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Journal of Cellular Biochemistry 113:1744–1753 (2012)

Journal of Cellular Biochemistry

Proteomic Identification of PSF and p54(nrb) as TopBP1-Interacting Proteins

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ABSTRACT

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 damage signalling. To further dissect its biological functions, we explored TopBP1-interacting proteins by co-immunoprecipitation assays and LC-ESI-MS-analyses. As TopBP1 binding partners we identified p54(nrb) and PSF, and confirmed the physical interactions by GST pull-down assays, co-immunoprecipitations and by yeast two-hybrid experiments. Recent evidence shows an involvement of p54(nrb) and PSF in DNA double-strand break repair (DSB) and radioresistance. To get a first picture of the physiological significance of the interaction of TopBP1 with p54(nrb) and PSF we investigated in real time the spatiotemporal behaviour of the three proteins after laser microirradiation of living cells. Localisation of TopBP1 at damage sites was noticed as early as 5 s following damage induction, whereas p54(nrb) and PSF localised there after 20 s. Both p54(nrb) and PSF disappeared after 200 s while TopBP1 was retained at damage sites significantly longer suggesting different functions of the proteins during DSB recognition and repair. J. Cell. Biochem. 113: 1744–1753, 2012.

KEY WORDS: p54(NRB)/PSF; DOUBLE STRAND BREAK; REPAIR; TopBP1; RECOGNITION

TopBP1 is a BRCT domain-rich protein that is structurally and functionally conserved throughout eukaryotic organisms. Though TopBP1 was initially identified as a DNA topoisomerase IIβ-binding protein (hence its name) [Yamane et al., 1997], its involvement in a number of cellular processes such as replication, transcription and DNA damage response was soon established [Mäkiniemi et al., 2001]. Moreover, very recent investigations of a conditional TopBP1 knockout mouse showed additional roles of the protein in cell proliferation and the maintenance of chromosomal integrity [Jeon et al., 2011]. The initiation of DNA replication requires the assembly of multiple protein complexes at the origins of replication. We and others have shown that TopBP1 is essential for chromatin loading of the MCM helicase activator Cdc45 at replication origins in metazoan cells [Van Hatten et al., 2002; Schmidt et al., 2008; Chowdhury et al., 2010] and hence for the formation of the pre-initiation complex. Human TopBP1 has nine BRCT domains, that originally were identified in a number of proteins involved in DNA repair and the cell cycle checkpoint [Caldecott, 2003; Rappas et al., 2011]. In response to UV-caused DNA damage, several checkpoint proteins, including Rad17, the

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: SFB 604/B02; Grant sponsor: Leibniz Institute for Natural Product Research and Infection Biology (Hans Knoell Institute).

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Received 22 July 2011; Accepted 21 December 2011 • DOI 10.1002/jcb.24045 • © 2011 Wiley Periodicals, Inc.
Published online 28 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

Rad9-Rad1-Hus1 complex, and the ATR/Atrip complex are recruited to RPA-coated single-stranded DNA [Zou et al., 2002, 2003], which leads to the subsequent phosphorylation of Chk1 by ATR [Zhao and Piwnica-Worms, 2001]. It was reported that TopBP1 plays a crucial role in the ATR signalling pathway by physically interacting with ATR, which dramatically enhanced the ATR kinase activity [Kumagai et al., 2006]. However, the important question of how TopBP1 recognises damaged DNA remained unresolved, since TopBP1 localises to the sites of DNA damage independently of ATR [Morishima et al., 2007]. Despite the fact that BRCT regions located in the N- and C-terminal halves of TopBP1 can bind in vitro to DNA with bulky base lesions [Choi et al., 2009] it is generally believed that TopBP1 interacts with proteins that sense DNA damage rather than directly participating in DNA damage detection [Garcia et al., 2005]. In support of this assumption we recently showed that TopBP1 interacts with PARP-1, an abundant nuclear protein involved in the DNA-base-excision-repair [Wollmann et al., 2007]. Another example of TopBP1 interactions with a DNA damage recognising protein is the Nijmegen breakage syndrome protein 1 (NBS1) [Morishima et al., 2007]. Therefore, there may exist a set of further proteins that interact with TopBP1 and mediate its recruitment to sites of DNA damage.

The polypyrimidine tract-binding-protein associated splicing factor (PSF) and the 54 kDa nuclear RNA binding protein [p54(nrb)] are members of a subfamily of RNA recognition motif (RRM) proteins defined by tandem RRMs flanked by an additional region of homology. PSF was initially regarded as a splicing factor [Patton et al., 1993] while p54(nrb) was thought to participate in transcriptional regulation [Sever et al., 2002]. Recent evidence shows that these proteins, which form a stable complex in vivo, exhibit multi-functional characteristics in a variety of nuclear processes [Shav-Tal and Zipori, 2002]. Both proteins are involved in DNA double-strand break (DSB) repair via nonhomologous end joining (NHEJ) as well as homologous recombination (HR) [Li et al., 2009; Salton et al., 2010]. Cells treated with siRNA against p54(nrb) expression exhibited a delay in DSB repair and showed increased radiosensitivity [Li et al., 2009]. Interestingly, downregulation of TopBP1 in mammalian cells using antisense oligonucleotides or siRNAs generated a very similar phenotype [Yamane and Tsuruo, 1999]. In line with these results TopBP1-ablated mouse cells from the TopBP1 knockout mice exhibited phosphorylation of H2AX and Chk2, indicating that the cells accumulate DNA breaks [Jeon et al., 2011].

In this study, we demonstrate that TopBP1 interacts with p54(nrb) and PSF both in vitro and in vivo. The proline/glutamin (P/Q)-rich N-terminal domain of PSF and the two RRMs of p54(nrb) are responsible for the interaction with TopBP1. All three proteins migrate to sites of DNA damage induced by a laser microbeam but display different retention times indicating different functions during DSB recognition and repair.

MATERIALS AND METHODS

CELL LINES

HEK293T (ATCC-CRL11268) cells were grown as monolayers in RPMI medium with L-glutamine (PAA) supplemented with 10% heat-inactivated foetal calf serum (FCS). Ls174T colon adenocarcinoma cells (ATCC-CL-188) were grown in RPMI-1640 with Glutamax I^{TM}, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 mg/ml streptomycin. U-2 OS cells (ATCC-HTB-96) were grown in DMEM, supplemented with 10% heat-inactivated FCS. All cell types were maintained at 37°C in an atmosphere of 5% CO_2.

MAMMALIAN EXPRESSION CONSTRUCTS AND TRANSFECTION

A full-length TopBP1 expression construct was kindly provided by J. Chen (University of Texas, Houston, USA). DNA insert corresponding to amino acids 1–473 of human p54(nrb) was amplified by PCR using the following primers: forward-5′-GCGAATTCACCATGCAGAGCAATAAAGCCTTTAACTTGGAG-3′, reversed-3′-GCGCTCGAGGTATCGGCGACCGTTTGTTTGG-3′, and plasmid pCR3.1HA-p54(nrb) (obtained from P.W. Tucker, University of Texas, Austin, USA) as a template. The PCR fragment was digested with *Eco*RI–*Xho*I and transferred into pcDNA3.1/myc-HisA (Invitrogen). To obtain EGFP- and DsRed2-p54(nrb) fusion constructs the same p54(nrb) DNA insert was amplified by PCR using the following primers: forward-5′-GCGAATTCTATGCAGAGCAATAAAGCCTTTAACTTGGAG-3′, the reversed primer above, and again pCR3.1HA-p54(nrb) as template. The *Eco*RI–*Xho*I-digested PCR fragment was transferred into the pEGFP-C1 and pDsRed2-C1 vectors (CLONTECH). To assemble the pDsRed2-p54(nrb) (aa 228–473) deletion construct PCR was performed using the following primers: forward-5′-GCGAATTCTCCTCCAAAAAAGAAGAGAAAGGTAGCTGACCAGTTAGATGATGAAGAG-3′, reversed-5′-GCGGTCGACTTAGTATCGGCGACGTTTGTT-3′, and the same template as above. The forward primer contains a nuclear localisation signal to ensure the import of the fusion protein into the nucleus. The *Eco*RI–*Xho*I-digested PCR fragment was cloned into the pDsRed2-C1 vector. The EGFP-PSF expression plasmid was kindly provided by J.G. Patton (Vanderbilt University, Nashville, Tennessee, USA). To obtain the full-length TopBP1-EGFP expression construct, a TopBP1 DNA fragment was amplified by PCR using the primers: forward-5′-GCGAATTCGCACCACCAGCGATGTGTCCCAAG-3′, reversed-5′-GCGGATCCTTAGTGTACTCTAGGTCGTTTGA-3′, and the full-length TopBP1 cDNA construct KIAA 0259 (a gift from T. Nagase, Kazusa DNA Research Institute, Chiba, Japan) as template. The *Eco*RI–*Bam*HI-digested PCR fragment was cloned into the pEGFP-C1 vector. HEK293T and U-2 OS cells were transfected with the FuGENE® HD transfection reagent according to the manufacturer's instructions (Roche).

YEAST TWO-HYBRID ASSAY

The full-length p54(nrb) DNA fragment (see above) was transferred into the GAL4 binding domain (BD) destination vector pGBKT7 (CLONTECH). DNA inserts corresponding to amino acids 1–780 of human TopBP1 (containing BRCT domains 0-5) and amino acids 534–1003 (BRCT domains 4–6) were amplified by PCR with the primers BRCT0-2for: 5′-GAACTTCCATATGTCCAGAAATGACAAAGAACCG-3′ and BRCT0-5rev: 5′-GCGGTCGACTGCAGTATCTGAATTTAGATT-3′ and BRCT4-5for and 6rev [Schmidt et al., 2008], correspondingly, and the full-length TopBP1 cDNA construct KIAA 0259 as template. The PCR fragments were accordingly digested

with *Nde*I–*Sal*I and *Eco*RI–*Xho*I and transferred into the GAL4 activation domain (AD) vector pGADT7 (CLONTECH). DNA inserts corresponding to BRCT 6, 7–8 and 6–8 were cut out from the previously described GST-fusion constructs [Wollmann et al., 2007; Schmidt et al., 2008] and introduced into pGADT7. For quantitative two-hybrid assays *Saccharomyces cerevisiae* Y190 (CLONTECH) was transformed with corresponding pGBKT7- and pGADT7-constructs. β-galactosidase liquid culture assays, using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate, were performed as described previously [Borth et al., 2010]. The β-galactosidase values represent mean numbers and standard deviations of three independent experiments.

Co-IMMUNOPRECIPITATION

Ls174T or HEK293T cells were harvested and lysed with 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100 containing a mixture of protease inhibitors (10 μg/ml leupeptin and aprotinin, 5 μg/ml pepstatin and 1 mM PMSF). Lysates were cleared by centrifugation at 11,000*g* at 4°C and either treated with 10 U of DNaseI for 15 min at 37°C in the presence of Mg^{2+} or not. A p54(nrb) monoclonal antibody (4 μg; BD Transduction Laboratories™), a TopBP1 monoclonal antibody (4 μg; BD Biosciences) or pre-immune serum were incubated with 20 μl (bed volume) of protein G-Sepharose (GE Healthcare) and 1% BSA in lysis buffer overnight at 4°C. Beads were incubated with the same buffer containing 1.5–3 mg pre-cleared lysate for 2 h at 4°C and with a constant rotation speed. After extensive washing with lysis buffer, co-immunoprecipitated proteins were eluted by boiling in SDS sample buffer at 95°C for 5 min.

IMMUNOBLOTTING

Co-immunoprecipitated proteins were resolved by sodium dodecyl sulphate (SDS)/10% polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Whatman). The membranes were blocked in 5% skim milk for 45 min, washed thrice with TBST and incubated with the primary antibodies: rabbit anti-p54(nrb) (1:200; Santa Cruz Biotechnology), rabbit anti-TopBP1 (1:200; Novus Biologicals) and rabbit anti-PSF (1:200; Santa Cruz Biotechnology) overnight at 4°C. After washing three times with TBST the membranes were incubated with horseradish peroxidase-conjugated goat anti-(rabbit-IgG) secondary antibody (Dianova) for 1 h at room temperature. Protein bands were detected with ECL Plus Western Blotting Detection Reagent (GE Healthcare) as described by the manufacturer.

GST PULL-DOWN

GST-tagged TopBP1 (aa1-1522) was kindly provided by W. C. Lin (University of Alabama, Birmingham, USA). GST fusion proteins were purified as formerly described [Borth et al., 2010]. [^{35}S]-methionine-labelled full-length p54(nrb) and PSF proteins were synthesised in vitro by coupled T7 RNA polymerase-mediated transcription and translation (IVT) in a reticulocyte lysate system as described by the manufacturer (Promega). L-[^{35}S]-methionine (specific activity: 1,175 Ci/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). pcDNA 3.1-PSF (kindly provided by H.H. Samuels, New York University School of Medicine, New York, USA), pGBKT7-p54(nrb) and the following partial constructs of p54(nrb) and PSF were used as templates in the IVT. DNA inserts corresponding to amino acids 75–473 and 228–473 of human p54(nrb) were amplified by PCR with the primers NONO1: 5′-GCGAATTCCGTAGCCGTCTCTTTGTGGGC-3′ and Nonrev: 5′-GCG-CTCGAGGTATCGGCGACGTTTGTTTGG-3′, and NONO2: 5′-GCGA-ATTCATGGACCAGTTAGATGATGAA-3′ and Nonrev and plasmid pCR3.1HA-p54(nrb) as template. The PCR fragments were digested with *Eco*RI–*Xho*I and transferred into the vector pGADT7 (CLONTECH). DNA inserts corresponding to amino acids 296–707 and 464–707 of human PSF were amplified by PCR with the primers PSF2: 5′-GGAATTCCCATATGTGTCGGTTGTTTGTTGGGAAT-3′ and PSFrev: 5′-GCCGTCGACCTAAAATCGGGGTTTTTTGTTTGGGCC-3′ and PSF1: 5′-GCGAATTCCAGAAGAATCCAATGTATCAA-3′ and PSFrev, correspondingly, and the EGFP-PSF plasmid as template. The PCR fragments were digested with *Nde*I–*Sal*I and *Eco*RI–*Sal*I, correspondingly, and transferred into the vector pGADT7 (CLONTECH). Glutathione–Sepharose beads (50 μl) liganded by either GST-tagged TopBP1 protein fragment or GST alone, were washed with HB-buffer (20 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, pH 7.4) and then resuspended in 50 μl HB-buffer containing 0.5% Igepal CA-630. To each sample, 10 μl [^{35}S]-methionine-labelled interaction partner protein(s) were added. After constantly rotating at 4°C for 2 h, the beads were washed extensively with HB-buffer. Bound proteins were eluted from the beads using SDS sample buffer. After separation by 10% SDS–PAGE, proteins were visualised by exposure to a KODAK X-OMAT AR film.

PROTEIN IDENTIFICATION

Proteins were identified by LC-ESI-MS/MS according to Baum et al. [2009]. Briefly, protein bands were excised from Coomassie-stained acryl amide gel and tryptic in-gel protein digests were analysed with an LC-ESI-MS equipment consisting of an Ettan micro LC-HPLC (GE Healthcare) and LTQ mass spectrometer (Thermo Scientific, Waltham, MA). For protein identification the Bio works 3.2 software (Thermo Scientific) and the NCBI human protein database were used.

INDUCTION OF DNA DAMAGE USING LASER MICROIRRADIATION

U-2 OS cells grown on chambered cover-slips (Nunc) were individually transfected with either EGFP-TopBP1, EGFP-p54(nrb) or EGFP-PSF. Alternatively, cells were co-transfected with EGFP-TopBP1 and DsRed2-p54(nrb), or EGFP-TopBP1 and DsRed2-p54(nrb) (aa 228-473). Laser microirradiation and live cell imaging were performed 24 h after transfection as described previously [Grigaravicius et al., 2009a,b]. Briefly, cells were damaged using a 350 nm UV-A laser (Nd:YLF; Spectra Physics) at a defined energy of 18 μJ and a repetition rate of 10 Hz for 10 s. Live cell imaging was done using a Zeiss laser scanning microscope (LSM 510) equipped with a 100×, NA 1.3 Plan Neofluar oil immersion objective (Zeiss). Images were collected at 3-s intervals.

RESULTS

TopBP1 INTERACTS WITH p54(NRB) AND PSF IN VIVO

Whole cell lysates of Ls174T colon adenocarcinoma cells were subjected to co-immunoprecipitation (CoIP) analyses using a

monoclonal anti-TopBP1 antibody. To remove unspecifically bound proteins, the protein G-Sepharose beads were extensively washed. As negative controls, the same procedure was repeated with beads only, and with pre-immune serum. The specifically bound proteins were eluted, resolved by 10% SDS–PAGE, and stained with Coomassie (Fig. 1A). Selected gel bands, which did not appear in the control lanes, were subjected to LC-ESI-MS/MS for protein identification. In total, 22 potential interaction partners of TopBP1 were found (Fig. 1B). Two of those, namely p54(nrb) and PSF, were already known to be involved in cellular processes comparable to TopBP1, such as DNA damage, checkpoint activation and transcription [Garcia et al., 2005; Li et al., 2009]. Therefore we focused our further analyses on the interaction of TopBP1 with p54(nrb) and PSF. To confirm the association of p54(nrb) and PSF with TopBP1 whole cell lysates of HEK293T cells were subjected to CoIP experiments using a polyclonal anti-p54(nrb) antibody. As expected the anti-p54(nrb) antibody but not a pre-immune serum co-precipitated both endogenous TopBP1 and PSF (Fig. 1C). To exclude that a contaminating DNA bridge is responsible for mediating the protein associations we co-transfected HEK293T cells with mammalian p54(nrb) and TopBP1 expression plasmids and performed CoIPs with anti-p54(nrb) antibodies in the presence of DNase I. As shown in Figure 1D also under this experimental conditions the anti-p54(nrb) antibody co-precipitated TopBP1 and endogenous PSF. We repeated the coIP experiments with lysates from UV-damaged cells but no increased interaction between p54(nrb), PSF and TopBP1 was found (Supplementary Fig. S1). Together, these data showed the ability of TopBP1 to associate with p54(nrb) and PSF in vivo in different cell lines.

FULL-LENGTH PROTEINS TopBP1, p54(NRB) AND PSF DIRECTLY INTERACT IN VITRO

To examine whether TopBP1, p54(nrb) and PSF interact in vitro we performed GST pull-down assays. Glutathione sepharose beads bound to GST-tagged full-length TopBP1 or GST alone (Fig. 2B) were incubated with either [^{35}S]-methionine-labelled full-length p54(nrb), with PSF or with both and processed as described in the Materials and Methods Section and analysed by autoradiography. As shown in Figure 2C,D, both full-length p54(nrb) and PSF alone or together specifically bound to the GST-TopBP1 full-length matrix, whereas no binding was detected using the GST control, suggesting a direct physical interaction of TopBP1 with p54(nrb) and PSF. We then performed deletion analyses to identify the TopBP1-interaction sites of p54(nrb) and PSF. For this purpose the GST pull-down experiments were repeated with [^{35}S]-methionine-labelled N-terminal deletion fragments of p54(nrb) and PSF. Deletion of the proline (P)-/glutamine (Q)-rich N-terminal region of PSF (aa 1–296, Fig. 2A) led to a complete loss of its interaction with TopBP1 (Fig. 2E, right panel). On the other hand, a p54(nrb) deletion fragment without the much shorter P/Q-rich domain (aa 1–75, Fig. 2A) still interacted with TopBP1 (Fig. 2E, left panel). But a further deletion of the two RRMs of p54(nrb) led to a complete loss of interaction with TopBP1 (Fig. 2E, left panel). p54(nrb) is highly homologous to the C-terminus of PSF, particularly to the two RRMs [Shav-Tal and Zipori, 2002]. Both RRMs of PSF were shown to be responsible for heterodimerisation with p54(nrb) [Dong et al., 2007]. This and our interaction studies suggest that TopBP1 directly interacts with p54(nrb) and PSF in vitro, where the P/Q-rich domain of PSF seems to permit the interaction with TopBP1 and the two RRMs of p54(nrb) promote heterodimerisation with TopBP1 and PSF.

p54(nrb) INTERACTS WITH BRCT DOMAINS 6–8 OF TopBP1 IN A YEAST TWO-HYBRID SYSTEM

In order to assess the protein domains of TopBP1 involved in interactions with p54(nrb), we employed yeast two-hybrid analyses. Full-length p54(nrb) was fused to the GAL4 BD and TopBP1 fragments covering the BRCT domains 0–5 (aa 1–780) and 6–8 (aa 793–1522) were fused to the GAL4 AD (Fig. 3). After transformation of the constructs into the *S. cerevisiae* reporter strain Y190, a β-galactosidase assay was performed. Significant transactivation was observed when the p54(nrb) construct was expressed in the presence of TopBP1 BRCT6-8, but not for TopBP1 BRCT0-5 (Fig. 3), indicating a physical interaction between p54(nrb) and the C-terminal BRCT6-8 domains of TopBP1. A further dissection of the p54(nrb) interacting region of TopBP1 using constructs harbouring only BRCT6 or BRCT7-8, resulted in a loss of p54(nrb) binding.

TopBP1, p54(NRB) AND PSF ARE RECRUITED TO SITES OF LASER-INDUCED DNA DAMAGE

Reduction of TopBP1, p54(nrb) or PSF expression after treatment with anti-sense oligonucleotides, siRNA or miRNA led to ultraviolet sensitive and less radioresistent cells [Yamane and Tsuruo, 1999; Li et al., 2009; Ha et al., 2011]. Moreover, for all three proteins an involvement in DSB recognition and/or repair was proposed [Garcia et al., 2005; Li et al., 2009; Salton et al., 2010]. To get a first picture of the physiological significance of the TopBP1 interaction with p54(nrb) and PSF we investigated the spatiotemporal behaviour of the three proteins in living cells after laser microirradiation. Laser microirradiation produces a variety of DNA lesions according to laser intensity and wavelength. In our study, in otherwise untreated cells DSBs were induced with a 350 nm UV-A laser. U-2 OS cells were transfected with EGFP-tagged constructs of either TopBP1, p54(nrb) or PSF and kinetic studies were performed to examine the association and dissociation of each of the proteins. As shown in Figure 4A recruitment of TopBP1 at damaged sites was noticed as early as 5 s following damage induction, whereas p54(nrb) and PSF clearly appeared later (20 s). On the other hand, both p54(nrb) and PSF disappeared after 200 s while TopBP1 was retained at damage sites significantly longer (at least 3 h, data not shown). Remarkably, all three proteins achieved their maximal fluorescence intensity at the points of damage almost at the same time (60 s) (Fig. 4B). This indicated an at least temporary co-localisation of all the three proteins at DNA lesions after UV-A treatment. EGFP and DsRed2 themselves did not accumulate at the damage site (Supplementary Fig. S2). To show more directly the co-localisation at sites of DNA damage we co-transfected U-2 OS cells with both EGFP-tagged TopBP1 and DsRed2-tagged p54(nrb) constructs and studied the behaviour of the proteins after irradiation. Prior to laser irradiation, fluorescence-tagged TopBP1 and p54(nrb) were equally distributed in the nucleoplasm, and to some extent, in discrete foci. The foci were more evident with p54(nrb) than with TopBP1. Sixty seconds

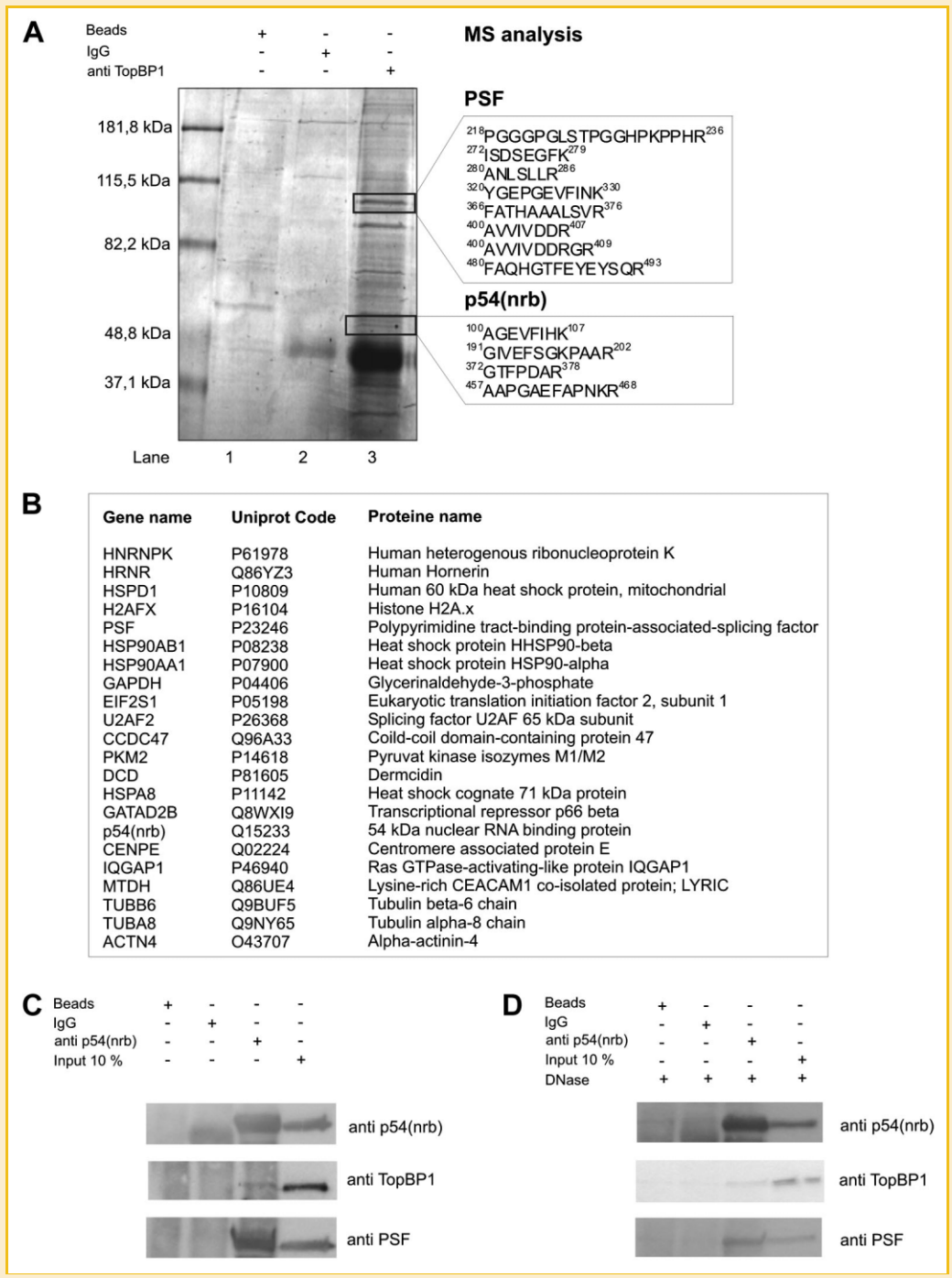

Fig. 1. PSF and p54(nrb) interact with TopBP1 in vivo. A: Whole cell lysate of Ls174T was subjected to co-immunoprecipitation experiments using an anti-TopBP1 antibody followed by SDS/10% PAGE and Coomassie staining. Controls were beads alone (lane 1) and pre-immune serum (IgG, lane 2). Proteins marked with rectangles in lane 3 were focused for further analyses. B: All proteins identified as potential TopBP1-interacting proteins in Ls174T lysate. C: p54(nrb) interacts with TopBP1 and PSF. Lysate of HEK293T cells was subjected to co-immunoprecipitation experiments using an anti-p54(nrb) antibody. Controls were beads alone and pre-immune serum (IgG). Input: 10% (300 μg) of the protein used for immunoprecipitation. D: Interaction between p54(nrb) and TopBP1 and PSF does not depend on the presence of DNA. Lysate of HEK293T cells co-transfected with mammalian expression constructs of p54(nrb) and TopBP1 was treated with 10 U DNase I at the presence of Mg^{2+} for 15 min at 37°C and subjected to co-immunoprecipitation experiments using anti-p54(nrb) antibody. Controls were beads alone and pre-immune serum (IgG). Input: 10% (150 μg) of the protein used for immunoprecipitation.

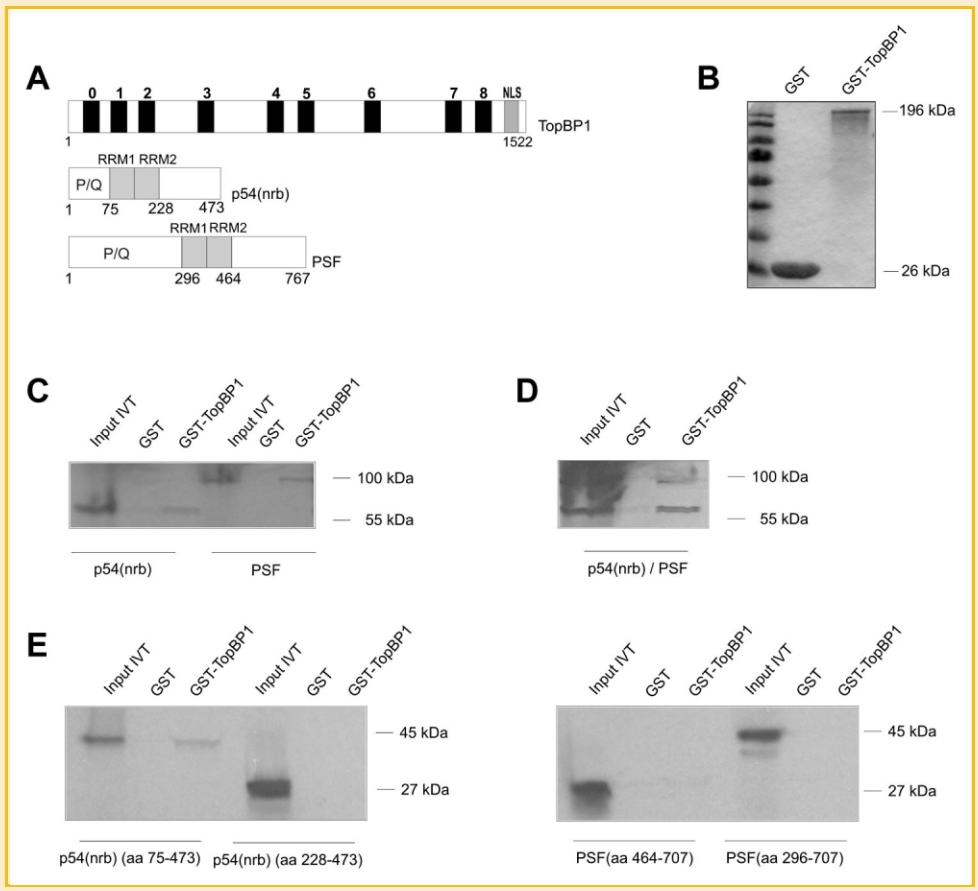

Fig. 2. TopBP1 interacts with p54(nrb) and PSF in vitro. A: Schematic representation of TopBP1, p54(nrb) and PSF. Abbreviations: NLS, nuclear localisation signal; RRM, RNA recognition motif; P/Q, proline/glutamine-rich region. B: Purified GST and GST-TopBP1 stained with Coomassie. C: Results of GST pull down experiments performed with TopBP1 fused to GST and either [^{35}S]-methionine-labelled full length p54(nrb) or PSF or (D) with both [^{35}S]-methionine-labelled full length p54(nrb) and PSF or (E) with [^{35}S]-methionine-labelled truncation constructs of p54(nrb) and PSF. GST served as control. Input IVT: 10 μl of the in vitro translation product.

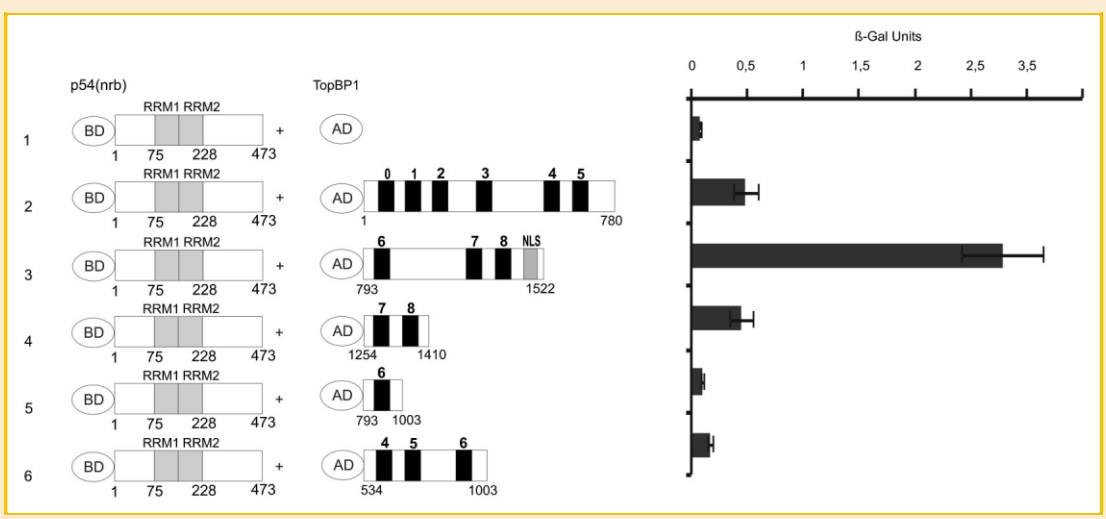

Fig. 3. The BRCT domains 6–8 are necessary and sufficient for the interaction of TopBP1 with p54(nrb). Specific interaction of p54(nrb) (aa 1–473) fused to GAL4 DNA-binding domain (BD) with TopBP1 truncation clones fused to GAL4 activation domain (AD) in yeast. Interactions were quantitatively assessed by β-galactosidase liquid culture activity. The given β-galactosidase units represent the mean number and standard deviation of three independent experiments.

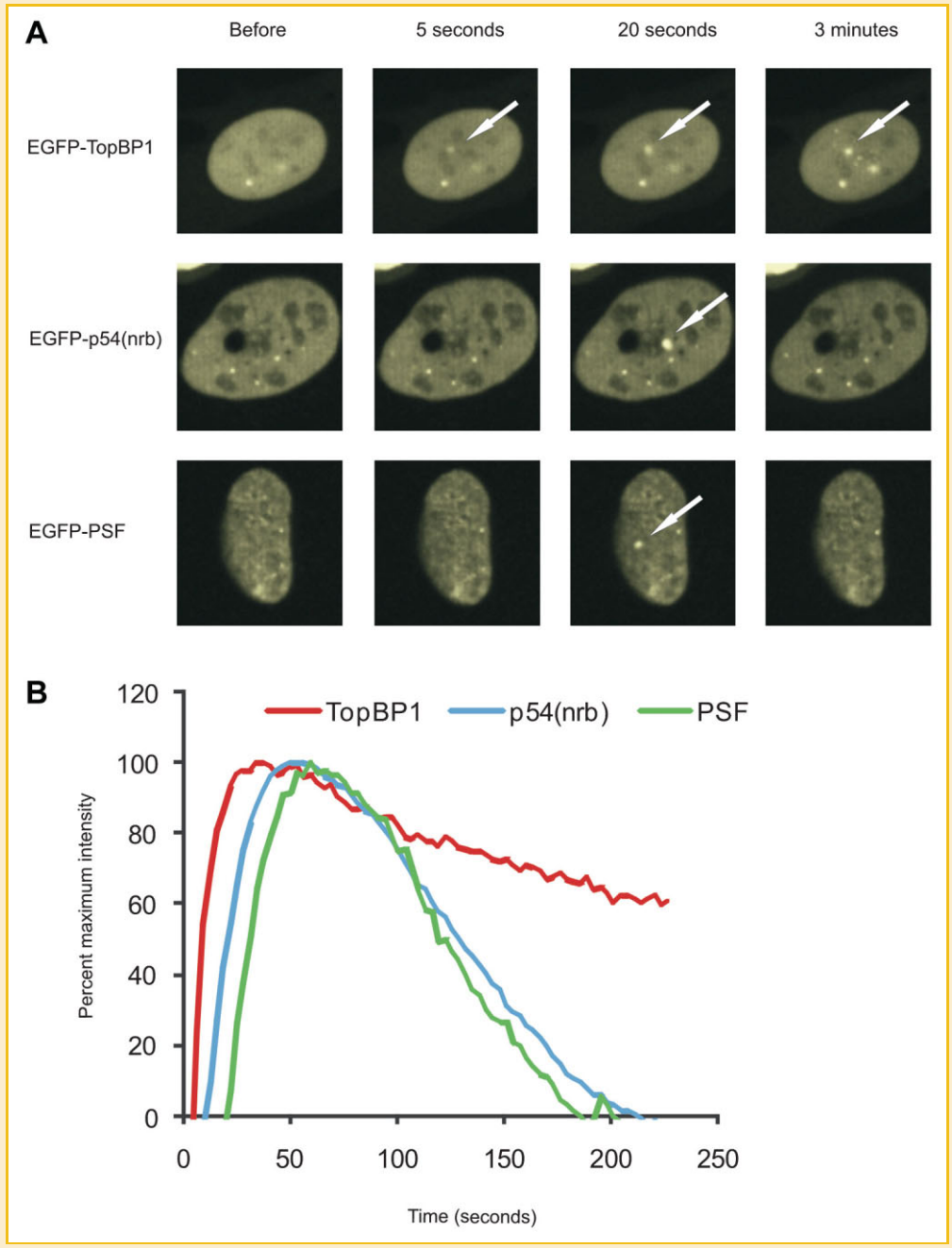

Fig. 4. Recruitment of TopBP1, p54(nrb) and PSF to sites of laser-induced DNA damages. A: U-2OS cells transfected with either EGFP-TopBP1, EGFP-p54(nrb) or EGFP-PSF were laser microirradiated within the nucleus using a 350 nm UV A laser at an energy of 18 μJ and a repetition rate of 10 Hz for 10 s. Arrows point to the sites of irradiation. B: Spatiotemporal behaviour of TopBP1, p54(nrb) and PSF in living cells after laser microirradiation. After background subtraction, obtained curves were normalised to the maximum. Mean number and standard deviation were calculated using at least 12 cells for each single protein. Data shown represent results from several independent experiments.

after laser treatment both proteins co-localised at sites of induced DNA damage, as shown in Figure 5A. Unfortunately, we were not able to perform the same experiment with DsRed2-tagged PSF due to the unstable expression of the fusion protein. To address the role of interaction between TopBP1 and p54(nrb) in recruitment of p54(nrb) to DNA damage sites we used fluorescent tagging of the p54(nrb) (aa 228–473) deletion construct that fails to interact with TopBP1 (Fig. 2D). In cells co-transfected with EGFP-TopBP1 and the p54(nrb) deletion construct only TopBP1 was recruited to sites of laser-induced DNA damage (Fig. 5B). Strikingly, the distribution in discrete foci was more pronounced in the case of p54(nrb) deletion construct in comparison with the full-length construct. To summarise, all three proteins were attracted to DNA damage sites induced by laser microdissection but displayed different retention times there indicating different functions during DSB recognition and repair. At least, TopBP1 and p54(nrb) co-localised at sites of

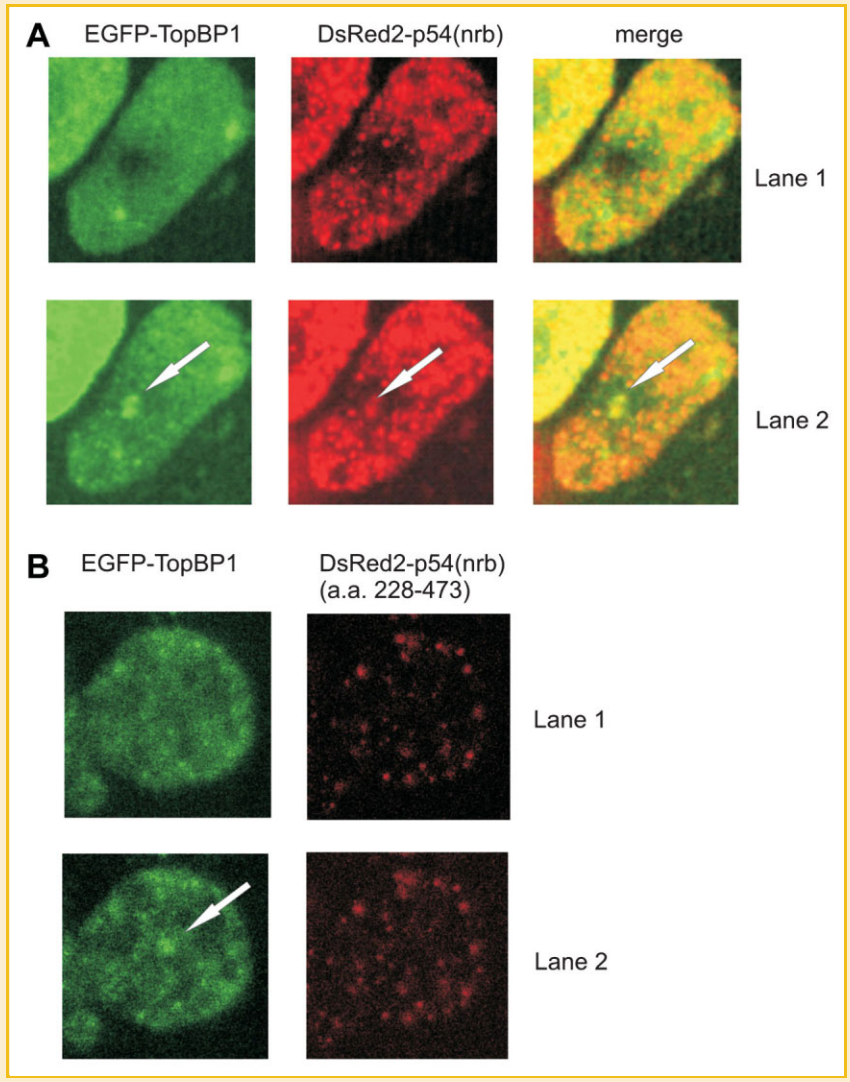

Fig. 5. Co-localisation of TopBP1 and p54(nrb) at sites of laser-induced DNA damage. A: Cells were co-transfected with EGFP-TopBP1 and DsRed2-p54(nrb) and treated as described above. Displayed are cells at the moment of (lane 1) and 60 s after (lane 2) laser irradiation. B: Co-localisation of TopBP1 and p54(nrb) depends on its interaction. Cells were co-transfected with EGFP-TopBP1 and a DsRed2-tagged deletion construct of p54(nrb) that does not interact with TopBP1 anymore. Imaged are cells during (lane 1) and after (lane 2) laser irradiation. Arrows represent the exact site of laser irradiation. Data shown represent results from several independent experiments.

DNA damage and the interaction with TopBP1 is required for relocalisation of p54(nrb) to sites of DNA damage.

DISCUSSION

DNA damage arises continuously as the result of intracellular metabolism and upon exposure of cells to a multitude of genotoxic agents [Ciccia and Elledge, 2010]. If left unrepaired, such insults can be life-threatening for cells and organisms as they alter the content and organisation of the genetic material. To overcome this challenge to genomic stability, cells have evolved a global signalling network known as DNA damage response (DDR) that senses different types of genotoxic stress to mount a coordinated and multi-faceted response, which includes modulation of cell cycle transitions and transcriptional processes, and stimulation of DNA repair [Bekker-Jensen and Mailand, 2010]. DNA can be damaged in many ways, ranging from relatively innocuous single base or nucleotide modifications and single strand breaks to highly cytotoxic lesions such as interstrand crosslinks and DSBs [Wyman and Kanaar, 2006]. DSBs arise from a number of endogenous and exogenous sources, such as ionising radiation and replication of damaged DNA. DSBs are repaired by two main mechanisms, NHEJ, the predominant mode of DSB repair in G0/G1 cells, and HR, which takes place only in S/G2 phase cells [Bekker-Jensen and Mailand, 2010]. While most of the DDR components are present at all times in the cell, activation of DDR is accompanied by a dramatic increase in the availability of this factors. The local up-concentration of DDR proteins into so-called IRIF (ionising radiation-induced foci) is a highly regulated yet dynamic process, where numerous proteins are recruited to sites of

DSBs. DSBs in mammalian cells are sensed by the MRN (MRE11-RAD50-NBS1) complex, which recruits the ATM kinase to the vicinity of the lesions [Lee and Paull, 2005]. The resulting ATM-mediated phosphorylation of the histone variant H2AX triggers an accumulation of the MDC1 protein, along with its binding partners [So et al., 2009]. These include the MRN complex and RNF8, a ubiquitin ligase, which initiates histone poly-ubiquitylation at sites of DNA damage. This chromatin modification allows for a second wave of protein accumulation, including factors such as 53BP1 [Mailand et al., 2007]. The number of proteins detected to be involved in IRIF is still increasing. We were interested in the potential role of TopBP1 in DSB sensing and repair. TopBP1 possesses nine BRCT domains and is structurally and functionally conserved throughout eukaryotic organisms. For *Xenopus* Cut5/TopBP1 several laboratories showed that the replication initiation function could be entirely separated from its checkpoint function. The N-terminal half fragment, that is, that part of Cut5 conserved through evolution, is sufficient for initiation of replication, but incapable of checkpoint activating; the C-terminal half fragment, which is unique in metazoans, is by itself capable of activating the checkpoint response without initiating replication. Upon activation of Chk1, the Ser1131 within the C-terminal region of Cut5 is phosphorylated, and this phosphorylation is critical for the checkpoint response [Hashimoto et al., 2006; Yan et al., 2006]. We show here that the BRCT domains 6-8 of human TopBP1 interact in vitro and in vivo with p54(nrb), a protein that together with PSF is involved in DSB repair and radioresistance. TopBP1 and p54(nrb) co-localised at sites of laser-induced DNA damage. Localisation of TopBP1 at damage sites was noticed as early as 5 s following damage induction whereas p54(nrb) and PSF clearly came later (20 s). In contrast, both p54(nrb) and PSF disappeared after 200 s while TopBP1 was retained at damage sites significantly longer. The recruitment kinetics of TopBP1, which to our knowledge was shown here for the first time, resembles that of Ku80, the major DSB-sensing protein in NHEJ [Mahaney et al., 2009; Ha et al., 2011]. This indicates a role of TopBP1 in immediate sensing DSBs besides of its already known functions in the ATR signalling pathway. This conclusion is supported by the fact that TopBP1 also interacts with Nbs1—an important constituent of the DNA sensing MRN complex [Kumagai et al., 2006; Ramirez-Lugo et al., 2011].

While the interaction of TopBP1 with p54(nrb) and PSF is a new result the heterodimerisation of p54(nrb) with PSF has been already described [Shav-Tal and Zipori, 2002]. Moreover, Li et al. [2009] found p54(nrb) and PSF to be involved in DSB repair. Very recently, Ha et al. [2011] showed that the P/Q-rich domain of PSF is responsible for radioresistance and recruitment of PSF-containing complexes to sites of dense, laser-induced DNA damage in living cells. Finally, PSF interacts via its P/Q-rich domain with RAD51 and modulates its homologous-pairing and strand-exchange activities [Morozumi et al., 2009; Rajesh et al., 2011]. We could show that the P/Q-rich domain of PSF permits the interaction with TopBP1 while the two RRMs of p54(nrb) may allow the heterodimerisation with both TopBP1 and PSF. Assuming that different PSF-containing protein complexes are required for different mechanisms of DSB repair, that is, PSF/RAD51 for HR and PSF/p54(nrb) for NHEJ, the interaction of TopBP1 with PSF and p54(nrb) may regulate the activation of HR or NHEJ during different stages of the cell cycle. But to support this hypothesis experiments to address whether in the presence or absence of TopBP1 interaction with PSF is cell cycle specific and could thereby favour one repair versus another have to be performed.

Taken together, the results of the present study identify PSF and p54(nrb) as TopBP1-binding proteins. This interaction involves the BRCT domains 6–8 of TopBP1, the P/Q-rich domain of PSF, and the two RRMs of p54(nrb). All three proteins are recruited to DNA damage sites after laser irradiation but display different retention times.

ACKNOWLEDGMENTS

We are indebted to J. Chen, P.W. Tucker, J.G. Patton, T. Nagase, W.C. Lin and H.H. Samuels for kindly providing materials. This work was supported by the Deutsche Forschungsgemeinschaft (to F.H. and F.G.: SFB 604/B02) and by the Leibniz Institute for Natural Product Research and Infection Biology (Hans Knoell Institute).

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