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The ePHD protein SPBP interacts with TopBP1 and together they co-operate to stimulate Ets1-mediated transcription

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ABSTRACT

SPBP (Stromelysin-1 PDGF responsive element binding protein) is a ubiquitously expressed 220 kDa nuclear protein shown to enhance or repress the transcriptional activity of various transcription factors. A yeast two-hybrid screen, with the extended plant homeodomain (ePHD) of SPBP as bait, identified TopBP1 (topoisomerase II β-binding protein 1) as a candidate interaction partner of SPBP. TopBP1 has eight BRCA1 carboxy-terminal (BRCT) domains and is involved in DNA replication, DNA damage responses and in the regulation of gene expression. The interaction between SPBP and TopBP1 was confirmed *in vitro* and *in vivo*, and was found to be mediated by the ePHD domain of SPBP and the BRCT6 domain of TopBP1. Both SPBP and TopBP1 enhanced the transcriptional activity of Ets1 on the *c-myc* P1P2- and matrix metalloproteinase-3 (MMP3) promoters. Together they displayed a more than additive effect. Both proteins were associated with these promoters. The involvement of TopBP1 was dependent on the serine 1159 phosphorylation site, known to be important for transcriptional activation. Depletion of endogenous SPBP by siRNA treatment reduced MMP3 secretion by 50% in phorbol ester-stimulated human fibroblasts. Taken together, our results show that TopBP1 and SPBP interact physically and functionally to co-operate as co-activators of Ets1.

INTRODUCTION

Stromelysin-1 PDGF responsive element binding protein (SPBP) is a 220 kDa ubiquitously expressed nuclear protein containing an N-terminal transactivation domain, three nuclear localisation signals, a DNA-binding domain with an AT-hook and a C-terminal extended PHD domain (ePHD) (Figure 1A) (1). Originally, SPBP was identified as a protein involved in transcriptional activation of the matrix metalloproteinase-3 (MMP3)/Stromelysin-1 promoter via the specific sequence element SPRE (Stromelysin-1 PDGF responsive element) (2). Later SPBP was found to act as a transcriptional co-activator since it enhanced the transcriptional activity of the positive co-factor and RING finger protein SNURF/RNF4 (3), and of certain transcription factors such as Sp1, Ets1, Pax6 and c-Jun (1). SPBP was also reported to interact with c-Jun *in vitro* (4). Recently, SPBP was found to act as a phosphoserine-specific repressor of oestrogen receptor α (ERα) (5). A region spanning NLS2 of SPBP (Figure 1A) bound directly to ERα phosphorylated on serine 104, 106 and 118, but not to the unphosphorylated form of ERα. Over-expression of SPBP inhibited the proliferation of an ERα-dependent breast cancer cell line (5). Thus, a picture is emerging of SPBP as a transcriptional co-regulator with both activating and inhibitory potential depending on the conditions.

The PHD finger is a common structural motif present throughout eukaryotic proteomes in nuclear, chromatin-associated proteins (6,7). PHD fingers are found to have both protein- and nucleosome-binding activity (7), and recently it was shown that the PHD finger is a highly specialized methyl-lysine-binding domain recognizing trimethylated lysine 4 on histone H3 (8). PHD fingers may co-operate with an adjacent domain to constitute a functional nucleosome-binding module (9,10). The ePHD domain of SPBP is related to the PHD finger motif. The PHD domains contain two zinc-fingers with the zinc ligands forming a Cys_4-His-Cys_3 signature. The ePHD domain has instead a Cys_4-His-Cys_2-His signature and an N-terminal extension that may form an additional zinc-finger with a Cys_2-His-Cys signature.

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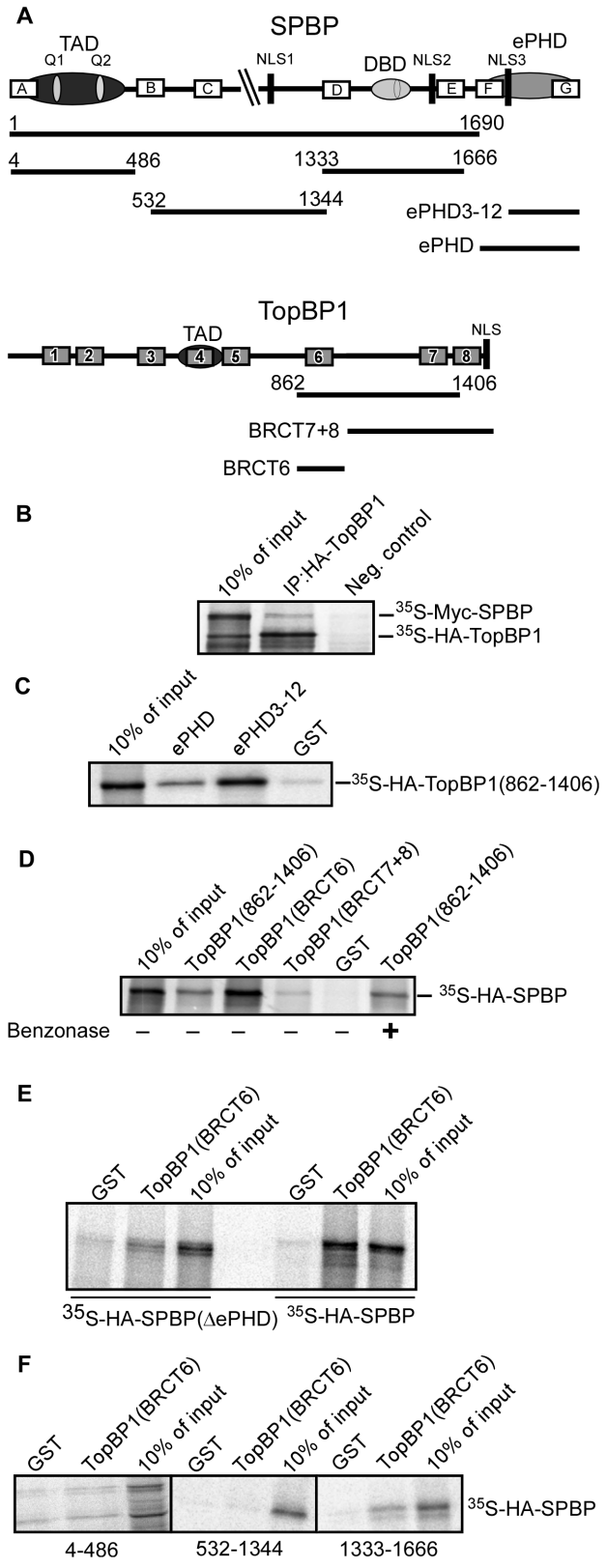

Figure 1. SPBP and TopBP1 interact *in vitro* via the ePHD and BRCT6 domains. (**A**) Schematic representation of the domain structure of human SPBP (1960 amino acids) and human TopBP1 (1522 amino acids). Deletion mutants of SPBP and TopBP1 used in this study and the TopBP1 region isolated in the yeast two-hybrid screen are indicated. The eight BRCT domains of TopBP1 are represented by numbered boxes. The open boxes designated A-G of SPBP represent regions with strong homology to human RAI1. TAD: trans-activation domain, DBD: DNA-binding domain, NLS: Nuclear Localization Signal, ePHD: extended PHD domain, Q1/Q2: Glutamine-rich stretches. (**B**) *In vitro* co-immunoprecipitation showing interaction between SPBP and TopBP1 full-length proteins. Full-length HA-TopBP1 was *in vitro* co-translated together with Myc-SPBP in the presence of [^{35}S]methionine. Immunoprecipitations were performed using an anti-HA antibody. Precipitated proteins as well as 10% input of *in vitro* translated proteins were resolved by SDS–PAGE (5%). Negative control is the HA-tag co-translated together with Myc-tagged full-length SPBP and immunoprecipitated with an anti-HA antibody. (**C**) GST pull-down assays demonstrating interaction both between the complete ePHD and the ePHD(3–12) domain of SPBP, and TopBP1(862–1406). HA-tagged TopBP1(862–1406) was *in vitro* translated in the presence of [^{35}S]methionine and incubated with equal amounts of either GST, GST-SPBP(ePHD) or GST-SPBP(ePHD3-12). The pulled down proteins, together with *in vitro* translated HA-TopBP1(862–1406) corresponding to 10% of the input were separated by SDS–PAGE (10%). (**D**) BRCT6 domain of TopBP1 binds strongly to SPBP, while BRCT7 + 8 shows weak affinity for SPBP. Radiolabelled *in vitro* translated full-length HA-SPBP was allowed to bind to equal amounts of the different GST-fused fragments of TopBP1. The interaction between TopBP1(862–1406) and full-length HA-SPBP was tested with, or without the nuclease benzonase. Samples and 10% of the input were resolved by SDS–PAGE (5%). (**E** and **F**) *In vitro* GST pull-down assays demonstrating that the SPBP(1333–1666) region interacts weakly with TopBP1. HA-tagged SPBP, SPBP(ΔePHD), SPBP(4–486), SPBP(532–1344) and SPBP(1333–1666) were *in vitro* translated in the presence of [^{35}S]methionine and incubated with equal amounts of either GST or GST-TopBP1(BRCT6). The pulled-down proteins, together with 10% of the input were separated by SDS–PAGE.

TopBP1 is an evolutionary conserved checkpoint mediator that binds to ATR (ataxia-telangiectasia and Rad3 related) and is a direct positive regulator of the ATR–ATRIP complex upon DNA damage (11). TopBP1 is recruited to sites of DNA damage and replication stress via its interaction with Rad9 (12,13). TopBP1, first identified as an interaction partner of topoisomerase II β (14), is associated with chromatin during DNA replication and important for genome integrity during normal S-phase (15). In addition to its roles in checkpoint signalling and genome maintenance, several reports implicate TopBP1 in transcriptional regulation. Metazoan TopBP1 contains five BRCT domains homologous to yeast Dpb11 (*Saccharomyces cerevisiae*) and Cut5/Rad4 (*Schizosaccharomyces pombe*), and three additional BRCT domains in the C-terminus that are found to interact with various transcription factors such as E2F1 (16), Miz-1 (17) and human papillomavirus type 16 (HPV16) transcription-replication factor E2 (18). Overexpression of TopBP1 enhanced the ability of HPV16 E2 to activate transcription and replication (18), while the Miz-1-TopBP1 interaction inhibited Miz-1's ability to activate its target genes *p15INK4B* and *p21Cip1* (17). TopBP1 interacts with E2F1 via its BRCT6 domain and recruits the chromatin modification complex Brg/BRM1 leading to repression of known E2F1 activities, including transcriptional activation and induction of apoptosis (16,19). This interaction is observed both after DNA damage and during G1/S transition, and believed to be crucial for the control of E2F1-dependent apoptosis. The interaction between TopBP1 and the three transcription factors HPV16 E2, Miz-1 and E2F1 involves the phosphoinositide 3-kinase (PI3K)—Akt/PKB signalling pathway (20). Akt/PKB

phosphorylation of TopBP1 at serine 1159 induces oligomerization of TopBP1, which is crucial for the observed interactions. Additionally, TopBP1 is reported to interact with the tyrosine kinase c-Abl (21). TopBP1 was demonstrated to be phosphorylated by c-Abl, and to repress c-Abl at the level of transcription, probably by recruiting histone deacetylases to the *c-abl* promoter. Similar to the repression activity involving E2F and Miz-1, the repression of the *c-abl* promoter is dependent on phosphorylation.

Here we show that TopBP1 and SPBP interact *in vitro* and *in vivo*. The interaction involved mainly the ePHD domain of SPBP and the BRCT6 domain of TopBP1. Reporter gene assays using the Ets1 regulated promoters *c-myc* P1P2 and MMP3, showed that TopBP1 and SPBP co-operate to enhance Ets1-mediated transcription. This co-operation requires their interaction domains and is dependent on the S1159 phosphorylation site of TopBP1. Both TopBP1 and SPBP were found to be associated with the *c-myc* P1P2 promoter and the MMP3 promoter. Mutation of the Ets1-binding sites in the MMP3 promoter inhibited the stimulatory effect of Ets1, SPBP and TopBP1. Finally, siRNA mediated knockdown of endogenous SPBP significantly reduced the expression of the metalloproteinase MMP3 in a human fibroblast cell line.

MATERIALS AND METHODS

Plasmids

cDNA constructs were subcloned into Gateway entry vectors and expression clones made as described (Invitrogen). Below, the numbers given in parantheses in the construct names refer to amino acid sequence positions. All constructs were verified by DNA sequencing (BigDye sequencing kit, Applied Biosystems).

The pSport1-SPBP plasmid encoding full-length human SPBP was generated by inserting a 1.3 kb KpnI fragment of the 5′RACE SPBP product (1) into the KpnI site of pSport1-KIAA0292 (kindly provided by T. Nagase, Kazusa DNA Research Institute). pcDNA3-HA-SPBP was obtained by inserting a PCR product generated from pSport1-SPBP using primers (5′-CAGTCCTTTCG GGAGCAAAGCAGTTAC-3′ and 5′-CTGGATCCT GT GCTTGCTGTCCTTTCCATT-3′) into the EcoRV site of pcDNA3-HA. pGBKT7-SPBP(ePHD3-12) was generated by inserting a PCR product obtained using the primers (5′-CTGGATCCTGTGCTTGCTGTCCTTTCCATT-3′) and (5′-CTGGATCCTGTGCTTGCTGTCCTTTCCAT T-3′) into the NcoI site (blunted) of pGBKT7 (Clonetech). pENTR-SPBP was made by a two-step cloning; first a 4.6 kb KpnI–XbaI fragment of pcDNA3-HA-SPBP was inserted into the KpnI and XbaI sites of pENTR1A (Invitrogen). Secondly, a PCR product obtained using primers (5′-CAGTCCTTTCGGGAGCA AAGCAGTTAC-3′ and 5′-ACTACTCAACCCAGGAT CTGTCAGTCG-3′) and pSport1-SPBP as template was digested with KpnI and DraI and inserted into the same sites of the plasmid from step 1. pENTR-SPBP(ΔePHD) was made using a PCR deletion strategy. The pENTR-SPBP plasmid was amplified by PCR using the primers (5′-GCGGCGGCCGCCTAGACCCAGCTT TCTTGTAC-3′ and 5′-GCGGCGGCCGCTCACTCTG TCACAACAGGTCCCTGC-3′) and cut with NotI and DpnI before ligation using T4 DNA ligase (Invitrogen). pENTR-SPBP (ePHD) was made by DraI and EcoRV digestion of a PCR product obtained using primers (5′-G AAAGCAAGGCGCTCCCGGCC-3′ and 5′-CTGGAT CCTGTGCT TGCTGTCCTTTCCATT-3′) and insertion into the DraI and EcoRV sites of pENTR1A. pDONR207-SPBP(1333–1666) was made by BP recombination of the PCR product obtained using primers (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTG CCCTGCTAAGACCAAAA TACTGCC-3′ and 5′-GGG GACCACTTTGTACAAGAAAGCTGGGTTGACCCT TCCTGCCCCTCACTA-3′) into the vector pDONR207 (Invitrogen).

The following TopBP1 constructs were made: pENTR-TopBP1, encoding full-length TopBP1 was made by inserting a 4.6 kb EcoRI fragment from pBluescript II SK$^+$-KIAA0259 (Kazusa DNA Research Institute, T. Nagase) into the EcoRI site of pENTR1A. pGST-TopBP1(862–1406) was generated by subcloning a 1.7 kb EcoRI–XhoI fragment from pGADGH-TopBP1(862–1406) into the EcoRI and XhoI sites of pGEX-4T-3 (Amersham Pharmacia Biotech). pENTR-TopBP1(862–1011), encoding BRCT domain 6, was made by inserting a 450 bp EcoRI–SalI fragment from pBluescript II SK + -KIAA0259 into the EcoRI and XhoI sites of pENTR2B. A 1.6 kb SalI–EcoRI fragment from pBluescript II SK + -KIAA0259 encoding BRCT domain 7 and 8, was ligated into the EcoRI and XhoI sites of pENTR2B, generating pENTR-TopBP1(1012-1522). pDestHA-TopBP1(ΔBRCT6) was made by inserting a blunt-ended 2.1 kb SalI–EcoRI fragment from pENTR-TopBP1 into pDestHA-TopBP1 cut with EcoNI (blunt-ended) and EcoRV. The TopBP1(S1159A) and the TopBP1(S1159D) mutants were generated by site-directed mutagenesis using the Quick Change Site Directed Mutagenesis Kit (Stratagene) and the sense primers (5′-GAGCAAGGCTTGCCGCCAATTTGC-3′) for S1159A and (5′-GAGCAAGGCTTGCCGACAATTTGC-3′) for S1159D.

Three different MMP3 reporter vectors were made (i) pGL3-MMP3(2298), a 2298 bp construct obtained by PCR on genomic DNA using the primers (5′-GACATG TAGAATCTGCAAATCTAG-3′ and 5′-GAGTGACA GTGTTTGTTTGGATCACC-3′) and cloned into the KpnI and BglII sites of pGL3Basic (Promega); (ii) pGL3-MMP3, a 1448 bp fragment obtained by SacI–BglII digestion of the 2298 bp MMP3 construct ligated into the SacI–BglII sites of pGL3Basic, (iii) pGL3-MMP3-EtsMut, obtained by mutating the two Ets1-binding sites in pGL3-MMP3 to AATC and AGTT and changing the region between them to TCGAGCCCGCTCGA.

pENTR-RAI1 was constructed using a three-step strategy: First, the 2500 most 5′-nucleotides were amplified by PCR on human genomic DNA using primers (5′-GCG GGATCCGGATGCAGTCTTTTCGAGAAAGGTGT GG-3′ and 5′-GCGGCGGCCGCGGAACAGCAGTGC CGGCTGTCCTC-3′) and inserted into the BamHI and NotI sites of pENTR1A. The next 2300 nucleotides were amplified by PCR on human genomic DNA using primers

(5′-CTGCAGTGCCCCGAGGTGGCCAAGGC-3′ and 5′-GGGTGGAGAAGCGAGACGCGTTCAC-3′) and inserted into the NotI and AgeI sites of the construct from step 1. Finally, the C-terminal ePHD domain was amplified by PCR using primers (5′-GGGTGGAAAGCG AGACGCGTTCAC-3′ and 5′-GCGGCGGCCGCCTAC GGCAGCCTCTTATCTTTGGGAC-3′) and IMAGE clone 2231442 as template, and inserted into the NdeI and NotI sites of the construct obtained in step 2. pENTR-RAI1(ePHD) was amplified by PCR using primers (5′-GC AGCCGCGGCCTCCTTGCCC-3′ and 5′-CGAATTCC GTTGGGGTGGATTACTACGGCAG-3′) on human pancreas Marathon Ready cDNA (Clonetech) and ligated into DraI and EcoRI sites of pENTR1A. pDestTet-EGFP-SPBP was constructed as follows: A puromycin resistance casette was cloned into the XhoI site of pUH10-3 (22), and the EGFP-tag (Eco47III–BamHI fragment of the pEGFP-C1 plasmid (Clontech)) was cloned into the EcoRI–BamHI sites. The Gateway reading frame cassette A was thereafter cloned into the EcoRI site and full-length human SPBP (2–1960) was recombined into the pDestTet-EGFP-expression vector.

Yeast two-hybrid screen

The yeast two-hybrid screen was carried out with the MATCHMAKER system (Clontech) according to the manufacturer's instructions, using the yeast strain PJ69-2a expressing the ePHD(3–12) zinc-finger domain of SPBP as bait [pGBKT7-SPBP(ePHD3–12)]. After mating with a HeLa two-hybrid library (Clontech), 2×10^6 transformants were screened and selection was performed on yeast minimal medium lacking Leu, Trp, His and Ade. Colonies were picked 4 days after transformation and confirmed by β-galactosidase filter lift assays. Plasmids from positive clones were purified and transformed into *Escherichia coli* strain DH5α. The sequenced cDNA inserts were identified by basic local alignment search tool (BLAST) searches of the National Center for Biotechnology Information (NCBI) database. In order to verify specific interactions clones were re-screened as described previously (23).

Antibodies

The following primary antibodies were used: Rabbit anti-SPBP antibody, raised against *E. coli* expressed GST-SPBP(1016–1335), -(1108–1335) and -(1535–1597), affinity-purified using the antigen-mix coupled to KHL (Biotrend Chemikalien GmbH, Koeln, Germany), and diluted 1:500 for western blots, 20 µl were used for immunoprecipitations (IP) and 25 µl for chromatin-immunoprecipitations (ChIP); rabbit anti-TopBP1 antibody (ab2402, Abcam) diluted 1:1.000 for WB, 6 µg for IP and ChIP; mouse anti-TopBP1 antibody (cat. no. 611874, BD Bioscience Pharmingen) diluted 1:500 for WB; rabbit anti-TopBP1 antibody (sc-32932, Santa Cruz Biotechnology) diluted 1:200 for WB; rabbit anti-Ets1 antibody (ab11912, Abcam) 15 µg for IP and ChIP; rabbit anti-acetylated histone H3 antibody (Upstate) 10 µg for ChIP; rabbit anti-GFP antibody (ab290, Abcam) diluted 1:5.000 for WB, 2 µl for IP; mouse anti-HA antibody (clone12CA5, Roche) diluted 1:1.000 for WB, 0.1 µg for IP; rabbit anti-MMP3 antibody (ab28948, Abcam) diluted 1:5.000 for WB; rabbit anti-β-actin antibody (Sigma) diluted 1:1.000 for WB.

Secondary antibodies used were: HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies (BD Bioscience Pharmigen) diluted 1:2.000 for WB; HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies (Pierce) diluted 1:5.000 for WB.

Cell culture

HeLa cells (ATCC CCL2) and IMR90 cells (ATCC CCL 186) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Invitrogen). All experiments using IMR90 cells were carried out between passages 12 and 18. For induction of MMP3, 4×10^4 cells/well were seeded in 24-well dishes 2 days before transfection. The cells were starved for 24 h in MEM with 0.1% FBS before being transfected twice with 50 nM SPBP siRNA or 50 nM scrambled siRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen). The cells were starved in MEM without serum 24 h post-transfection. The medium was then replaced with 200 µl fresh MEM supplemented with 1 ng/ml Phorbol 12-myristate 13-acetate (PMA) (P1585, Sigma) followed by 20 h incubation after which 21 µl of the medium was harvested for western blotting. U2OS-TA cells, a kind gift from Dr Jiri Lukas, Danish Cancer Society, Denmark, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and the antibiotics described above. In addition, 400 µg/ml geneticin, (G418, Sigma) was added to the medium. The Tet-off gene expression system (22), was used for the regulated expression of GFP-SPBP in U2OS-TA cells, a human osteosarcoma cell line with stably integrated tetracycline-regulated transcriptional activator (tet-VP16) and a neomycin resistance gene (24). Following transfection of these cells with pDestTet–GFP–SPBP stably transfected clones were selected in medium containing puromycin dihydrochloride (1 µg/ml). Single colonies were picked and the expression of GFP-tagged SPBP was verified using fluorescence microscopy and western blotting. For repression of the transgene, cell monolayers were washed three times with PBS, trypsinated and plated into media with 2 µg/ml doxycycline.

GST pull-down assays

Expression of GST-fusion proteins (1) and GST-pull-down assays (25) were as described previously. For expression of GST fusion proteins containing the ePHD domain of SPBP, 0.1 mM $ZnCl_2$ was added to the growth medium. When benzonase was used 0.5 µl of 520 U/µl benzonase was added to the binding reactions followed by incubation for 10 min at room temperature before incubation at 4°C for 1 h.

IP and immunoblotting

In vitro co-IP assays were described previously (23). Co-IP of over-expressed and endogenous proteins were

performed as follows: 6×10^6 U2OS cells per antibody were harvested in 1 ml lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) (13) supplemented with protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (Calbiochem) and sonicated briefly. The cell lysates were pre-cleared by incubation with 20 µl Protein-A Sepharose beads (Amersham) for 30 min, 4°C on a rotating wheel. The pre-cleared cell-lysates were incubated with the appropriate antibody overnight at 4°C on a rotating wheel. BSA-saturated Protein-A Sepharose beads (30 µl) were added and the incubation continued for 1 h. The beads were washed 3×1 ml in lysis buffer, re-suspended in 20 µl $2 \times$ SDS loading buffer and resolved on SDS–PAGE gels. Co-precipitated proteins were detected by western blotting. Biotinylated Protein Ladder (Cell Signaling) was run in parallel for determination of MW. Detection was performed using ECL- (Santa Cruz Biotechnology) or ECL SuperSignal Femto Molar Detection kits (Pierce) and a LumiAnalyst imager (Roche Applied Sciences).

Reporter gene assays

HeLa cells were seeded at a density of 10^5 cells/well in six-well plates and transfected 24 h later using the calcium phosphate co-precipitation method. Transfections were performed with 1 µg of various SPBP and TopBP1 expression plasmids together with 0.1 µg of pRc-Ets68 (26) or 0.5 µg pCMV-Tag2a-Ets-1 (27). The empty expression vector pcDNA3-HA (23) was used as control. The plasmids p1p2mycluc (28) (0.5 µg) or pGL3-MMP3 (0.5 µg) were used as reporters, and the β-galactosidase expressing pCMV-β-gal vector (0.1 µg) (Stratagene) was included to determine transfection effeciency. Cells were harvested 24 h post-transfection and luciferase activities measured using the Dual Light luciferase and β-galactosidase kit (TROPIX) on a Luminoskan RT dual injection luminometer (Labsystems). All reporter gene assays were carried out in three parallel experiments and repeated several times. The luciferase values varied 1–12% between the parallels.

Reporter gene assays involving siRNA-mediated knock down of SPBP or TopBP1 were performed as follows: HeLa cells seeded in 24-well dishes at 2×10^5 cells/well the day before transfection were transfected using Lipofectamine PLUS (Invitrogen) with reporter vectors (30 ng), expression vectors pRc-Ets68 (15 ng) and pcDNA3-HA-TopBP1 (60 ng) and 50 nM of SPBP siRNA oligos (siGENOME SMARTpool TCF20 siRNAs, Dharmacon), 50 nM of Scrambled siRNA oligos (Dharmacon) or 50 nM of TopBP1 siRNA oligos [synthesized by Ambion, sequence as described in (15)]. pCMV-β-gal (10 ng) was included to determine transfection efficiency. The cells were harvested 2 days post-transfection and the luciferase and β–galactosidase activities determined.

ChIP

ChIP was performed mainly as described in the Chromatin Immunoprecipitation Assay Kit (Upstate catalog no. 17–295). 1.5×10^7 U2OS–GFP–SPBP cells were used for each tested condition. Crosslinking with 1% formaldehyde was performed for 10 min. at room temperature. This was stopped by adding 0.125 M glycine for 5 min. Harvested cells were re-suspended in 500 µl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1, protease inhibitor cocktail), and incubated 10 min on ice before sonication for 15 min in a Bioruptor (Diagenode). The DNA fragments were purified using a QIAquick Spin Kit (QIAGEN). One to 5 µl of a 30 µl DNA purification was used for 30–35 cycles of PCR amplification. PCR primers used to amplify the *c-myc* P2 promoter were (5′-TCGGGGCTTTATCTAACTCG-3′) and (5′-GC TGCTATGGGCAAAGTTTC-3′), while the MMP3 primers used were (5′-TCCAGTTTTCTCCTCTA CCAAGA C-3′) and (5′-TTGCTTTCATCCAAATGGCAGCAG3′). Primers for the control PCRs were: cathepsin D control (5′-TCTGGCCCCACTGGCCATCCGCAC-3′) and (5′-C TTTCCAGTGGGCTGGGATCTTGG-3′), MMP3 control (5′-CAGGTGTGCACTGCCACAAGTGGC-3′) and (5′-AACCTAGCCAAGGAAAGAATCCC-3′), c-myc control (5′-GAGTTTGAGACCAGCCTGGGCAAC) and (5′-CCTGGACTCAGGTGATCCTCCTAC-3′).

RESULTS

SPBP and TopBP1 interact *in vitro* via the ePHD and BRCT6 domains

The ePHD domain (amino acids 1690–1960) of SPBP can be viewed as composed of a 'core PHD domain' with eight zinc ligands (ePHD5–12) with four additional ligands N-terminal to the core making up a third finger. A yeast two-hybrid screen using the C-terminal ePHD3-12 region of SPBP (amino acid 1837–1960) as bait (Figure 1A), identified the DNA damage checkpoint mediator TopBP1 as a putative interaction partner. We denote the bait fragment ePHD3-12 since it contains the 10 C-terminal zinc ligands (3–12). This region has previously been called the ZNF2 domain and is conserved between SPBP and members of the trithorax family proteins such as MLL, MLL2 and −4 (1). Nearly 60% (165 of 280) of the positive clones sequenced encoded BRCT domains 6 and 7 and part of BRCT domain 8 of TopBP1 [TopBP1(862–1406)] (Figure 1A). Co-immunoprecipitation assays of *in vitro* translated HA-tagged TopBP1 (HA-TopBP1) and Myc-tagged SPBP (Myc-SPBP) demonstrated interaction between the full-length proteins *in vitro* (Figure 1B). To verify the interaction *in vitro* and to determine whether the complete ePHD domain of SPBP also could interact with TopBP1, GST pull-down assays using GST-fusions of SPBP(ePHD3-12) and SPBP(ePHD), and *in-vitro* translated HA-tagged TopBP1(862–1406) were performed. As shown in Figure 1C, TopBP1 (862–1406) binds to both SPBP(ePHD) and SPBP(ePHD3-12) with the strongest interaction observed with SPBP(ePHD3-12).

To map the domain of TopBP1 responsible for the interaction with full-length SPBP, two deletion mutants of TopBP1 were constructed. A region containing the BRCT6 domain of TopBP1 (amino acids 862–1011) and

a region containing BRCT domains 7 and 8 (amino acids 1012–1522) (Figure 1A) were produced as GST fusion proteins and tested for interaction with *in vitro* translated HA-tagged SPBP (HA-SPBP). As seen in Figure 1D, full-length SPBP bound to BRCT domain 6 of TopBP1. Since the BRCT domains of TopBP1 are shown to bind double- and single-stranded DNA (29), we wanted to test whether the interaction between SPBP and TopBP1 is indirect via DNA. Complete removal of DNA by treatment with the nuclease benzonase had no effect on the interaction between SPBP and TopBP1(862–1406). Clearly, DNA is not a 'bridging factor' between the two proteins (Figure 1D).

Next, the interaction between TopBP1 and a deletion mutant of SPBP lacking the ePHD domain was investigated. Surprisingly, SPBP(ΔePHD) bound both to TopBP1(BRCT6) (Figure 1E) and TopBP1(BRCT7 + 8) (data not shown), even though the interaction was clearly weaker than for the full-length SPBP (Figure 1E). To further delineate which region of SPBP that was involved in this additional interaction, three deletion constructs of SPBP (amino acids 4–486, −532–1344 and −1333–1666) were *in vitro* translated and assayed by GST-pulldown with GST-TopBP1(BRCT6) and GST-TopBP1(BRCT 7 + 8). These studies showed that the SPBP region containing amino acids 1333–1666 interacted weakly both with BRCT6 (Figure 1F) and BRCT7 + 8 of TopBP1 (data not shown). This region of SPBP encompasses the DNA-binding domain and a bipartite nuclear localization signal (1) (Figure 1A). In conclusion, SPBP and TopBP1 interact *in vitro* primarily via the ePHD domain of SPBP and the BRCT6 domain of TopBP1. This interaction is not dependent on DNA. There are also weaker contributing interactions involving amino acids 1333–1666 of SPBP and BRCT7 + 8 domains of TopBP1.

SPBP and TopBP1 interact *in vivo*

To investigate the SPBP–TopBP1 interaction *in vivo* we first established a U2OS-TA cell line stably expressing *GFP-SPBP* (U2OS-GFP-SPBP) as described in Materials and Methods section. U2OS-TA is a human osteosarcoma cell line with a stably integrated tetracycline regulated transcriptional activator (tet-VP16) (24). This system allows regulated expression of the transgene in a doxycycline-repressible manner. A clone expressing GFP-SPBP at the level of endogenous SPBP that displayed efficient doxycycline-mediated repression of GFP-SPBP expression was used for the further study (Figure 2A). HA-tagged TopBP1 was transiently expressed in the U2OS-GFP-SPBP cell line, and an anti-GFP antibody was used to immunoprecipitate GFP-SPBP from total cellular extracts. As shown in Figure 2B, HA-TopBP1 was co-precipitated with GFP-SPBP. Next, we wanted to determine whether endogenous TopBP1 was co-precipitated with SPBP. SPBP was immunoprecipitated from total U2OS-GFP-SPBP cellular extracts with anti-SPBP antibodies, and the associated TopBP1 was detected by anti-TopBP1 antibodies on a western blot. Figure 2C shows that endogenous TopBP1 was co-precipitated with SPBP. The weak band of co-precipitated TopBP1 may

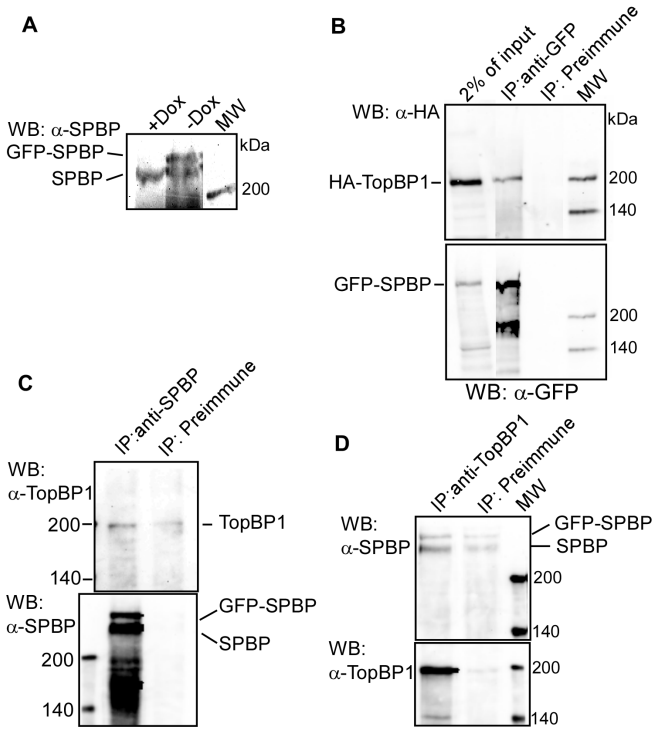

Figure 2. SPBP and TopBP1 interact *in vivo*. (**A**) Expression of GFP-SPBP stably integrated into the genome of the U2OS-TA cell line. Growing the cells in the presence of doxycycline represses the expression of the integrated GFP-SPBP (left lane). Removal of doxycycline induces expression of GFP-SPBP at a similar level as the endogenous protein (middle lane). Total cellular extracts from U2OS-TA cells containing the GFP-SPBP minigene grown in the presence or absence of doxycycline were separated by SDS–PAGE (5%) and immunoblotted using an anti-SPBP antibody. (**B**) GFP-SPBP and HA-TopBP1 interact in U2OS-GFP-SPBP cells. U2OS-GFP-SPBP cells were transfected with a HA-TopBP1 expressing plasmid. One day after transfection immunoprecipitations were performed using an anti-GFP antibody. The co-precipitated proteins were detected by western blotting using an anti-TopBP1 antibody (upper panel). Immunoprecipitated SPBP was visualized using the anti-GFP antibody (lower panel). (**C**) SPBP co-precipitate endogenous TopBP1. Immunoprecipitations from U2OS-GFP-SPBP cells were performed using an anti-SPBP antibody. The co-precipitated proteins were detected by western blotting using an anti-TopBP1 antibody (upper panel). Immunoprecipitated SPBP was visualized using an anti-SPBP antibody (lower panel). The two SPBP bands correspond to GFP-SPBP (upper band) and endogenous SPBP (lower band). (**D**) Endogenous TopBP1 co-precipitate SPBP. Immunoprecipitations were performed using an anti-TopBP1 antibody. The co-precipitated proteins were detected by western blotting using an anti-SPBP antibody (upper panel). Immunoprecipitated TopBP1 was visualized using the anti-TopBP1 antibody (lower panel). The two SPBP bands correspond to GFP-SPBP (upper band) and endogenous SPBP (lower band).

reflect the expression level of TopBP1 in an extract from unsynchronised cells. TopBP1 generally displays a low expression level, except during S-phase when its expression is induced (19,30). Similarly, IP of endogenous TopBP1 using TopBP1 antibodies showed that both endogenous SPBP and the fusion protein GFP-SPBP were associated with TopBP1 (Figure 2D). Although the co-immunoprecipitations of endogenous proteins displayed some background with pre-immune serum, the SPBP and TopBP1 signals were reproducibly above background in several independent experiments.

TopBP1 co-operates with SPBP to stimulate Ets1-mediated transcriptional activation

TopBP1 is a checkpoint control protein shown to re-localize to DNA repair foci containing ATR and BRCA1 upon induction of double-strand breaks (20,30). However, in contrast to TopBP1, SPBP did not re-localize to foci or stripes of DNA double-strand breaks induced by X-ray irradiation (10 Gy) or local laser micro-irradiation of HeLa or HT1080 cells (data not shown). Furthermore, the protein level of SPBP did not change upon DNA damage or during S-phase as is the case for TopBP1 (data not shown). We therefore chose to focus our study on the interplay between SPBP and TopBP1 in regulation of gene expression. SPBP has been shown to enhance the transcriptional activity mediated by Ets1 on the *c-myc* P1P2 promoter (1). Hence, we decided to investigate whether TopBP1 would modulate the positive effect of SPBP on Ets1-mediated transcriptional activation. Transient co-transfection studies in HeLa cells using a luciferase reporter vector containing the *c-myc* P1P2 promoter (28) and the p68 chicken form of Ets1 (Ets68) were performed. As seen in Figure 3A, TopBP1 stimulates Ets68-mediated transcriptional activation of the *c-myc* P1P2 promoter, but to a lesser extent than SPBP. However, a more than additive activation of the P1P2 promoter was observed when both SPBP and TopBP1 were co-expressed with Ets68.

The occupancy of SPBP and TopBP1 on the *c-myc* P2 promoter was examined by ChIP assays using the U2OS-GFP-SPBP cells. As shown in Figure 3B, *c-myc* P2 promoter sequences were detected in both SPBP- and TopBP1 immunoprecipitates strongly suggesting that both SPBP and TopBP1 are located on the *c-myc* P2 promoter. IP of acetylated histone H3 was used as a positive control confirming that the endogenous *c-myc* P2 promoter was transcriptionally active. As a control, PCR analyses did not detect any binding of SPBP- or TopBP1 to a region ~4100 bp upstream the *c-myc* P2 promoter. We also included a region around 3 kb upstream of the cathepsin D gene promoter as an additional negative control (Figure 3B; lower panels).

Ets1 was recently shown to be a main activator of the MMP3 (Stromelysin-1) promoter via binding to tandem Ets1-binding sites (EBS) at position −216 and −201 (Figure 4A) (31). To test whether the stimulation of Ets1-mediated transactivation by SPBP and TopBP1 observed with the *c-myc* promoter could be a more general phenomenon, similar co-transfection experiments were performed using the MMP3 promoter. As seen in Figure 4B, SPBP and TopBP1 co-operated to enhance Ets68-mediated activation of the MMP3 promoter. Similar results were obtained when the p54 form of murine Ets1 (27) was co-expressed instead of the chicken p68 Ets1 (Figure 4C). To confirm that the MMP3 promoter-region harbouring the EBS could be occupied by SPBP and TopBP1 in addition to Ets1 itself we performed ChIP assays using the U2OS–GFP–SPBP cells. As shown in Figure 4D, in addition to Ets1, both SPBP and TopBP1 bind to the MMP3 promoter. The specific binding of these proteins to the MMP3 promoter

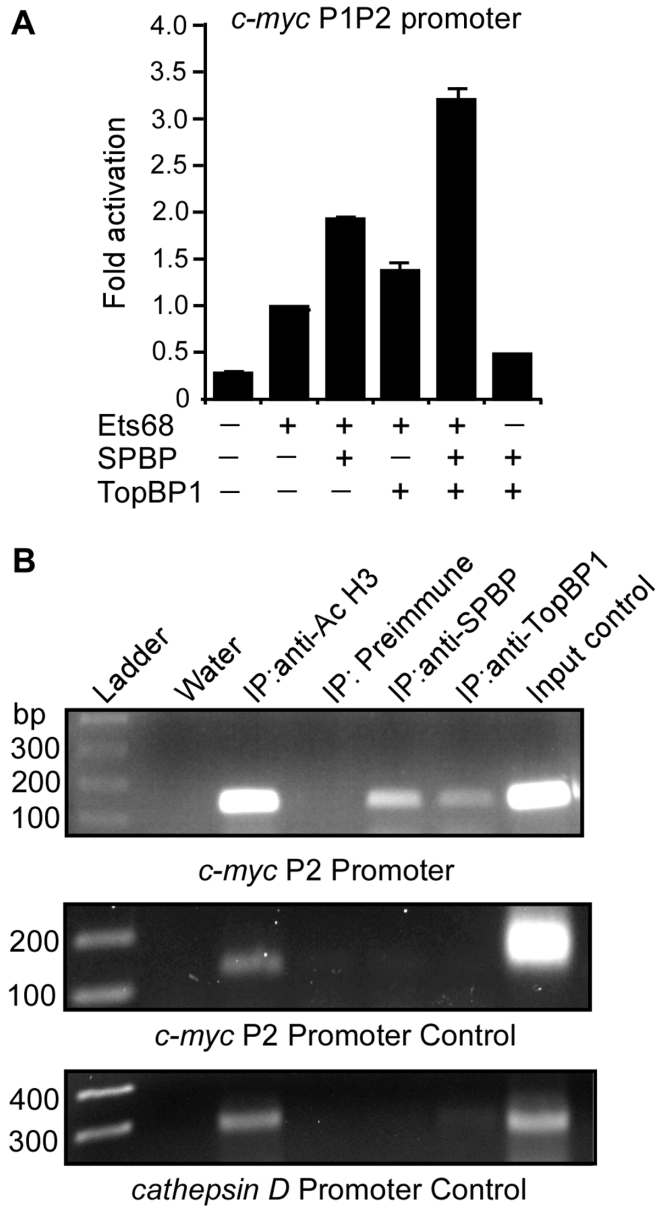

Figure 3. SPBP and TopBP1 associate with the *c-myc* promoter and co-operate to enhance Ets1-mediated transcriptional activation of this promoter. (**A**) When SPBP and TopBP1 are co-expressed with Ets68, a more than additive activation of Ets68-mediated transcription of the *c-myc* P1P2 promoter is observed. Transient transfection assays were carried out in HeLa cells using 0.5 μg of the reporter p1p2mycluc, together with expression vectors for Ets68 (0.1 μg), HA-SPBP (1.0 μg) and/or HA-TopBP1 (1.0 μg) as indicated. The data represent the mean of three independent experiments, each performed in triplicate. (**B**) Chromatin immunoprecipitation assays show that both SPBP and TopBP1 associate with the *c-myc* promoter. Extracts from U2OS-GFP-SPBP cells (1.5×10^7 cells per antibody) were immunoprecipitated with preimmune serum (IgG), polyclonal anti-SPBP antibody, polyclonal anti-TopBP1 antibody and anti-acetylated histone H3 antibody (positive control). Input control (1:50) was included (lane 7). PCR analyses on the immunoprecipitated chromatin were carried out using primers flanking the *c-myc* P2 promoter (position −28 and +200 respectively), primers aligning to positions −4107 and −3949 of the *c-myc* P2 promoter (*c-myc* P2 Promoter Control), and primers aligning to positions −3351 and −3069 of the cathepsin D promoter (*cathepsin* D Promoter Control). The 1 kb DNA ladder is shown to the left.

was verified by control PCR analyses of a region ~3.4 kb upstream the MMP3 promoter. No binding was detected to that region. We also included a region around 3 kb upstream the cathepsin D gene promoter as an additional negative control (Figure 4D, lower panels). To further test the role of SPBP in Ets1-mediated transcriptional activation of the MMP3 promoter, endogenous SPBP was depleted by transfecting HeLa cells with SPBP siRNAs. Of four different siRNAs tested, all caused a significant knockdown of SPBP (Figure 4E). Reporter gene assays performed upon knockdown of SPBP using siRNA oligo 2 (Figure 4F) or −1 (data not shown) showed a small but significant reduction of the transcriptional activation potential of over-expressed Ets1 on the MMP3 promoter (Figure 4F). Importantly, the co-activation potential of TopBP1 was completely lost when SPBP was knocked down (Figure 4F). Similar results were obtained using the *c-myc* P1P2 promoter (data not shown). These results indicate that SPBP acts as a co-activator of Ets1, and that the TopBP1-mediated enhancement of Ets1 activity is strongly dependent on SPBP.

Next, we addressed the role of TopBP1 in Ets1-mediated transcriptional activation of the MMP3 and the *c-myc* P1P2 promoters. Endogenous TopBP1 expression was knocked down using a previously published TopBP1 siRNA (15) (Figure 4G). A small but reproducible reduction in Ets1 transcriptional activity was seen when TopBP1 was knocked down while the co-activation potential of SPBP was strongly reduced (Figure 4H). Similar results were obtained using the *c-myc* P1P2 promoter (data not shown). Thus, these data suggest that TopBP1 may act as a co-activator of Ets1, and that the co-activation potential of SPBP is significantly enhanced by the presence of TopBP1.

To confirm that the induction of the MMP3 promoter was via Ets1, the tandem EBS in the MMP3 promoter (Figure 4A) were mutated and the mutant promoter tested in reporter gene assays. As shown in Figure 5, mutation of the tandem EBS abolished both Ets1 activation of the MMP3 promoter and the stimulatory effect of SPBP or TopBP1 on this promoter. When all three proteins were co-expressed, the mutated promoter was induced but to a much lower extent than the wild-type promoter. This residual induction is very likely due to activation via weaker Ets1 responsive sites at position −92/−73, −344/−355 and −72/−48 (32). Hence, the SPBP- and TopBP1-mediated stimulatory effect on the MMP3 promoter is mainly dependent on Ets1 binding to the tandem EBS.

Lowering the level of SPBP reduces phorbol ester induced MMP3 secretion in IMR90 fibroblasts

Recently it was reported that down-regulation of Ets1 in HIG-82 cells reduced the phorbol ester-induced expression from the MMP3 promoter (31). To further characterize the effect of SPBP on Ets1-mediated stimulation of the MMP3 promoter, we determined the impact of SPBP down-regulation on phorbol ester-induced MMP3 secretion in the human fibroblast cell line IMR90. The IMR90 cells were transfected with SPBP siRNA or control siRNA, serum starved and stimulated with phorbol ester before performing western blot on the cell medium to determine the level of secreted MMP3 (Figure 6A). The level of secreted MMP3 was normalized to the level of actin of harvested cells (Figure 6B). Effective knockdown of SPBP was monitored by immunoblotting (insert in Figure 6B). Strikingly, siRNA-mediated knockdown of SPBP lead to a 50% reduction in the level of secreted MMP3. These results clearly demonstrate the importance of endogenous SPBP for Ets1-mediated activation of MMP3 expression in IMR90 cells.

The related ePHD domain-containing protein RAI1 does not enhance Ets1 transcription or interact with TopBP1

RAI1 (retinoic acid-induced 1) is a nuclear retinoic acid-induced protein containing seven short regions with strong homology to SPBP (denoted A–F in Figure 1A) (1,33,34). Importantly, RAI1 also possesses a C-terminal ePHD domain, an N-terminal transactivation domain and three nuclear localisation signals (35,36). The gene organization of SPBP and RAI1 is remarkably similar suggesting that they represent duplicated genes with a common ancestor (36). RAI1 is implicated in Smith–Magenis syndrome, a syndrome with multiple congenital anomalies and mild mental retardation (35,36). The ePHD domain of RAI1 is organized similarly to the SPBP ePHD domain with an extended loop region between the two most N-terminal zinc-ligands and the conserved region containing the PHD fingers. We decided to test whether RAI1 had the ability to enhance Ets1-mediated transactivation of the MMP3 promoter similar to SPBP. However, comparative reporter gene assays in HeLa cells showed that RAI1 neither stimulated Ets1-mediated transcriptional activation of the MMP3 promoter nor co-operated with TopBP1 (Figure 7A and B). On the contrary, RAI1 repressed Ets1-mediated transcriptional activation. Next, we asked whether the ePHD domain of RAI1 had the ability to interact with TopBP1. Interestingly, a yeast two-hybrid assay revealed that RAI1 has very low affinity for TopBP1 compared to the SPBP ePHD domain (Figure 7D). This was confirmed by GST pull-down assays (data not shown). In contrast to RAI1, the ePHD domains from the trithorax group proteins MLL, MLL2 and -4 can bind to TopBP1 suggesting that this is a more general feature of some ePHD domains. However, the RING finger protein hRNF4, known to interact with SPBP and carrying a zinc-finger domain related to the ePHD domain (3), does not bind to TopBP1 (Figure 7D).

The ePHD domain of SPBP and the BRCT6 domain of TopBP1 are required for their ability to co-operate in stimulating Ets1-mediated transcription

As described above the interaction between SPBP and TopBP1 is mainly mediated by the ePHD domain of SPBP and the BRCT6 domain of TopBP1. To determine the importance of these regions for their ability to co-activate Ets1-mediated transcription, a C-terminal deletion mutant of SPBP lacking the ePHD domain, SPBP(ΔePHD), and a TopBP1 deletion mutant lacking BRCT6, TopBP1(ΔBRCT6), were tested in reporter gene assays

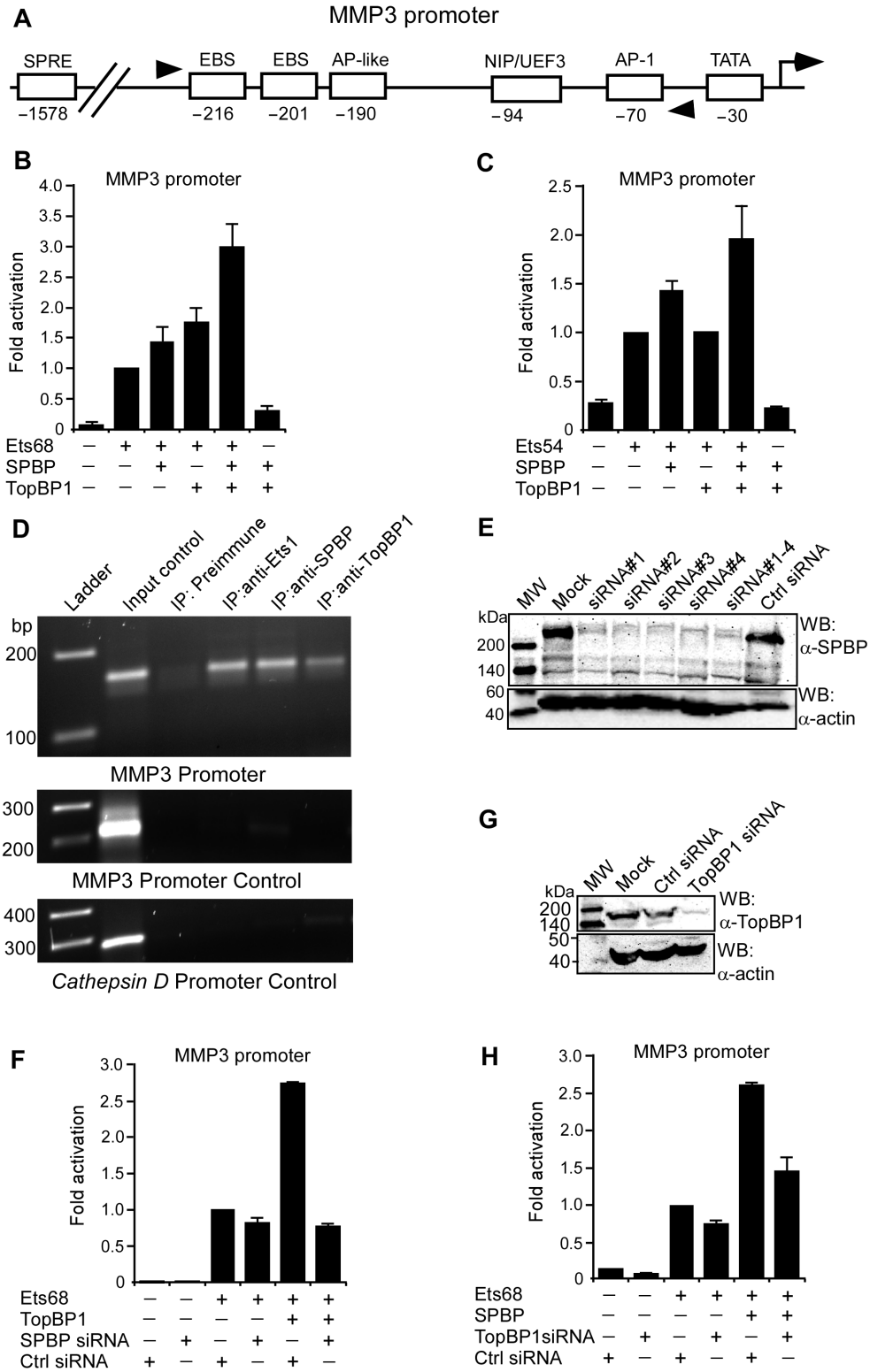

Figure 4. SPBP and TopBP1 co-operate to enhance Ets1-mediated transcriptional activation of the MMP3 promoter. (**A**) Schematic representation of the human MMP3 promoter. Arrowheads indicate the position of forward and reverse primers (−231 and −65, respectively) used for ChIP. EBS: Ets1-binding sites, NIP/UEF3: nuclear inhibitory protein/urokinase-type plasminogen activator enhancer factor 3, SPRE: Stromelysin-1 PDGF-responsive element. (**B**) SPBP and TopBP1 co-operate to enhance Ets68-mediated activation of the MMP3 promoter. Transient transfections were carried out in HeLa cells as described in Figure 3A except that the reporter plasmid contains 1448 bp of the human MMP3 promoter in front of the luciferase gene. The data represent the mean of four independent experiments, each performed in triplicate. (**C**) SPBP and TopBP1 co-operate to enhance Ets54-mediated transcriptional activation of the MMP3 promoter. Transient transfections were carried out in HeLa cells as described in (B) except that an expression vector for murine Ets54 (27) was used instead of the Ets68 expression vector. The data represent the mean of

using the MMP3 promoter as read-out. Figure 8A shows that both SPBP(ΔePHD) and TopBP1(ΔBRCT6) enhanced Ets1-mediated transcriptional activity similar to the full-length proteins. However, SPBP(ΔePHD) co-operated less efficiently with TopBP1 than full-length SPBP did. Likewise, TopBP1(ΔBRCT6) co-operated less efficiently with SPBP than full-length TopBP1 did. Also, when deletion mutants of both SPBP and TopBP1 are over-expressed together with Ets1, co-operation between TopBP1 and SPBP is completely abolished, leaving only the additive effect. Western blots and immunostaining showed that the deletion mutants are expressed at similar levels as the full-length proteins (Figure 8B) and are present in the cell-nucleus (data not shown). These results indicate that the co-operation between SPBP and TopBP1 is dependent on interactions involving the ePHD domain of SPBP and the BRCT6 domain of TopBP1. The weak co-operation displayed when only the ePHD domain or only the BRCT6 domain was deleted may either be due to (i) the region encompassing amino acids 1333–1666 of SPBP and BRCT7 + 8 of TopBP1 or (ii) the fact that both TopBP1(20) and SPBP(data not shown) have the ability to oligomerize. Hence, the weak effect may be caused by endogenous proteins recruited to the promoters by interaction with the deletion mutants.

Phosphorylation-induced oligomerization of TopBP1 is required for co-operation with SPBP

Recently it was reported that serine 1159 of TopBP1 is an Akt/PKB substrate (20). Phosphorylation of S1159 induces oligomerization of TopBP1, and this oligomerization is specifically required for its interaction with the transcription factors E2F1, Miz-1 and HPV E2. Liu *et al.* (20) suggested that TopBP1 oligomerization could be a general requirement for its involvement in transcriptional regulation. To test whether TopBP1 oligomerization is required for co-operation with SPBP to enhance Ets1 activity, serine 1159 of TopBP1 was mutated to alanine and co-expressed with Ets1 or Ets1 and SPBP in HeLa cells. Reporter gene assays using the MMP3 promoter revealed that TopBP1(S1159A) had a reduced ability to enhance Ets1-mediated transcriptional activity and to co-operate with SPBP (Figure 8C). To further verify that this S1159 phosphorylation site is important phosphorylation at this site was mimicked by expressing a TopBP1(S1159D) mutant. TopBP1(S1159D) displayed an even stronger enhancement of Ets1 activation together with SPBP than wild-type TopBP1 (Figure 8C). These results strongly suggest that co-operation between SPBP and TopBP1 to stimulate Ets1-mediated transactivation is dependent on phosphorylation-induced oligomerization of TopBP1.

DISCUSSION

In this study, we present evidence for physical and functional interactions between SPBP and TopBP1. The physical interaction is mainly mediated by the ePHD domain of SPBP and the BRCT6 domain of TopBP1. There is also a weaker interaction between BRCT6 of TopBP1 and a region encompassing amino acids 1333–1666 of SPBP. Similarly, the BRCT domains 7 and -8 of TopBP1 could also interact weakly with the ePHD of SPBP. Upon induction of DNA double-strand breaks, TopBP1 is recruited to sites of damage where it plays an important role as an adaptor protein to facilitate phosphorylation of Chk1 by the ATR kinase (11,37). However, in contrast to TopBP1, SPBP is not recruited to sites of DNA double-strand breaks (data not shown). Hence, we have so far no evidence for a role of SPBP in DNA damage responses, but this issue deserves further investigation. Here we decided to focus on functional studies of the SPBP-TopBP1 interaction in gene regulation since both SPBP and TopBP1 are implicated in transcriptional regulation (1,17,18,20,21,38). Reporter gene assays using two different Ets1-regulated promoters demonstrated that TopBP1 and SPBP co-operate to enhance Ets1-mediated transcriptional activation. This co-operation was dependent on their interaction domains (the ePHD domain of SPBP and the BRCT6 domain of TopBP1) in addition to phosphorylation of serine 1159 of TopBP1 (20). ChIP showed that both TopBP1 and SPBP are associated with these Ets1-regulated promoters, and siRNA-mediated knockdown of SPBP in a fibroblast cell-line revealed that the endogenous levels of SPBP affected the expression of the Ets1-regulated matrix metalloproteinase MMP3. Mutation of the tandem EBS in the MMP3 promoter demonstrated that the stimulatory effect

two independent experiments, each performed in triplicate. (**D**) Chromatin immunoprecipitation assays show that Ets1, SPBP and TopBP1 are associated with the MMP3 promoter. Extracts from U2OS-GFP-SPBP cells (1.5×10^7 cells per antibody) were immunoprecipitated with preimmune serum (IgG), polyclonal anti-Ets1 antibody, polyclonal anti-SPBP antibody and polyclonal anti-TopBP1 antibody. Input control (1:50) was included (lane 2). PCR analyses on the immunoprecipitated chromatin were carried out using primers aligning at position −231 and −65 of the MMP3 promoter. Control PCR analysis was carried out using primers aligning to positions −3537 and −3326 of the MMP3 promoter, and positions −3351 and −3069 of the cathepsin D promoter. The 1 kb DNA ladder is shown to the left. (**E**) Western blot showing knockdown of SPBP in HeLa cells using four different siRNA oligos. Transient transfections were carried out using siRNA oligos 1–4 alone and mixed (50 nM) as indicated. Cell lysates were separated by SDS–PAGE (6%), and immunoblotted using the anti-SPBP antibody (upper panel). The blots were re-probed with an anti-β-actin antibody in order to determine the amount of loaded protein (lower panel). (**F**) Down-regulation of SPBP using siRNA decreased the transcriptional activation of MMP3 promoter mediated by either Ets1 alone or Ets1 and TopBP1 together. Transient transfections were carried out in HeLa cells using the pGL3-MMP3 reporter plasmid (30 ng), together with the expression plasmids for Ets68 (10 ng) and HA-TopBP1 (50 ng), and a SPBP-specific siRNA (50 nM) or scrambled siRNA as indicated. Cells were harvested 40 h post-transfection. The data represent the mean of three independent experiments performed in triplicate. (**G**) Western blot showing siRNA-mediated knockdown of TopBP1 in HeLa cells. Transient transfections were carried out using 50 nM TopBP1 siRNA or 50 nM Control siRNA. Cell lysates were separated by SDS–PAGE (6%), and immunoblotted using an anti-TopBP1 antibody (upper panel). β-actin was used as loading control (lower panel). (**H**) Down-regulation of TopBP1 using siRNA decreased the transcriptional activation of the MMP3 promoter mediated by either Ets1 alone or Ets1 and SPBP together. Transient transfections were carried out as described in (F) except that an expression plasmid for SPBP was used instead of the TopBP1 expression plasmid, and TopBP1 specific siRNA was used instead of the SPBP siRNA. The data represent the mean of two independent experiments performed in triplicate.

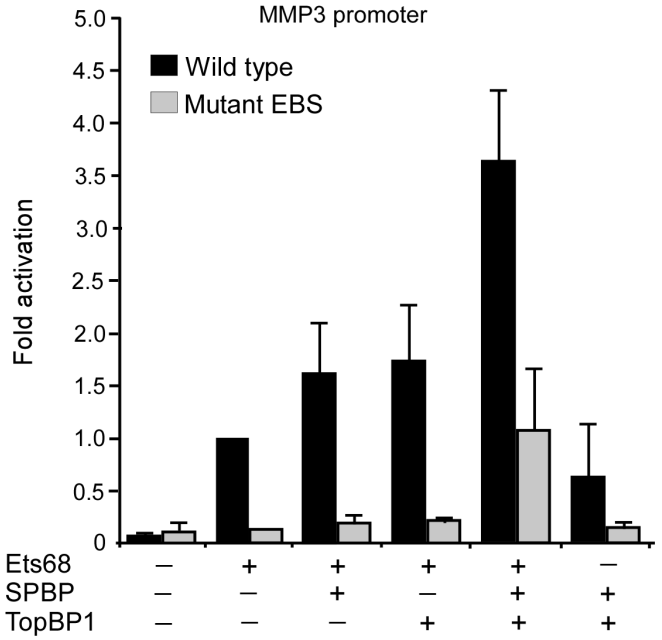

Figure 5. The stimulation of Ets1 activation of the MMP3 promoter by SPBP and TopBP1 is dependent on the tandem Ets1-binding sites. Transient transfections were carried out in HeLa cells as described in Figure 3A except that two different luciferase reporter gene plasmids were used, one containing the wild-type human MMP3 promoter (1448 bp upstream sequence), and one containing the human MMP3 promoter with the tandem Ets1-binding sites mutated. The data represent the mean of two independent experiments, each performed in triplicate.

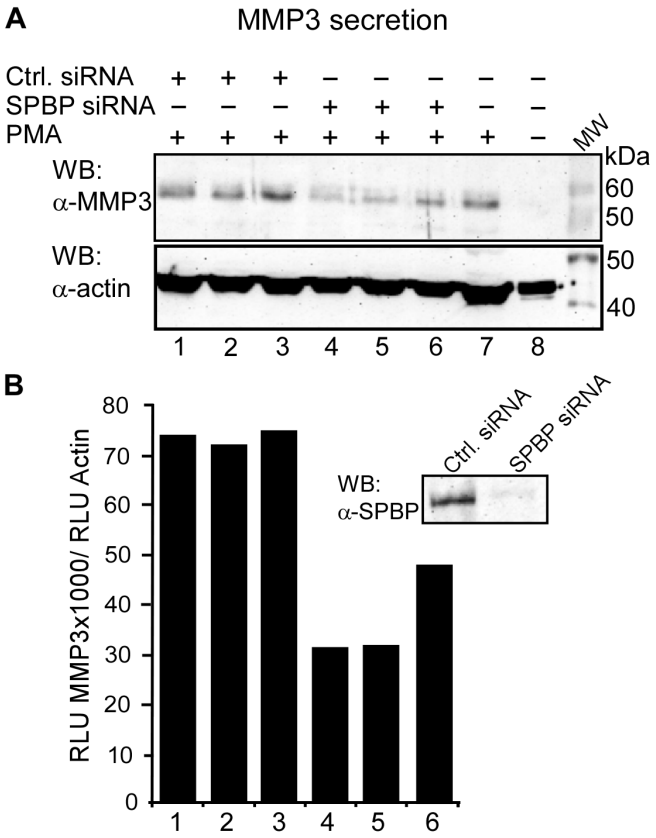

Figure 6. The level of endogenous SPBP affects the level of phorbol ester-induced MMP3 secretion. (**A**) A western blot showing the effect of down-regulation of endogenous SPBP on the level of secreted MMP3 induced by PMA in IMR90 fibroblasts. Cells were starved in MEM with 0.1% FBS for 24 h before being transfected two following days with SPBP siRNA (50 nM) or scrambled siRNA (50 nM). The cells were starved in MEM without serum for 24 h post-transfection before stimulation with MEM containing 1 ng/ml PMA for 20 h. Medium samples were analysed by SDS–PAGE (7.5%) and immunoblotting with an MMP3 antibody. Medium from three parallel wells per siRNA are shown (lanes 1–3 and 4–6, respectively) in addition to untreated cells showing the induction of MMP3 in the presence of PMA (compare lanes 7 and 8). The levels of β-actin in the cells from which the medium were harvested are shown in the lower panel. The cells in each well were harvested in 30 µl 2× SDS loading buffer and the extract separated by SDS–PAGE (10%). (**B**) Knockdown of endogenous SPBP reduces the MMP3 secretion in PMA-stimulated IMR90 cells by 50%. The amount of secreted MMP3 was normalized to the level of β-actin expression in each well. Quantification was performed using the LumiAnalyst software (Roche). A western blot of SPBP expression in total cellular extract from IMR90 cells transfected with scrambled siRNA and SPBP siRNA are shown in the inserted panel.

of SPBP and TopBP1 is mediated via Ets1 binding to these sites.

The interaction between SPBP and TopBP1 shows similarity to the E2F1–TopBP1 interaction (20). The interaction is dependent on the BRCT6 domain of TopBP1 and on oligomerization of TopBP1 induced by phosphorylation on serine 1159 (20). Liu *et al.* (20) suggested that oligomerization of TopBP1 was specifically required for TopBP1 to interact with transcription factors. Our results are in line with this hypothesis. BRCT domains have been identified as phosphopeptide-binding modules (39–41). BRCT domain 6 of TopBP1 binds preferentially to an E2F1 peptide phosphorylated at a serine residue, rather than the unphosphorylated counterpart (41). The ePHD domain of SPBP has several potential serine-, threonine- and tyrosine phosphorylation sites as predicted by the NetPhos 2.0-(42) and ScanSite (43) servers. However, phosphorylation of the ePHD domain of SPBP is not essential for binding to TopBP1 since an *E. coli*-produced GST fusion of the ePHD domain is capable of interacting with *in vitro*-translated TopBP1.

TopBP1 is reported to act both as a repressor and as an activator of transcriptional activity (16–18,21). Here we present evidence that TopBP1 has a stimulatory effect on Ets1 transcriptional activity of the *c-myc* P1P2 promoter and the MMP3 promoter. Co-overexpression of TopBP1 and SPBP resulted in a more than additive enhancement of transcriptional activation mediated by Ets1 on both these promoters. Interestingly, preliminary results suggest that SPBP and TopBP1 can also co-operate to stimulate c-Jun transcriptional activity (data not shown). Thus, their co-operation in activating transcription is most likely not limited to Ets1. The stimulatory effect of SPBP and TopBP1 is significantly reduced when the level of endogenous SPBP or endogenous TopBP1 is knocked down by specific siRNAs. SPBP is reported to bind to the SPRE-element at position −1578 of the MMP3 promoter and stimulate its transcriptional activity (Figure 4A) (2).

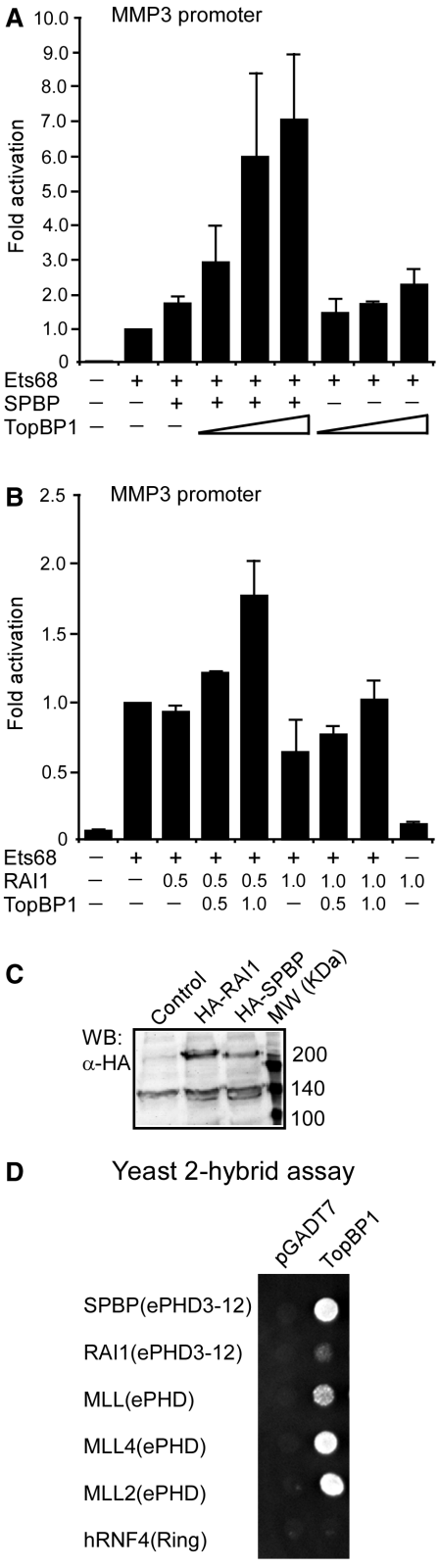

Figure 7. The SPBP homologue RAI1 does not stimulate Ets1-mediated transcriptional activation of the MMP3 promoter or co-operate with TopBP1. (**A**) TopBP1 potentiates the SPBP-enhanced Ets1 transactivation in a dose-dependent manner. Transient transfections were carried out in HeLa cells as described in Figure 4 except that 0.5 μg of the SPBP expression plasmid and increasing amounts of TopBP1 expression plasmids (0.5, 1.0 and 1.5 μg, respectively) were used. The data represent the mean of two individual experiments performed in triplicate. (**B**) RAI1 does not enhance Ets1 transactivation or act together with TopBP1 to enhance Ets1-mediated transcription. Transient transfections were carried out in HeLa cells as described in (A) except that 0.5 μg or 1.0 μg of RAI1 expression plasmid was used instead of the SPBP expressing plasmid. The data represent the mean of two individual experiments performed in triplicate. (**C**) Western blot documenting similar expression levels of HA-SPBP and HA-RAI1 in HeLa cells. Total cellular extracts from HeLa cells transfected with expression plasmids for HA-RAI1 or HA-SPBP as indicated were analysed by 6% SDS–PAGE and immunoblotted using an anti-HA antibody. (**D**) Yeast two-hybrid assay showing that the ePHD domain of RAI1 interacts very weakly with TopBP1 compared to the SPBP ePHD domain, and the ePHD domains of MML protein family. One colony containing AD or DBD plasmids was picked and re-suspended 200 μl dH_2O. Ten microlitres of the cell suspension was spotted on QDO medium and allowed to grow at 30°C for 3 days.

However, the stimulatory effect of SPBP reported here is not dependent on this SPRE element since: (i) mutation of the tandem EBS in the MMP3 promoter inhibited the stimulatory effect of SPBP; (ii) the MMP3 promoter construct used in this study (containing 1448 bp of the MMP3 upstream region) does not include the SPRE element; (iii) a MMP3 promoter construct containing the SPRE site (2298 bp of the MMP3 upstream region) gave similar results as the 1448 bp construct (data not shown) and (iv) no such SPRE element has been identified in the *c-myc* P1P2 promoter.

We found that SPBP, TopBP1 and Ets1 are all associated with a short fragment of the MMP3 promoter, and that SPBP and TopBP1 interacts *in vivo*. However, we have not been able to co-immunoprecipitate endogenous Ets1 and SPBP, or endogenous Ets1 and TopBP1, suggesting that any physical interactions between Ets1 and these two proteins are weak and/or may be dependent on an unknown factor. Hence, the mechanism for SPBP and TopBP1-mediated stimulation of Ets1 has to be elucidated. At least three mechanistic models can be suggested. First, SPBP contains a DNA-binding domain with an AT-hook motif which binds sequence specifically to AT-rich DNA-binding motifs (1). The EBS in the MMP3-promoter and the P1P2 *c-myc* promoter are flanked with AT-rich sequences. Hence, SPBP may recognize these sequences, and when Ets1 is bound at a proximal site, the binding of both proteins is stabilized. Other transcription factors have been shown to modulate the DNA-binding affinity of Ets1, and thereby regulate its transcriptional activity (44). The Ets1–SPBP–DNA complex may recruit oligomerized TopBP1 to the promoter via the ePHD domain of SPBP. Second, PHD domains are known to contain nucleosome-binding activity (7), specifically recognizing methylated histone tails (8). Hence, SPBP may be recruited to the promoter sites via its ePHD domain. The ePHD domain which also contains protein-binding activity (7), may recruit or stabilize Ets1 and TopBP1 at the promoters. Third, SPBP may relocalize TopBP1 from repressor complexes. It is shown that E2F1 and Ets1 compete for the same binding site in the P1P2 *c-myc* promoter (45,46). E2F1 represses expression from the *c-myc* promoter (45), while Ets1 act as an activator (46,47). TopBP1 binds to E2F1 and repress its activity (16). Thus, SPBP associated with Ets1 may recruit TopBP1 from the repressive E2F1–TopBP1 complexes to

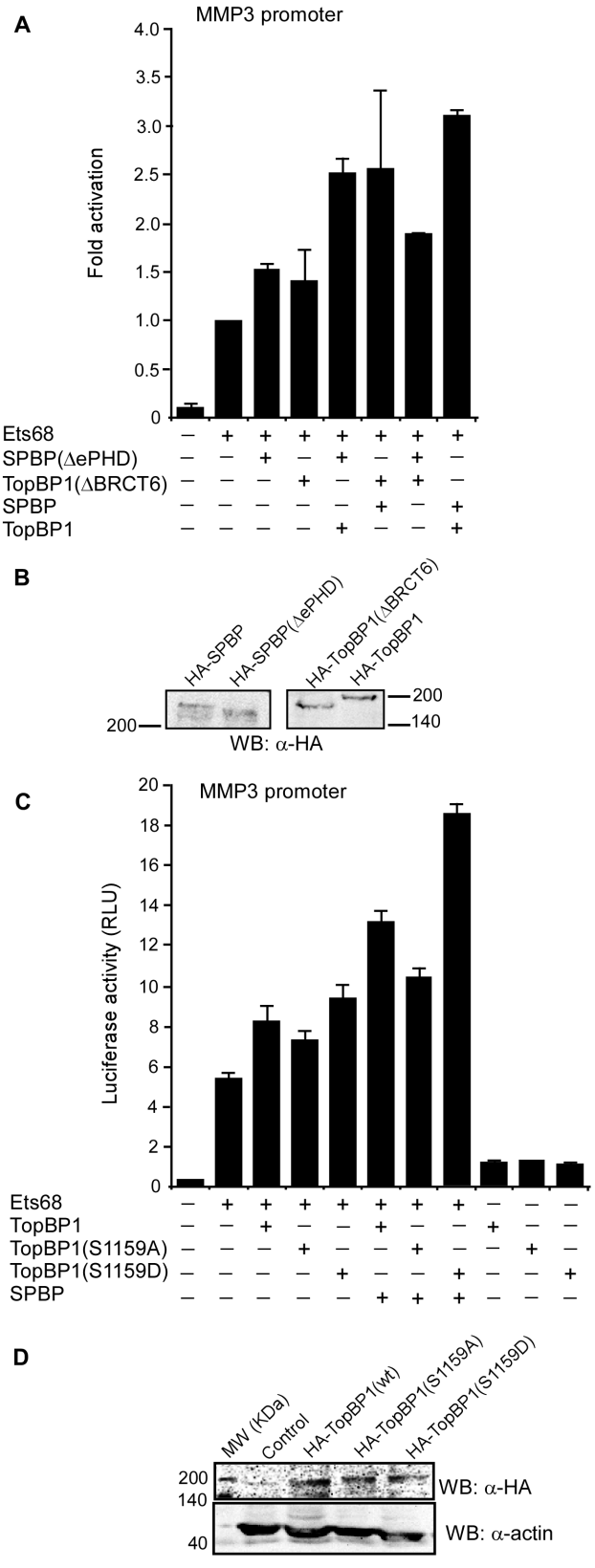

Figure 8. The co-operation between SPBP and TopBP1 is dependent on the interaction between the ePHD- and BRCT6 domains and on TopBP1 oligomerization. (**A**) Deletion of the SPBP ePHD domain or the BRCT6 domain of TopBP1 abolishes their ability to co-operate to enhance Ets1 transactivation. Transient transfections were carried out in HeLa cells using the pGL3-MMP3 reporter (0.5 μg), together with expression vectors for Ets68 (100 ng), HA-SPBP (1 μg), HA-SPBP(ΔePHD) (1 μg), HA-TopBP1 (1 μg) and/or HA-TopBP1(ΔBRCT6) (1 μg) as indicated. Cells were harvested 20 h post-transfection. The data represent the mean of four independent experiments, each performed in triplicate. (**B**) Western blot showing similar expression levels of the full-length- and deletion constructs of SPBP and TopBP1. Total cellular extracts from HeLa cells transfected with expression plasmids for HA-SPBP, HA-SPBP(ΔePHD), HA-TopBP1 or HA-TopBP1(ΔBRCT6) as indicated were analysed by 6% SDS–PAGE and immunoblotted using an anti-HA antibody. (**C**) TopBP1-mediated enhancement of Ets1 transcriptional activity and the co-operation with SPBP is dependent on phosphorylation and oligomerization of TopBP1. HeLa cells were transfected with the pGL3-MMP3 reporter (0.5 μg), expression vectors for Ets68 (100 ng), HA-TopBP1(S1159A) (1 μg), HA-TopBP1(S1159D) (1 μg), HA-TopBP1(1 μg) and/or HA-SPBP(1 μg) as indicated. Cells were harvested 20 h post-transfection. The mean luciferase activities obtained in a representative experiment performed in triplicate is shown. (**D**) Western blot showing similar expression levels of wild-type and mutated HA-TopBP1. Anti-HA immunoblot performed as in (B).

the stimulatory co-operation of Ets1, SPBP and TopBP1, and thereby switch the regulation of the P1P2 *c-myc* promoter from off to on. It is reported that Akt/PKB induces expression of *c-myc* (48). This is in line with our results showing that stimulation of the P1P2 *c-myc* promoter by Ets1, SPBP and TopBP1 is dependent on the S1159 phosphorylation site of TopBP1 shown to be an Akt/PKB substrate (20).

SPBP is found to be 4- to 6-fold up-regulated in arthrinogenic synovial fibroblasts (49), which are the cells mainly responsible for hyperplasia and chronic inflammation in rheumatoid arthritis. Activated fibroblast-like synoviocytes (FLS) are characterized by an up-regulated expression of adhesion molecules, chemokines, proinflammatory molecules, oncogenes and matrix metalloproteinases (50). Interestingly, both *c-myc* (51) and MMP3 (52) are shown to be extensively expressed in FLSs. Hence, SPBP expression correlates well with *c-myc* and MMP3 expression, and also with the invasive properties of the FLSs. Furthermore, two recent papers show that MMP3 expression is induced via the PI3K/Akt pathway in fibroblasts (53) and microglia (54). These reports correspond well with our results showing that the more than additive enhancement of the MMP3 promoter by SPBP and TopBP1 is abolished if a S1159A mutant of TopBP1 is co-expressed with SPBP. FLS exhibit characteristics of metastatic cells, such as decreased adhesion, increased proliferation and migration. Interestingly, SPBP expression is shown to be up-regulated in small-cell lung cancer and advanced adenocarcinoma cells from patients receiving extensive chemotherapy treatment (55) and in desmoid tumours (56). Hence, together with our data these results suggest that a co-operation between SPBP and TopBP1 may contribute to the metastatic process by up-regulating c-*myc* and MMP3 expression.

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