Council [BBSRC] and MMD is supported by Cancer Research UK. We thank these organisations for their support and also Dr Winifred Boner and Professor Saveria Campo for critical reading of the manuscript. We also thank Dr Stefan Roberts, University of Manchester, for the Gal4 antibody.

ABBREVIATIONS

BRCT, BRCA1 carboxyl terminus; DNA, deoxyribonucleic acid; DTT, dithiothreitol; HDAC, histone deacetylase; HPV, human papillomavirus; ONPG, O-Nitrophenyl β-D-galactopyranoside; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl [lauryl] sulfate-polyacrylamide gel electrophoresis; TCA, tri-chloro acetic acid; TSA, trichostatin acid; UVB, ultra-violet B irradiation.

FIGURE LEGENDS

Figure 1. Assaying for transcriptional regulatory domains encoded by TopBP1. a] Fusion proteins between the TopBP1 sequences shown and the Gal4 DNA binding domain were constructed in order to assay for transcriptional control domains of intact TopBP1 and the shown regions. To the right of the figure is the predicted size of the fusion proteins. Boxes 1 to 8 represent the BRCT domains. b] The plasmids encoding the proposed fusion proteins described in a] were transfected into 293T cells. Protein extracts were prepared from these cells and western blots using an anti-Gal4 antibody were carried out. A representation of the resultant blot is shown. To the left of the blot are numbers representing the marker proteins in kDa. c] The ability of these proteins to activate transcription from a Gal4 DNA binding site containing promoter, pG5luc, was assayed in 293T cells. The results are expressed relative to the Gal4 DNA binding domain expressing plasmid pBIND being equivalent to 1.

Figure 2. TopBP1 encodes a transcriptional activation domain. a] Further deletion mutants of TopBP1 were prepared as shown and to the right of the figure is the predicted size of the fusion proteins. b] The ability of these proteins to activate transcription from a Gal4 DNA binding site containing promoter, pG5luc, was

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assayed in 293T cells. The results are expressed relative to the Gal4 DNA binding domain expressing plasmid pBIND being equivalent to 1. c] The plasmids encoding the proposed fusion proteins described in a] were transfected into 293T cells. Protein extracts were prepared from these cells and western blots using an anti-Gal4 antibody were carried out. A representation of the resultant blot is shown. To the left of the blot are numbers representing the marker proteins in kDa.

Figure 3. The TopBP1 repressor domain is able to repress the strong transcriptional activation domain of VP16. a] This diagram is a representation of the plasmids encoding Gal4 DNA binding domain fusion proteins with the VP16 activation domain with and without the TopBP1 repressor domain. b] The ability of these proteins to activate transcription from a Gal4 DNA binding site containing promoter, pG5luc, was assayed in 293T cells. The results are expressed relative to the Gal4 DNA binding domain expressing plasmid pBIND being equivalent to 1. c] The plasmids encoding the proposed fusion proteins described in a] were transfected into 293T cells. Protein extracts were prepared from these cells and western blots using an anti-Gal4 antibody were carried out. A representation of the resultant blot is shown. To the left of the blot are numbers representing the marker proteins in kDa. d] Similar 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 460 and 500 amino acids incorporating the amino terminal part of BRCT4. a] A schematic representation of Gal4 DNA binding domain 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 293T cells. The results are expressed relative to the Gal4 DNA binding domain expressing plasmid pBIND being equivalent to 1. c] The plasmids encoding the proposed fusion proteins described in a] were transfected into 293T cells. Protein extracts were prepared from these cells and western blots using an anti-Gal4 antibody were carried out. A representation of the resultant blot is shown. To the left of the blot are numbers representing the marker proteins in kDa. d] An alignment of the

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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 star highlights identical residues, the colon conserved substitutions and the full stop semi-conserved substitutions.

Figure 5. The TopBP1 transcriptional activation and repressor domains function in yeast. a] A schematic representation the Gal4 DNA binding domain 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 media, where they should all grow due to the presence of the plasmid, and Sc-trp his ade media where growth should only occur following transcriptional activation by the Gal4-TopBP1 fusion proteins. Growth is only seen on the triple dropout media 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 O.D. 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 shown on the left of the figure correspond to the marker sizes in kD. The 2-258 fusion protein major band has a higher molecular weight than prediceted. The predicted size is marked by an * where a protein can be detected. This is discussed in the text.

Figure 6. An additional repression domain between amino acids 586 and 635. a] To confirm that the 586-1435 fusion protein was a repressor an experiment with a titration of plasmid concentrations was carried out and the results shown show the ability of these proteins to activate transcription from a Gal4 DNA binding site containing promoter, pG5luc in 293T cells. The results are expressed relative to pG5luc being equivalent to 1. 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 293T cells. The results are expressed relative to the Gal4 DNA binding

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domain expressing plasmid pBIND being equivalent to 1. d] The plasmids encoding the proposed fusion protein described in b] was transfected into 293T cells. Protein extracts were prepared from these cells and western blots using an anti-Gal4 antibody were carried out. A representation of the resultant blot is shown. To the left of the blot are numbers representing the marker proteins 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.

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Figure 1:

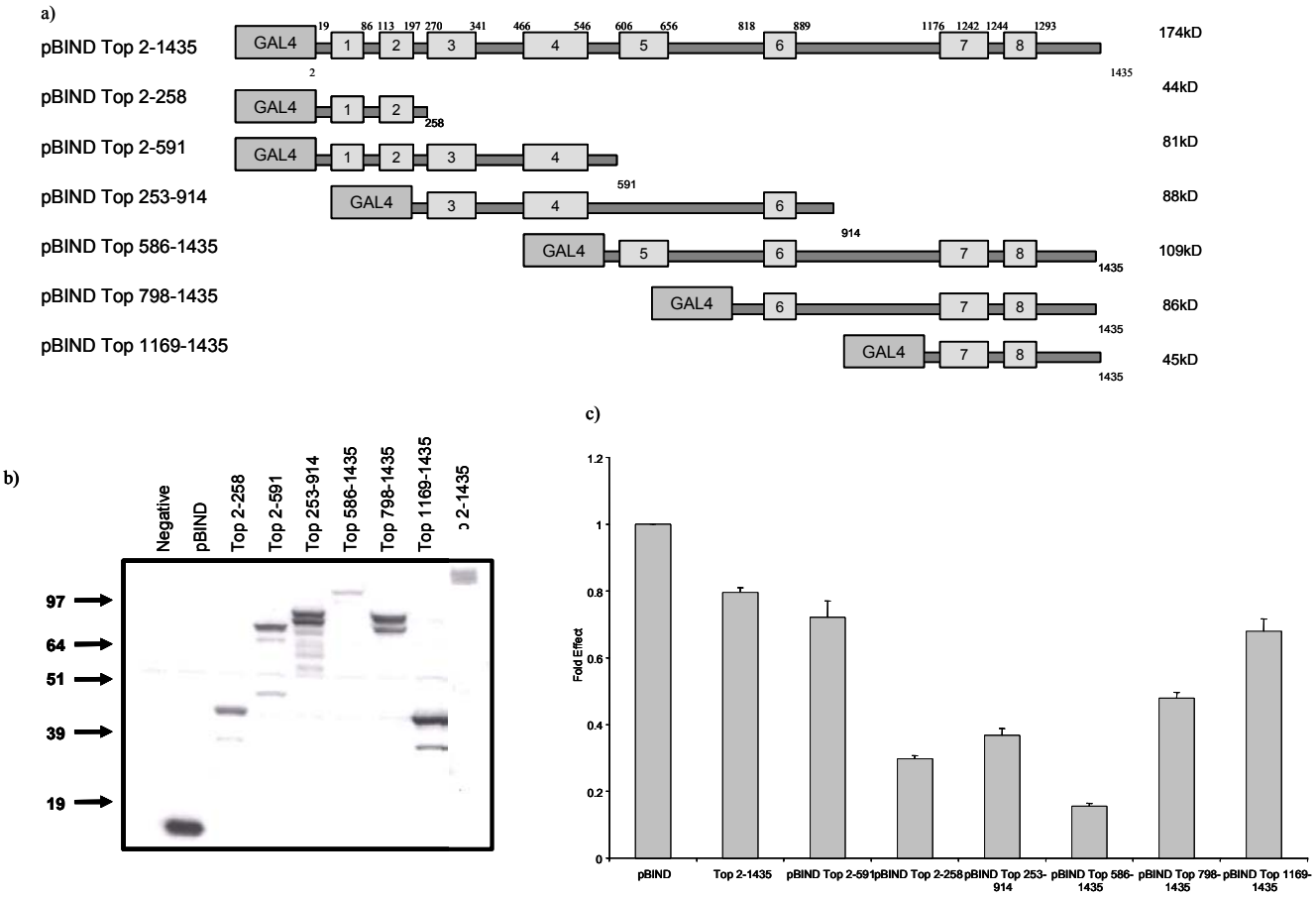

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Figure 2:

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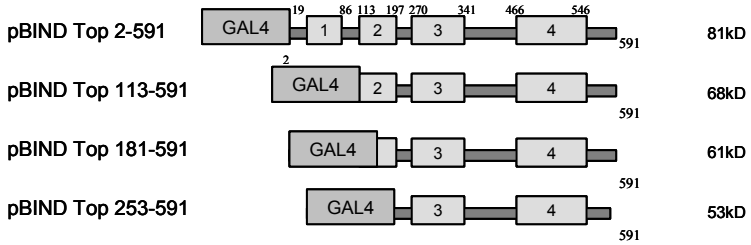

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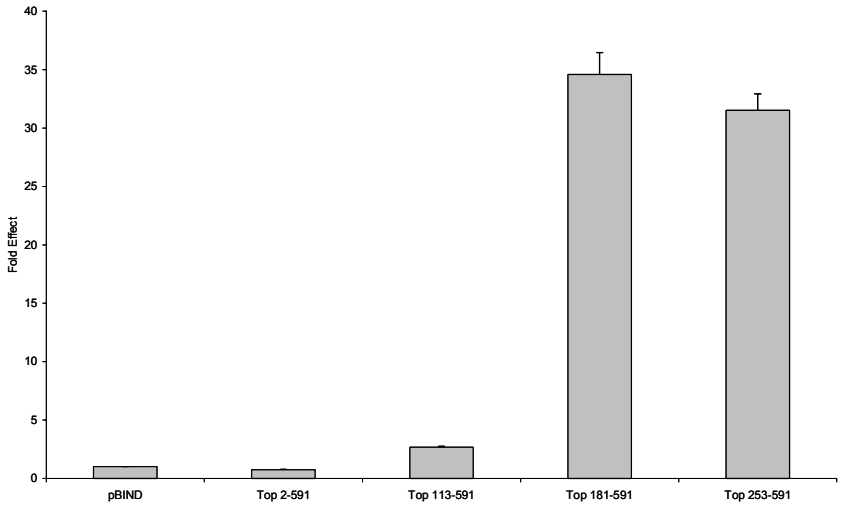

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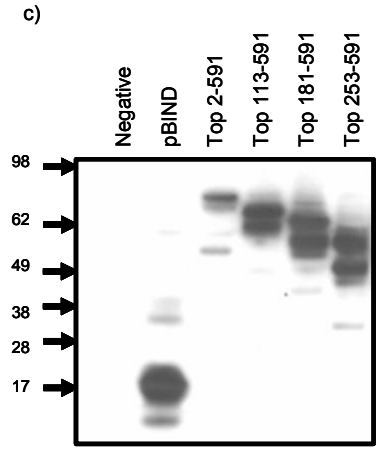

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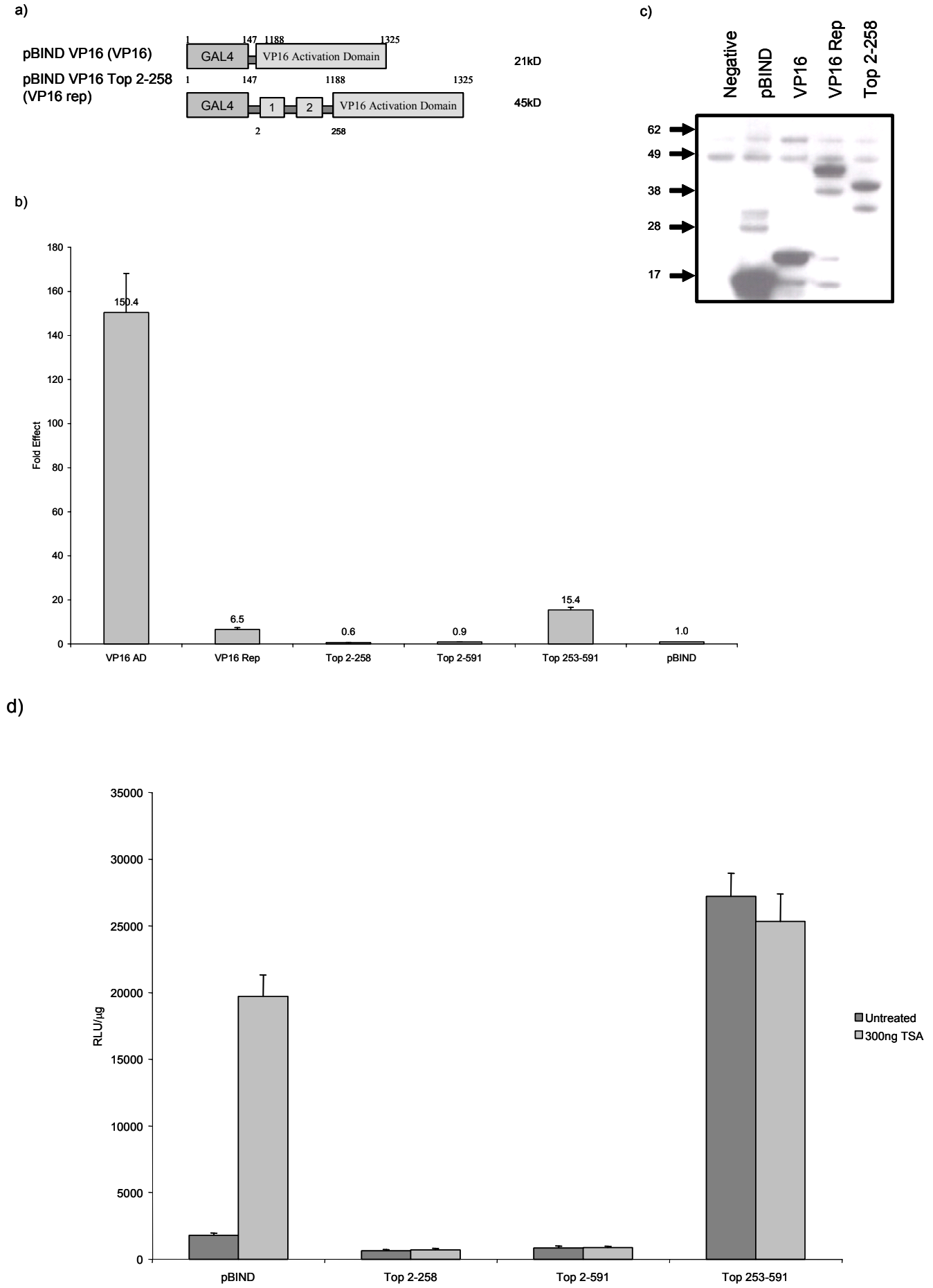

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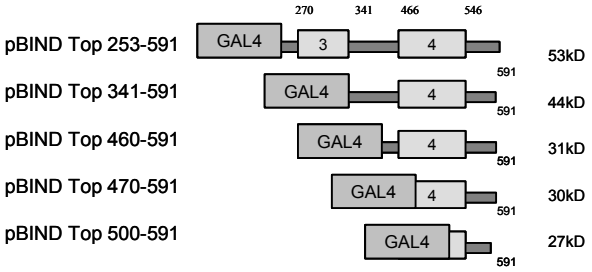
Figure 4:

a)



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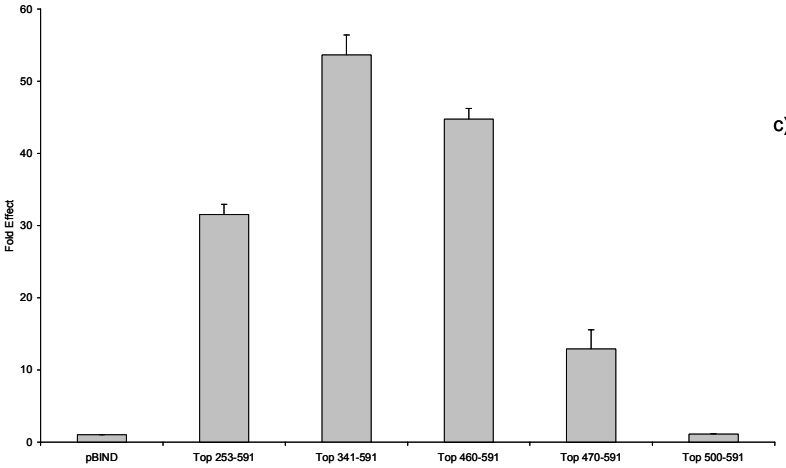

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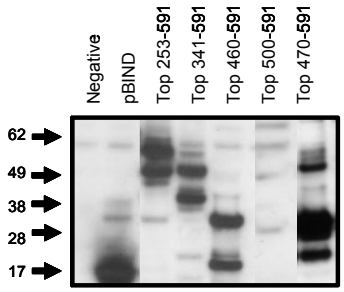

d) Sequence Alignment

```
                    •    ••    •••• •      •• ••   •  •• ••
Homo sapiens      TITEEGLFSQKSFLVLGFSNENESNIANIIKENAGKIMSLLSR 500
Pan troglodytes   TITEEGLFSQKSFLVLGFSNENESNIANIIKENAGKIMSLLSR
Canis familiaris  TTTEEGLFSQKSFLVLGFSNENESNIANIIRENAGKVVSLQSR
Mus musculus      TVREEGLFSQKSFLVLGFSVENKCNIVDIIREHAGKIVSLPSR
Rattus rattus     TVPEEGLFSQKSFLVLGFSVENECNIVNIIREHAGKIVSLPSR
Gallus gallus     SALDEGLFARKRFLLLGFGEEDESCIADLIKEHAGKTVPLQSR
                  :  :****::* **:***. *::. *.::*:*:*** :.* **
```

- Indicates hydrophobic residue

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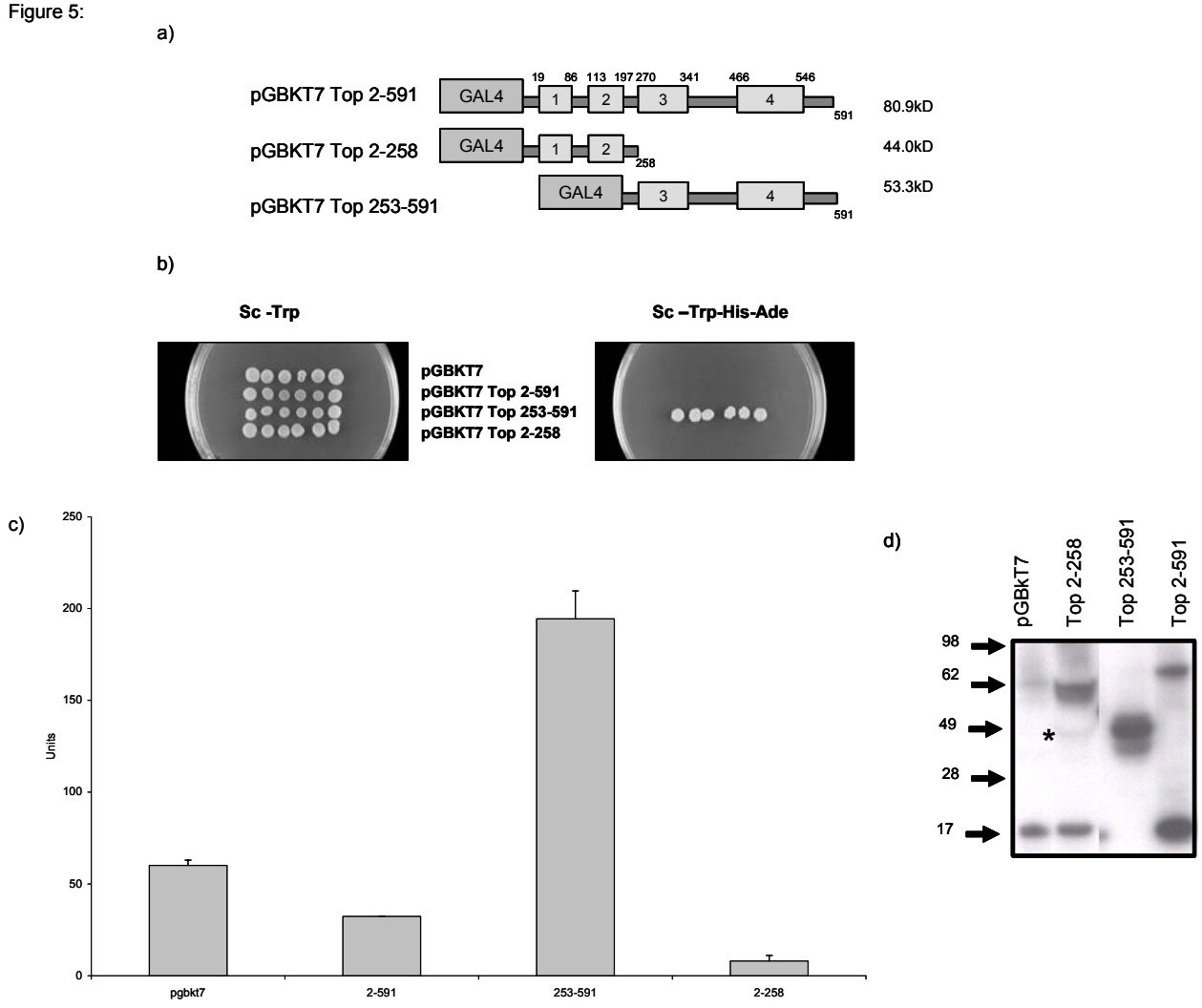
Figure 5:



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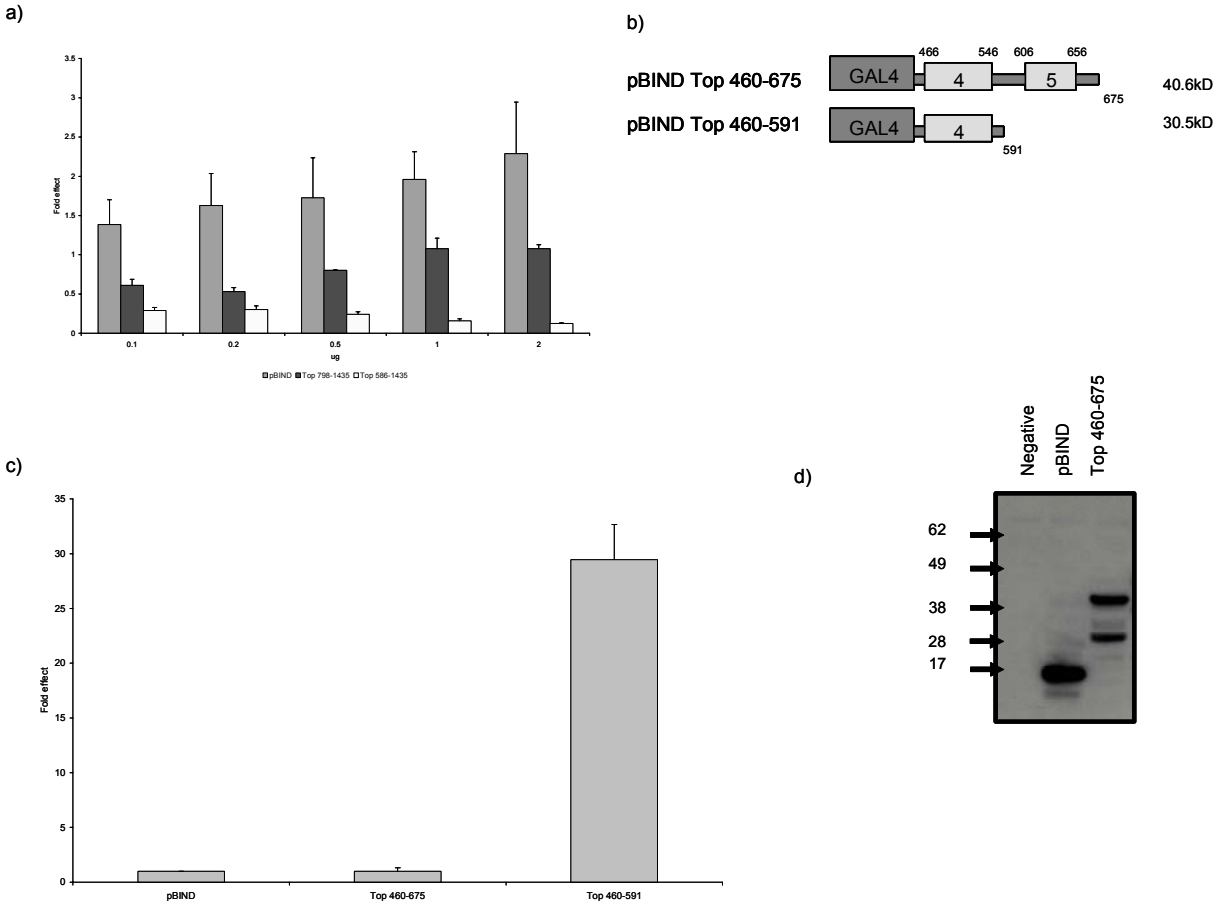

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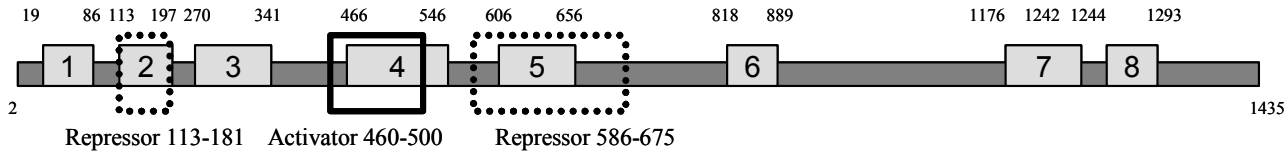
Figure 7



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