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## Characterization of the interaction between the human DNA topoisomerase II $\beta$ binding protein 1 (TopBP1) and the cell division cycle protein 45 (Cdc45)

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Running title: TopBP1 interacts with Cdc45

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ABBREVIATIONS USED: TopBP1, topoisomerase II $\beta$  binding protein 1; Cdc45, cell division cycle protein 45; Mus, mutagen-sensitive; GST, glutathione S-transferase; BRCT, BRCA1 carboxyl-terminal; ATM, ataxia-telangiectasia mutated kinase; ORC, origin recognition complex; MCM, minichromosome maintenance; Chk1, checkpoint kinase 1; ATR, ATM and Rad3-related; pre-RC, pre-replicative complex; IC, initiation complex; S-CDKs, S-phase cyclin dependent kinases; DDK, Cdc7-Dbf4 kinase; DMEM, Dulbecco's modified Eagle's medium; TdR, thymidine desoxyribose; HEPES, N-(2-hydroxyethyl)piperazine-N(2-ethanesulfonic acid); DTT, dithiothreitol; DAPI, 4',6-Diamidino-2-phenyleindole-dihydrochlorid; Cy5, cyanine 5; BrdU, 5-bromodeoxyuridine; RRX, Rhodamine X; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; IPTG, isopropylthiogalactopyranoside; PBS, phosphate buffered saline; ECL, enhanced chemoluminescence reaction; ONPG, o-nitrophenyl  $\beta$ -D-galactopyranoside; GINS, go ichi ni san.

## SYNOPSIS

TopBP1 is a BRCT-domain rich protein that is structurally and functionally conserved throughout eukaryotic organisms. It is required for the initiation of DNA replication and for DNA repair and DNA damage signaling. Experiments with fission yeast and *Xenopus* revealed that the TopBP1 homologues of these organisms are required for chromatin loading of the replication protein Cdc45. To improve our understanding of the physiological functions of human TopBP1 we investigated the interplay between human TopBP1 and Cdc45 proteins in synchronized HeLa-S3 cells. Using glutathione S-transferase (GST) pull-down and co-immunoprecipitation techniques we showed a direct interaction between TopBP1 and Cdc45 *in vitro* and *in vivo*. The use of deletion mutants in GST pull-down assays identified the first and second as well as the sixth BRCT domains of TopBP1 to be responsible for the functional interaction with Cdc45. Moreover, the interaction between Cdc45 and the first and second BRCT domains of TopBP1 inhibited their transcriptional activation both in yeast and mammalian one-hybrid systems. Both proteins interacted exclusively at the G1/S boundary of cell cycle, only weak interaction could be found at the G2/M boundary. The overexpression of the sixth BRCT domain led to diminished loading of Cdc45 onto chromatin. These data suggest that human TopBP1 is involved in the formation of the initiation complex of replication in human cells and is required for the recruitment of Cdc45 to origins of DNA replication.

## INTRODUCTION

TopBP1 was initially identified as a DNA topoisomerase II $\beta$ -interacting protein [1]. Human TopBP1 possesses eight BRCA1 carboxyl-terminal (BRCT) domains, a motif which was first described at the C terminus of the breast cancer susceptibility gene product, BRCA1, and which is conserved in many proteins related to the cell cycle checkpoint and DNA damage response [2]. TopBP1 shares sequence homology with *Saccharomyces cerevisiae* Dbp11, *Schizosaccharomyces pombe* Rad4/Cut5, *Drosophila melanogaster* Mutagen-sensitive-101 (Mus101), and *Xenopus laevis* Xmus 101/Cut5. Like their yeast counterparts, human TopBP1 is required for cell survival, DNA replication, resistance to DNA damage and checkpoint control. The literature on TopBP1 also suggests a function as a transcriptional regulator [3]. In response to ionizing radiation, TopBP1 is phosphorylated by the ataxia-telangiectasia mutated kinase (ATM) [4, 5], implying a role of TopBP1 in the DNA damage checkpoint control. This role was directly demonstrated by a later study using TopBP1 antisense oligonucleotides, showing that ionizing radiation-induced G2/M checkpoint and checkpoint kinase 1 (Chk1) phosphorylation was partially abrogated in the absence of TopBP1 [6]. Moreover, in a very recent paper it was shown that TopBP1 physically interacts with the ATM and Rad3-related (ATR) gene product and greatly enhances ATR's kinase activity [7].

While it is clear that human TopBP1 participates in the DNA damage checkpoint control, in human cells, however, the role of TopBP1 in DNA replication or S phase progression is poorly understood. The human transcript encoding TopBP1 and TopBP1 protein levels both increase during S phase [4, 8], suggesting involvement in replication. Neutralizing TopBP1 with a polyclonal antiserum raised against the sixth BRCT-repeat inhibited replicative DNA synthesis in HeLa cell nuclei *in vitro* [9]. Replication was inhibited more effectively by the recombinant protein fragment itself. This may indicate that the sixth BRCT domain is critical for replication activity, possibly interacting with a crucial replication factor [9]. The physical interaction between TopBP1 and DNA polymerase  $\epsilon$ , found by the same authors, also implies an involvement of TopBP1 in replication [9]. Moreover, Jeon and co-workers very recently showed, that human TopBP1 is required for G1 to S progression in a normal cell cycle. TopBP1 deficiency inhibited cells from entering S phase by up-regulating p21 and p27, resulting in down-regulation of cyclin E/CDK2 [10]. In contrast to these findings other authors propose that TopBP1 is not essential for DNA replication [11]. They found that TopBP1-deficient cells show increased histone H2AX phosphorylation and ATM-Chk2

activation, suggesting the accumulation of DNA double-strand breaks in the absence of TopBP1. This leads to formation of gaps and breaks at fragile sites, 4N accumulation, and aberrant cell division. Therefore Kim and colleagues propose that the cellular function of TopBP1 is to monitor ongoing DNA replication [11].

Yeast Rad4/Cut5<sup>TopBP1</sup> and Dbp11<sup>TopBP1</sup> are essential for the initiation of DNA replication. Our detailed knowledge of replication initiation has been established from work realized mainly in the budding yeast and *Xenopus* systems. During G1 phase, origin recognition complex (ORC), Cdt1, Cdc6 and the minichromosome maintenance proteins 2-7 (MCM<sub>2-7</sub>) proteins are sequentially assembled on the origin to form a pre-replicative complex (pre-RC). Then the pre-RC is converted to an initiation complex (IC) at the onset of S phase by S-phase cyclin dependent kinases (S-CDKs) and the Cdc7-Dbf4 kinase (DDK). This leads to the binding of Cdc45 and to the unwinding of the DNA at the replication origin [12]. Cdc45 is an essential replication factor that has been reported to load the initiating DNA polymerase  $\alpha$ -primase complex [13], and to act as a processivity clamp for the putative DNA helicase MCM 2-7 [14, 15, 16]. Furthermore, human Cdc45 may play an important role in elongation of DNA replication by bridging the processive DNA polymerases  $\delta$  and  $\epsilon$  with the replicative helicase in the elongation machinery [17]. In *Xenopus*, Xmus101/Cut5 protein is required for the recruitment of Cdc45 to origins of DNA replication [18].

To improve our understanding of the physiological functions of human TopBP1 during the initiation step of DNA replication we investigated the interplay between TopBP1 and Cdc45 proteins in synchronized HeLa-S3 cells. Using glutathione S-transferase (GST) pull-down and co-immunoprecipitation techniques we showed a direct interaction between TopBP1 and Cdc45 *in vitro* and *in vivo*. The use of deletion mutants in GST pull-down assays identified the first and second as well as the sixth BRCT domains of TopBP1 to be responsible for the functional interaction with Cdc45. Moreover, the interaction between Cdc45 and the first and second BRCT domains of TopBP1 inhibited their transcriptional activation both in yeast and mammalian one-hybrid systems. Both proteins interacted exclusively at the G1/S boundary of cell cycle, only weak interaction could be found at the G2/M boundary. The overexpression of the sixth BRCT domain led to diminished loading of Cdc45 onto chromatin. These data suggest that human TopBP1 is involved in the formation of the initiation complex of replication in human cells and is required for the recruitment of Cdc45 to origins of DNA replication.

## MATERIALS AND METHODS

### Cell culture and synchronization

HeLa and HeLa-S3 cells were grown as monolayers in Dulbecco's modified Eagle's medium with GlutaMAX<sup>TM</sup>I (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 U ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin (Biochrom). Cells were maintained in an atmosphere of 5% CO<sub>2</sub> at 37°C. To create stable cell lines HeLa-cells were transfected with derivatives of the expression vector pCEP4 (Invitrogen), containing the hygromycin resistance gene for selection of stable cell lines using hygromycin B. 48 hours after transfection HeLa cells were transferred to a medium containing 300 µg hygromycin B. After 6 to 8 days of hygromycin B selection cells were harvested and used for cell fractionation and chromatin isolation (see below).

Synchronization at G1/S was performed for 16 hours using L-mimosine (Sigma) at a final concentration of 0,3 mM. After 16 hours of growth cells were directly harvested (0 h) or released into fresh medium (without L-mimosine) and allowed to proceed into S phase by incubation for 1, 3 and 6 hours. Cells were harvested and used for Flowcytometry analysis and immunoprecipitation. Cell cycle arrest at early S phase was achieved by a double thymidine (TdR, Sigma)-block as previously described [19]. Briefly, cells were cultivated to a confluence of 60% and first blocked by adding 5 mM TdR. After 16 hours of growth cells were removed from the TdR-block and cultivated in complete medium for additional 10 hours. Then a second TdR-block was performed as described above. 16 hours later the cells were removed from the second TdR-block and harvested 0, 1, 3 and 6 hours after release into S phase. To synchronize HeLa-S3 cells in G2/M, cells were pre-synchronized by a single TdR block. Six hours after TdR release, nocodazole (Sigma) was added for a final concentration of 100 ng ml<sup>-1</sup> for 3 hours. Cells were directly harvested (0 h) or released into fresh medium (without nocodazole) and allowed to proceed into M phase by incubation for 3 hours.

### Flowcytometry analysis

For cell sorting 10<sup>6</sup> HeLa-S3 cells after synchronization (see above) were fixed in 70% ethanol for one hour at 4°C, resuspended in a freshly prepared propidium iodide solution (40 µg ml<sup>-1</sup> propidium iodide, 250 µg ml<sup>-1</sup> RNase A) and incubated for 30 min in the dark. Flowcytometry analysis was performed with a FACS-Calibur (Becton Dickinson) using the CellQuest Pro software (Becton Dickinson).

### Cell fractionation and chromatin isolation

Cell fractionation was performed as previously described [20]. To isolate chromatin, cells were resuspended ( $4 \times 10^7$  cells  $\text{ml}^{-1}$ ) in buffer A (10 mM N-(2-hydroxyethyl)piperazine-N(2-ethanesulfonic acid (HEPES), pH 7.9; 10 mM KCl; 1.5 mM  $\text{MgCl}_2$ ; 0.34 M sucrose; 10% glycerol, 1 mM dithiothreitol (DTT);  $5 \mu\text{g ml}^{-1}$  aprotinin;  $5 \mu\text{g ml}^{-1}$  leupeptin;  $0.5 \mu\text{g ml}^{-1}$  pepstatin A; 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). Triton X-100 (0.1 %) was added, and the cells were incubated for 5 min on ice. Nuclei were collected in pellet 1 (P1) by low-speed centrifugation (4 min,  $1300 \times g$ ,  $4^\circ\text{C}$ ). Supernatant (S1) was further clarified by high-speed centrifugation (15 min,  $20000 \times g$ ,  $4^\circ\text{C}$ ) to remove cell debris and insoluble aggregates, resulting in the S2 fraction. Nuclei were washed twice in buffer A, and then lysed in buffer B (3 mM EDTA; 0.2 mM EGTA; 1 mM DTT; protease inhibitors as described above). Insoluble chromatin was collected by centrifugation (4 min,  $1700 \times g$ ,  $4^\circ\text{C}$ ) and the supernatant represented the soluble nucleic fraction (S3). The chromatin pellet was washed once in buffer B, and centrifuged again under the same conditions. The final chromatin pellet (P3) was resuspended in Laemmli buffer and sonicated for 15 s at “low power” using Labsonic U equipment (Schütt Labor Technik). Fractions S2, S3 and P3 were analyzed by Western blotting.

### Immunoprecipitation

HeLa-S3 cells were harvested, and lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% Nonidet-P40,  $10 \mu\text{g ml}^{-1}$  leupeptin and aprotinin,  $5 \mu\text{g ml}^{-1}$  pepstatin, 1 mM PMSF. The lysate was cleared by centrifugation at  $11000 \times g$  at  $4^\circ\text{C}$ . TopBP1 monoclonal antibody ( $1 \mu\text{g ml}^{-1}$ , BD Biosciences) or control IgG was incubated with 15  $\mu\text{l}$  (bed volume) protein G Sepharose and 3% Perfect Block (Mabtec) in lysis buffer overnight at  $4^\circ\text{C}$ . The beads were then washed twice with the same buffer, containing 1 mg precleared lysate (300  $\mu\text{l}$ ) and incubated with constant rotation at  $4^\circ\text{C}$  for 2 hours. After extensive washing, proteins were eluted from the beads by boiling in SDS sample buffer at  $95^\circ\text{C}$  for 5 min. Proteins were detected by immunoblotting as described below.

Alternative of the procedure described above co-immunoprecipitation with  $\mu\text{MACS}$  protein G microbeads was performed as described by the manufacturer (Miltenyi Biotec). Briefly, Cdc45 monoclonal antibody C45-3G10 (kindly provided by E. Kremmer, GSF München) [21] bound to  $\mu\text{MACS}$  protein G microbeads was added to the cell lysate. After 1,5 h of incubation at  $4^\circ\text{C}$ , magnetic separation using a  $\mu$  column and the  $\mu\text{MACS}$  separator was performed.

After extensive washing, co-immunoprecipitated proteins were eluted from the column with SDS gel loading buffer and detected by immunoblotting as described below.

### Western blots

Cell lysates or eluted proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane (Millipore). The membranes were blocked overnight in Tris-buffered saline (pH 7.5) containing 0.2% Tween 20, 5% skim milk (Merck) and incubated with primary antibodies: anti-Cdc45 (1:5), anti-TopBP1 (1:10000, BD Biosciences), anti-Mek (1:2500, BD Biosciences), anti-Histone H1 (1:100, BD Biosciences), anti-Lamin B1 (1:100, BD Biosciences) and anti-His-tag (1:2000, Cell Signaling Technology) for 1 hour. After 3 times washing filters were incubated with horseradish peroxidase-conjugated goat anti-(mouse IgG), anti-(rabbit IgG) or anti-(rat IgG). Protein bands were visualized by Enhanced Chemoluminescence Reaction (ECL)-Plus (GE Healthcare) and following exposure to a X-OMAT AR film (Kodak).

### Plasmid construction and purification of recombinant Cdc45 and TopBP1 proteins

To produce GST-tagged Cdc45 and TopBP1 protein fragments polymerase chain reaction (PCR) experiments were performed and resulting PCR products were inserted into expression vector pGEX-4T-1 (GE Healthcare), encoding GST. The full-length Cdc45L cDNA construct pRSETC-Cdc45L (kindly provided by A. Dutta, Boston, USA) and the full-length TopBP1 cDNA construct KIAA 0259 (a kind gift of T. Nagane, Chiba, Japan) were used as a PCR template. Primer sequences were the following:

GCGAATTCACCATGTTTCGTGTCCGATTTC	(Cdc45for);	5'
GGCGTCGACCTAGGACAGGAGGGAAATAAGTGC	(Cdc45rev);	5'
GGCGTCGACCCACAGCTTGAACCTGGCTGC	(Cdc45Nrev);	5'
GCGAATTCATGTCTGTGCATGGACAGAAGCGG	(Cdc45Cfor);	5'
GCGAATTCATGCACCACCAGCGATGTGTC	(TopBP1-BRCT1-2for);	5'
GCCGTCGACAAGTGAATTACATATTGATTC	(TopBP1-BRCT1-2rev);	5'
GCGGAATTCAACAGCAAACCTGGAGCCTACAC	(TopBP1-BRCT3for);	5'
GCCGTCGACCTTCATCAGCTTGCTCATGCT	(TopBP1-BRCT3rev);	5'
GCGAATTCAGTCTTCTGTCAGTCATTGTG	(TopBP1-BRCT4-5for);	5'
GCCGTCGACAGGTGTAACGACGGTTTTTCTG	(TopBP1-BRCT4-5rev);	5'
GCGAATTCGCTGTGGTCTCACAAACATGCCA	(TopBP1-BRCT6for);	5'
GCCGTCGACTGCGCTGATATCCAAGCTCATT	(TopBP1-BRCT6rev);	5'

GCGAATTCATTATAACGATAGAGGAGACTC (TopBP1-BRCT7-8for); 5'-  
GCGGATCCTCGAGACTGATCCACATGTAA (TopBP1-BRCT7-8rev). Plasmids  
containing sequences encoding GST, GST-Cdc45 (aa 1-566), GST-Cdc45 (aa 1-303), GST-  
Cdc45 (aa 304-566), GST-TopBP1-BRCT1-2 (aa 1-246), GST-TopBP1-BRCT3 (aa 247-  
401), GST-TopBP1-BRCT4-5 (aa 447-693), GST-TopBP1-BRCT6 (aa 706-916), GST-  
TopBP1-BRCT7-8 (aa 1167-1323) and GST-TopBP1 (aa 1-1435) (kindly provided by W.-C.  
Lin, Alabama, USA), were transformed into *E. coli* strain BL21-CodonPlus-RIL (Stratagene).  
5 ml of over-night cultures were transferred into 20 ml LB medium containing 100 µg of  
ampicillin ml<sup>-1</sup>. After 3 hours GST fusion derivatives were induced with 1 mM  
isopropylthiogalactopyranoside (IPTG) and incubation was continued for 2 hours. Cells were  
collected by centrifugation, resuspended in 1 ml PBS containing 1% TritonX-100, 1 mM  
PMSF, 10 mM DTT, and then sonicated. Upon sonification the lysate was cleared by  
centrifugation and the supernatant was subjected to Glutathione-Sepharose affinity  
chromatography for purification of the GST fusion proteins as described by the manufacturer  
(GE Healthcare).

### GST Pull-Down Assays

<sup>35</sup>S-Methionine- or <sup>35</sup>S-Cysteine-labeled TopBP1- and Cdc45-full-length proteins were  
synthesized *in vitro* by coupled T7 RNA polymerase-mediated transcription and translation in  
a reticulocyte lysate system as described by the manufacturer (Promega). Glutathione-  
Sepharose beads (50 µl) liganded by either GST-tagged Cdc45 protein fragments or TopBP1  
protein fragments or GST alone, were washed with HB-buffer (20 mM HEPES, 100 mM KCl,  
5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, pH 7.4) and then resuspended in 100 µl HB-buffer  
containing 0.5% Igepal CA-630 and 10 µl <sup>35</sup>S-methionine- or <sup>35</sup>S-Cysteine-labeled TopBP1-  
and Cdc45-full-length proteins. After incubation at 4°C by constantly rotating for 2 hours, the  
beads were washed with HB-buffer extensively. Proteins were eluted from the beads using  
SDS sample buffer. After separation by 10% SDS-PAGE, TopBP1 and Cdc45 were revealed  
by exposure on KODAK X-OMAT AR film (Kodak).

### Yeast one-hybrid assay

The TopBP1-BRCT1-2 (aa 1-246) fragment was ligated to DNA encoding the C-terminus  
of the GAL4 DNA-binding domain of the pGBKT7 vector (carrying a TRP1 marker,  
Clontech Laboratories). Full-length Cdc45-cDNA was isolated as an EcoRI-SalI-fragment  
from the plasmid pRSETC-Cdc45L (see above) and ligated to the yeast expression vector

p423TEF (carrying a HIS3 marker, 22). Yeast strain Y190 (Clontech Laboratories), which has the reporter genes *HIS3* and *lacZ* under the control of GAL4-responsive promoters was transformed with the TopBP1-BRCT1-2-plasmid alone or with both plasmids and  $\beta$ -galactosidase liquid culture assays, using *o*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) as substrate were performed according to the manufacturer's instructions (Clontech Laboratories) [23]. The  $\beta$ -galactosidase values represent the mean numbers and standard deviations of three independent experiments.

### Mammalian one-hybrid and Dual-luciferase assays

A PCR-fragment enclosing the TopBP1-BRCT1-2 (aa 1-246)-fragment was inserted into the pCMV-BD vector (Mammalian Two-Hybrid Assay Kit, Stratagene) resulting in the plasmid pUS6. Furthermore, the full-length Cdc45-cDNA from pRSETC-Cdc45L (see above) was inserted into pcDNA3 vector (Invitrogen) resulting in the plasmid pcDNA3-Cdc45L. HeLa cells ( $1 \times 10^5$  cells) were co-transfected with the following combinations of plasmids: (1) pCMV-BD (Stratagene), pFR-Luc-luciferase reporter vector (Stratagene) and pHRLTK Renilla control vector (Promega) as a negative control, (2) pUS6, pFR-luc and pHRLTK Renilla as mammalian one-hybrid system, and (3) pUS6, pcDNA3-Cdc45L, pFR-luc and pHRLTK Renilla to investigate the influence of Cdc45-interaction on transactivation by TopBP1-BRCT1-2 (aa 1-246) in mammalian cells. After 36 hours cells were lysed in NP-40 lysis buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 0.25% NP-40) on ice. The extracts were cleared by centrifugation at 11000 x g for 15 min at 4°C. Relative luciferase activity of cell extracts was analysed using the Dual-luciferase assay system (Promega) according to the manufacturers instructions and a Luminoscan luminometer (Labsystems). The Firefly luciferase activities of the reporter constructs were normalised to the *Renilla* luciferase activity.

## RESULTS

### TopBP1 and Cdc45 interact *in vivo* exclusively at the G1/S boundary of cell cycle

To investigate the interplay between human TopBP1 and Cdc45 proteins during early and late phases of cell cycle, HeLa-S3 cells were synchronized either at G1/S boundary by mimosine treatment, or at the beginning of S phase with a double thymidine desoxyribose (TdR)-block [19], or at the G2/M boundary by nocodazole treatment. Cell cycle distribution was confirmed by Flowcytometry analysis (Fig. 1B and D). Cell extracts from synchronized HeLa-S3 cells at different time points after release from the mimosin-block were

immunoprecipitated with anti-Cdc45 and  $\mu$ MACS protein G microbeads and analyzed by Western blotting. As shown in Fig. 1A, endogenous TopBP1 could be co-precipitated together with Cdc45 by an anti-Cdc45 antibody at G1/S boundary (0h) and one hour after release from the mimosine-block. At later time points (3 and 6h) when more than half of the cell population was in the S phase co-precipitation efficiency dramatically declined. All control experiments, i.e. beads alone or beads with control serum, confirmed specificity of the above precipitations. Only weak co-precipitation could be obtained with nocodazole treated cells at the G2/M boundary (Fig. 1C). *Vice versa*, Cdc45 could be co-precipitated together with endogenous TopBP1 using anti-TopBP1 antibody bound to Sepharose beads in cell extracts from synchronized HeLa-S3 cells in early S phase 3 hours after release from the double TdR-block (Fig. 1E). The immunoprecipitation studies demonstrated that a protein-protein complex between TopBP1 and Cdc45 is most abundant in cells enriched for the G1/S boundary of cell cycle.

### **TopBP1 and Cdc45 proteins physically interact *in vitro***

We next performed GST pull-down assays to investigate whether TopBP1 and Cdc45 directly interact *in vitro*. Glutathione-Sepharose beads bound to either GST-tagged Cdc45 or GST alone were incubated with  $^{35}$ S-methionine-labeled full-length TopBP1, processed as described in Materials and Methods and analyzed by autoradiography. As shown in Fig. 2B (lane 5) full-length TopBP1 bound specifically to the GST-Cdc45 matrix, no binding was detected using GST as a control, suggesting a direct physical interaction between TopBP1 and Cdc45. Cdc45 does not contain any characteristic protein domains, but a central region of human Cdc45 showed significant homology to E1 proteins of human papillomaviruses [13]. To further narrow down the TopBP1 interaction domain of Cdc45, two GST-tagged partial constructs of Cdc45 were used in further GST pull-down experiments: an N-terminal construct, containing the E1-homology domain (aa 1-303) and a C-terminal construct (aa 304-566) (Fig. 2A). As shown in Fig. 2B (lanes 3 and 4), TopBP1 specifically bound to the N-terminal protein fragment of Cdc45. The binding efficiency was quite high – about 70% of the included TopBP1 bound to the GST-Cdc45(aa1-303)-matrix (Fig. 2B, lower part). The above results showed that TopBP1 and the N-terminus of Cdc45 physically interact *in vitro*.

### **Cdc45 interacts both with the first and second and with the sixth BRCT domains of TopBP1 *in vitro***

Human TopBP1 protein contains 8 BRCT domains. To find out which domains were involved in the interaction with Cdc45, GST-tagged TopBP1 protein fragments were purified from *E. coli*. We performed GST pull-down experiments involving the purified first and second BRCT domains of TopBP1 (aa 1-246), the third BRCT domain (aa 247-401), the 4<sup>th</sup> and 5<sup>th</sup> BRCT domains (aa 447-693), the 6<sup>th</sup> BRCT domain (aa 706-916), the 7<sup>th</sup> and 8<sup>th</sup> BRCT domains (aa 1167-1323) as well as the full-length protein (aa 1-1435) as GST-fusion constructs (Fig. 3A and B). Glutathione-Sepharose bound to each of the above GST-fusions was incubated with <sup>35</sup>S-methionine-labeled full-length Cdc45 and processed as described above. As shown in Fig. 3C, Cdc45 did not bind to the third, the fourth and the fifth and the seventh and eighth BRCT domains of TopBP1, neither to GST alone, whereas the first and the second as well as the sixth BRCT domain were sufficient for Cdc45-binding. Fig. 3D shows a quantitative interpretation of the interaction data shown in Fig. 3C. Although the involvement of a rabbit reticulocyte intermediate cannot be absolutely excluded, the above results suggest that Cdc45 directly interacts both with the first and second and with the sixth BRCT domains of TopBP1 both *in vitro* and *in vivo*.

### **Cdc45 inhibits the transcriptional activity of TopBP1 in yeast and in mammalian cells**

Several lines of evidence suggest that human TopBP1 can function as a transcriptional regulator [3]. Furthermore, it was found that full-length TopBP1 as well as the tandem BRCT domain repeats 1 and 2 fused to the DNA binding domain LexA activated *lacZ*-reporter construct in *Saccharomyces cerevisiae* [9]. To get data further supporting the interaction between Cdc45 and TopBP1 *in vivo* we decided to prove whether the interaction of Cdc45 with the first and second BRCT domains of TopBP1 had any influence on the ability of TopBP1 to activate transcription. The TopBP1-BRCT1-2 (aa 1-246) fragment was ligated to DNA encoding the C-terminus of the GAL4 DNA-binding domain in the pGBKT7 vector. The *S. cerevisiae* strain Y190 was transformed with the construct and a  $\beta$ -galactosidase assay was performed. As expected a significant transactivation of the reporter construct was observed (Fig. 4A, lane 2). For evaluation, the absolute value of  $\beta$ -galactosidase activity (14 U ml<sup>-1</sup>) was taken as 100%. The co-transformation of Y190 with the BRCT 1-2-GAL4 DNA-binding domain-construct and a Cdc45 expression vector led to a 50% reduction of the transcriptional activity of TopBP1 in yeast (Fig. 4A, lane 3). This result showed that Cdc45 was able to repress the transcriptional activity of TopBP1 in bakers yeast.

To show the repression of transcriptional activity of TopBP1 by Cdc45 also in mammalian cells a PCR-fragment enclosing the TopBP1-BRCT1-2 (aa 1-246)-fragment was inserted into the pCMV-BD vector encoding the C-terminus of the GAL4 DNA-binding domain resulting in plasmid pUS6. HeLa cells were co-transfected respectively with pFR-Luc-luciferase reporter vector and pHRLTK Renilla control vector and additionally with the following plasmids: (1) pCMV-BD as a negative control, (2) pUS6 and pFR-luc as mammalian one-hybrid system, and (3) pUS6 and pcDNA3-Cdc45L to investigate the influence of Cdc45-interaction on transactivation by TopBP1-BRCT1-2. The relative luciferase activity of cell extracts was analysed using the Dual-luciferase assay system. As expected a significant transactivation of the luciferase reporter construct by the pUS6-construct was observed (45 times of the negative control luciferase activity of which was taken as 100) (Fig. 4B, lane 2). The co-transfection of HeLa cells with pUS6 and pcDNA3-Cdc45L led to a 90 % reduction of the transcriptional activity in HeLa cells (Fig. 4B, lane 3). This results show that Cdc45 was able to repress the transcriptional activity of TopBP1 not only in bakers yeast but also in mammalian cells and speak in favour of an *in vivo* interaction between TopBP1 and Cdc45.

### **Overexpression of the 6<sup>th</sup> BRCT domain in HeLa cells leads to diminished binding of Cdc45 to chromatin**

In two recent papers authors conclude that the N-terminal half of *Xenopus* Cut5 protein (a TopBP1-like protein), containing BRCT domains 1 to 5, is sufficient to complement a replication defect caused by depletion of Cut5 in a *Xenopus in vitro* replication system [24, 25]. This raises the question whether in human cells the interaction between Cdc45 and the 6<sup>th</sup> BRCT domain of TopBP1 is associated with a function of both proteins in replication. For this purposes we cloned DNA fragments encoding either His-tagged 6<sup>th</sup> or 4<sup>th</sup> and 5<sup>th</sup> BRCT domains of TopBP1 into the expression vector pCEP4 (Invitrogen), containing the hygromycin resistance gene for selection of stable cell lines using hygromycin B. HeLa cells were transfected with the above constructs and after 6 to 8 days of hygromycin B selection cells were harvested and fractionated into cytoplasmic fraction, nucleoplasmic fraction and chromatin pellet as described in Materials and Methods. All fractions were analyzed by Western blotting. The upstream activator kinase Mek-2, which is only localized in the cytoplasm over the whole cell cycle [26], Lamin B1, a component of the inner nuclear membrane and Histone H1, a part of nucleosomes, were used as loading controls for normalization of Cdc45 band intensities. As shown in Fig. 5A and B, overexpression of the 6<sup>th</sup> BRCT domain of human TopBP1 resulted in diminished binding of Cdc45 (44% of control)

replication protein Cdc45 onto chromatin [31, 18]. For budding yeast Zegerman and Diffley [32] and Tanaka and co-workers [33] very recently showed that Dbp11 connects phosphorylated Sld2 and Sld3 to facilitate interactions between replication proteins, such as Cdc45 and GINS. However, orthologs of yeast Sld2 and Sld3 in metazoans are not known until now. The N terminus of human RecQL4, a helicase, shares homology with the N terminus of Sld2, suggesting that RecQL4 is a metazoan ortholog of yeast Sld2. However, RecQL4 is not required for the Cdc2-dependent Cdc45 loading step [34].

Here we show a direct interaction between human TopBP1 and Cdc45 *in vitro* and *in vivo* using glutathione S-transferase (GST) pull-down and co-immunoprecipitation techniques. Moreover, Cdc45 was able to repress the transcriptional activity of TopBP1 in bakers yeast and in mammalian cells. This is an additional indication of an *in vivo* interaction between TopBP1 and Cdc45. Both proteins interacted exclusively at G1/S boundary and in early S phase of the cell cycle, no interaction could be found in the M phase. If one acts on the assumption that the interaction between TopBP1 and Cdc45 takes place on chromatin [3] this results are in accordance with previous data from Saha and co-workers showing that the affinity of CDC45L protein to a nuclear tether is significantly diminished as DNA replication proceeds [35]. Moreover, in a very recent paper it was shown that the human Cdc45 protein was localized to the nucleus from late G1 to G2 phases but was distributed throughout the cell and no longer associated with DNA in M phase [17]. TopBP1 shows a similar distribution during cell cycle. Kim and co-workers showed that human TopBP1 was only detected in the chromatin-containing fractions after cells left mitosis and entered S phase [11]. Our results support the idea that human TopBP1 is a Cdc45 loading factor but might be dispensable for DNA replication after initiation had been completed. This would be comparable to the role of the TopBP1 analogues Rad4/Cut5 in budding yeast and Xmus101 in *Xenopus*. Cdc45, on the other hand, was found to have a function in both replication initiation as well as elongation where it possibly functions as a helicase processivity factor [14, 16, 17].

However, does the here observed physical interaction between TopBP1 and Cdc45 really suggest that TopBP1 is also involved in the human replication process? We have demonstrated that Cdc45 binds to the sixth BRCT domain of TopBP1. This is an interesting region with regard to a possible involvement in DNA replication for two reasons. Firstly, it was shown that an antiserum raised against the sixth BRCT-repeat inhibited replicative DNA synthesis in HeLa cell nuclei *in vitro*. From this the conclusion was drawn that an unknown protein involved in replication may interact with this region of TopBP1 [9]. Secondly, it was shown that the transcription factor E2F1 interacts with the sixth BRCT domain of TopBP1

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## LEGENDS TO FIGURES

**Fig. 1.** TopBP1 and Cdc45 interact *in vivo* exclusively at the G1/S boundary of cell cycle.

Whole cell lysates were derived from synchronized HeLa-S3 cells (A) at the G1/S1 transition, using L-mimosine at a final concentration of 0.3 mM, (C) in G2/M, performing a single TdR block and after that nocodazole treatment at a final concentration of 100 ng ml<sup>-1</sup> and (E) at early S phase performing a double thymidine-block. Lysates (800 µg) derived at different time points after cell cycle block (as indicated) were subjected to co-immunoprecipitation experiments using antibodies against Cdc45 (A, C) and TopBP1 (E). Controls were beads alone (C1) or pre-immune serum (C2). Input represents 15% (120 µg) of the protein used for IP (A) or 10% of protein (80 µg) (C). An unsynchronized cell population was used as control (as). Cell cycle distributions were determined by Flowcytometry analysis (B, D). The precipitates were analyzed by immunoblotting experiments using antibodies against TopBP1 and Cdc45.

**Fig. 2.** Full-length TopBP1 interacts with the N-terminal part of Cdc45 *in vitro*. (A) GST, GST-Cdc45(aa 1-303), GST-Cdc45(aa 304-566) and GST-Cdc45(full-length) were visualized by Coomassie staining. (B) The results obtained from GST-pull down experiments with the GST fusion of full-length Cdc45 (lane 5) and N- and C-terminal protein fragments of Cdc45 (lanes 3 and 4) are shown. Unfused GST served as a control (lane 2). Interaction assays were performed with <sup>35</sup>S-cysteine-labeled full-length TopBP1. TopBP1 was detected by autoradiography. First lane: input, 10 µl of the *in vitro* translation product used for the interaction assay. (B, lower part): Quantification of the GST-pull down experiments. Intensity of the input band was taken as 1. The values represent the mean numbers and standard deviations of three independent experiments.

**Fig. 3.** Cdc45 interacts with the first and second as well as with the sixth BRCT domain of TopBP1 *in vitro*. (A) Schematic representation of the TopBP1 truncation clones fused to GST.

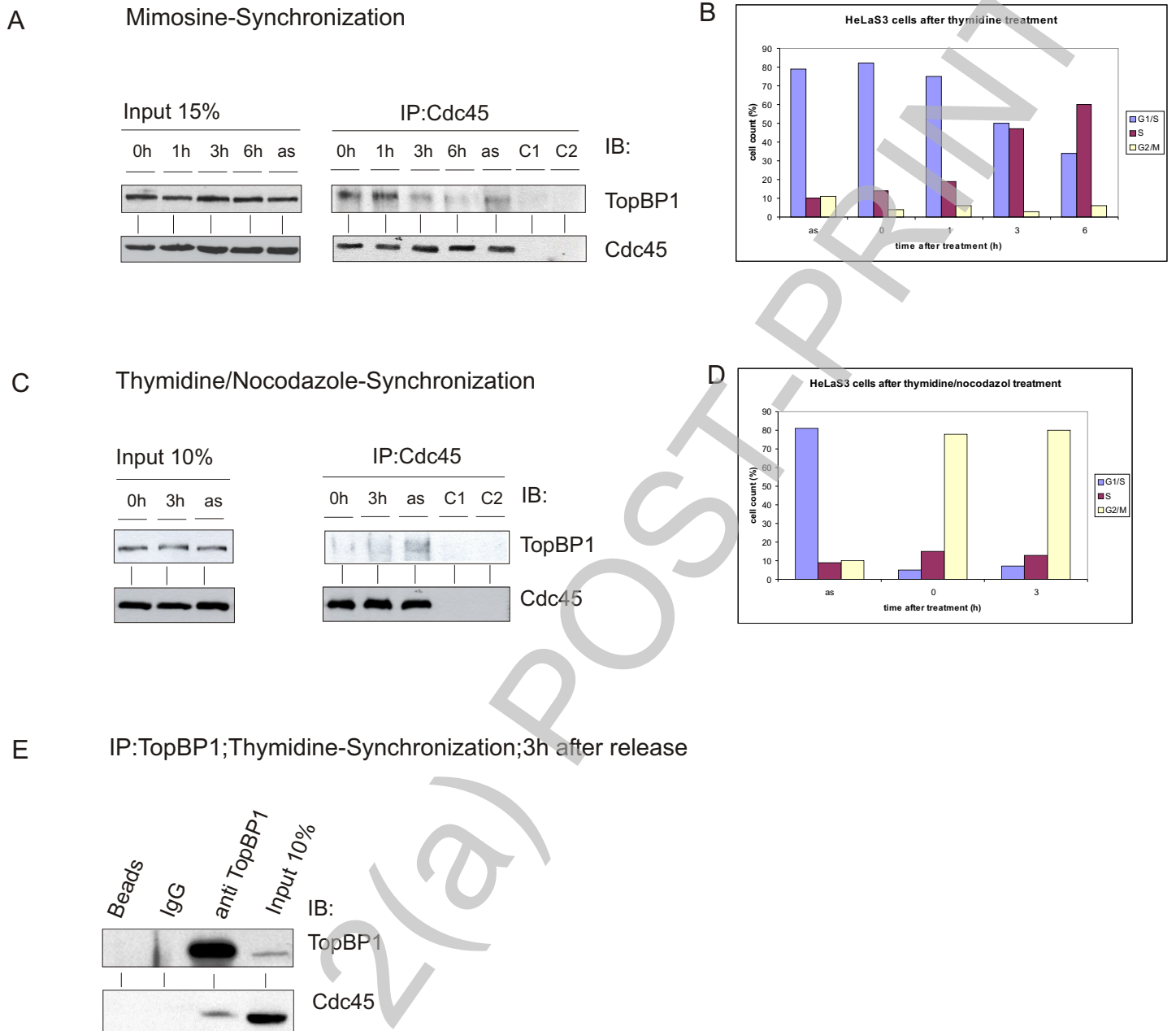
The numbers of BRCT domains are indicated. NLS: nuclear localization signal. (B) GST and the TopBP1 truncation clones fused to GST (see Fig. 5A) were visualized by Coomassie staining. (C) Results of GST-pull down experiments performed with the TopBP1 truncation clones fused to GST and  $^{35}\text{S}$ -methionine-labeled full-length Cdc45 protein. GST served as control. First lane: input, 10  $\mu\text{l}$  of the in vitro translation product used for the interaction assay. (D) Quantification of the GST-pull down experiments. Intensity of the input band was taken as 1. The values represent the mean numbers and standard deviations of three independent experiments.

**Fig. 4.** Cdc45 represses the transcriptional activity of TopBP1 in yeast and mammalian cells. (A) The TopBP1-BRCT1-2 fragment was ligated to the DNA encoding the C-terminus of the GAL4 DNA-binding domain of the pGBKT7 vector. Full-length Cdc45-cDNA was ligated into the yeast expression vector p423TEF. Yeast strain Y190, which has the reporter genes *HIS3* and *lacZ* under the control of GAL4-responsive promoters was transformed with the pGBKT7 vector alone as a control (1), TopBP1-BRCT1-2-plasmid alone (2) or with both plasmids (3);  $\beta$ -galactosidase liquid culture assays using o-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) as a substrate were performed. The absolute value of  $\beta$ -galactosidase activity of (2) was taken as 100%. The  $\beta$ -galactosidase digits represent the mean values and standard deviations of three independent experiments. (B) A PCR-fragment enclosing the TopBP1-BRCT1-2 (aa 1-246)-fragment was inserted into the pCMV-BD vector resulting in the plasmid pUS6. HeLa cells ( $1 \times 10^5$  cells) were co-transfected with the following combinations of plasmids: (1) pCMV-BD, pFR-Luc-luciferase reporter vector and pHRLTK Renilla control vector as a negative control, (2) pUS6, pFR-luc and pHRLTK Renilla as mammalian one-hybrid system, and (3) pUS6, pcDNA3-Cdc45L, pFR-luc and pHRLTK Renilla to investigate the influence of Cdc45-interaction on transactivation by TopBP1-BRCT1-2 (aa 1-246) in mammalian cells. After 48 hours relative luciferase activity of cell extracts was analysed using the Dual-luciferase assay system. The Firefly luciferase activities of the reporter constructs were normalised to the *Renilla* luciferase activity. The relative luciferase units of the negative control was set to 100. The values represent the mean numbers and standard deviations of four independent experiments.

**Fig. 5.** Overexpression of the 6<sup>th</sup> BRCT domain in HeLa cells leads to diminished binding of Cdc45 to chromatin. DNA fragments encoding either His-tagged 6<sup>th</sup> or 4<sup>th</sup> and 5<sup>th</sup> BRCT domains of TopBP1 into the expression vector pCEP4, containing hygromycin resistance

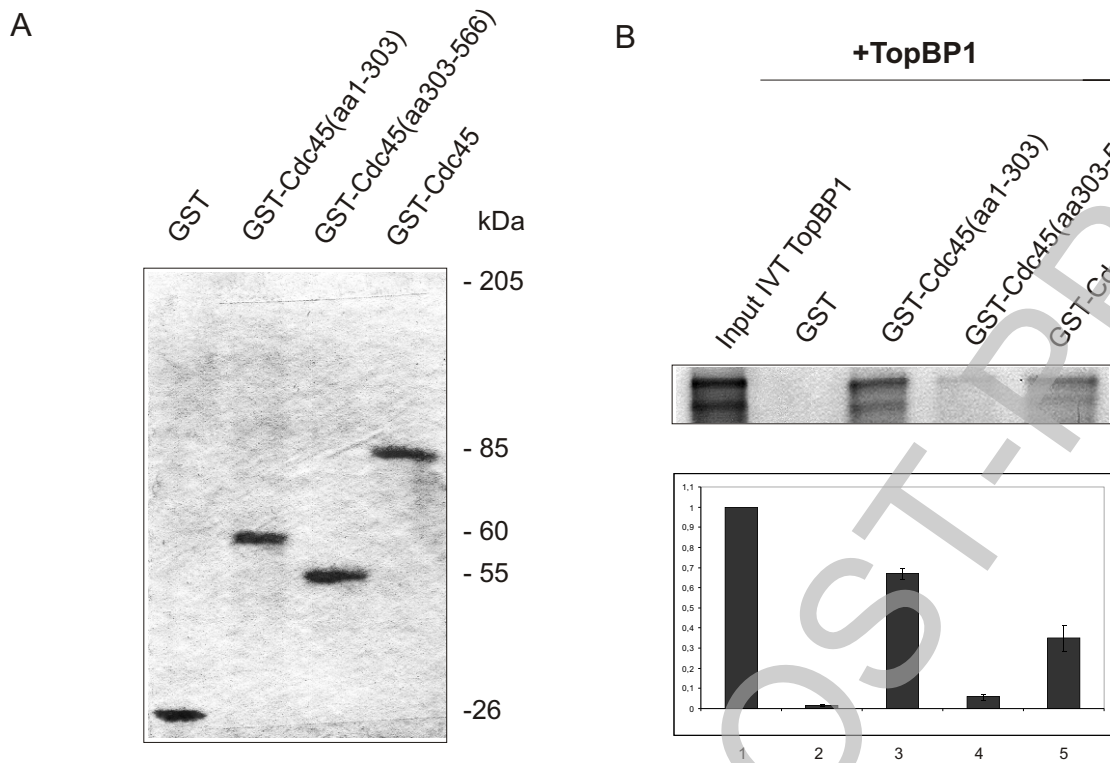
gene for selection of stable cell lines using hygromycin B. HeLa-cells were transfected with pCRP4-TopBP1-BRCT4-5 (1), pCRP4-TopBP1-BRCT6 (2) and the pure vector as a control (3). After 6 to 8 days of hygromycin B selection cells were harvested and fractionated into cytoplasmic fraction (S2), nucleoplasmic fraction (S3) and chromatin pellet (P3) as described in Materials and Methods. (A) Fractions S2, S3 and P3 were analyzed by Western blotting using antibodies against Cdc45, TopBP1, Mek2, Lamin B1 and Histone H1 (B) Quantification of the Cdc45 blots from all three fractions from (A). Intensity of Cdc45 bands was normalized with regard to the intensity of the loading control (Ctrl) bands. Intensity of Cdc45 band in lane 3 was taken as 1. The values represent the mean numbers and standard deviations of three independent experiments. (C) Expression of the His-tagged 4<sup>th</sup> and 5<sup>th</sup> (1) or 6<sup>th</sup> (2) BRCT domains of TopBP1 detected with an anti-His antibody.

Fig. 1



THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20070872

Fig.2



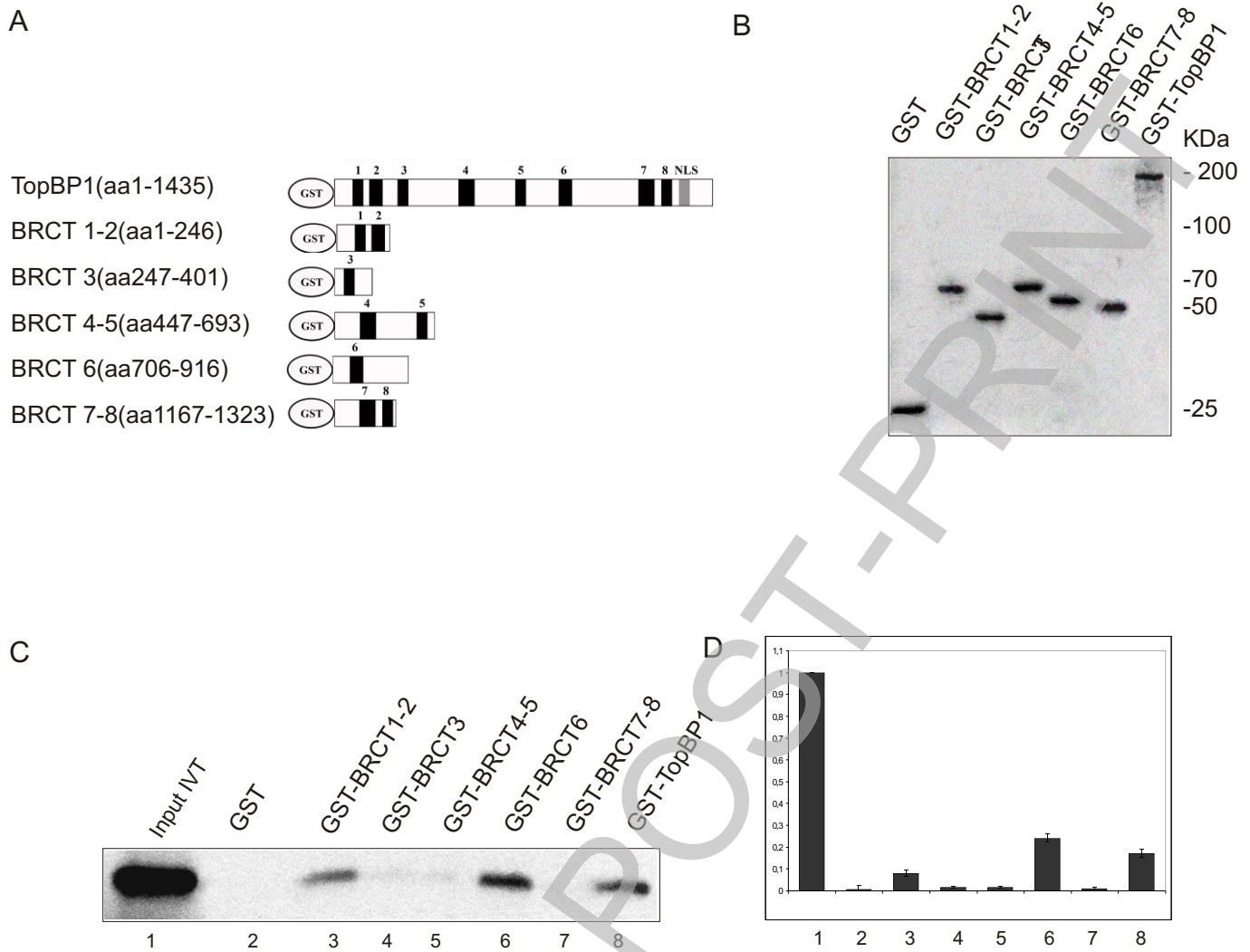
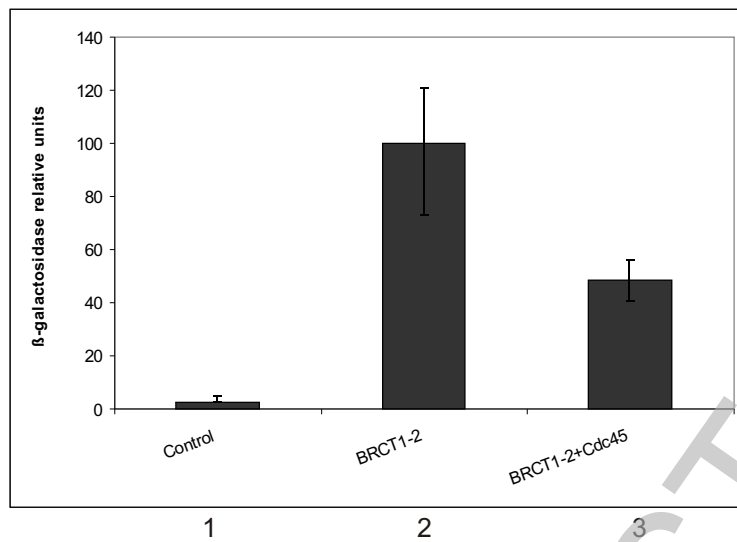


Fig.4

## A Yeast cells



## B HeLa cells

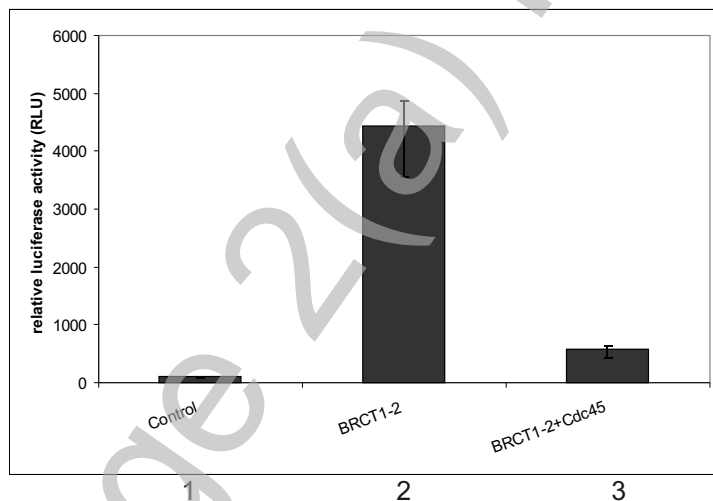


Fig.5

