



<b>Title</b>	XRCC1 interacts with the p58 subunit of DNA Pol -primase and may coordinate DNA repair and replication during S phase
<b>Authors(s)</b>	Levy, N., Oehlmann, M., Delalande, F., et al.
<b>Publication date</b>	2009-03-21
<b>Publication information</b>	Levy, N., M. Oehlmann, F. Delalande, and et al. "XRCC1 Interacts with the P58 Subunit of DNA Pol -Primase and May Coordinate DNA Repair and Replication during S Phase." Oxford University Press, March 21, 2009. <a href="https://doi.org/10.1093/nar/gkp144">https://doi.org/10.1093/nar/gkp144</a> .
<b>Publisher</b>	Oxford University Press
<b>Item record/more information</b>	<a href="http://hdl.handle.net/10197/4999">http://hdl.handle.net/10197/4999</a>
<b>Publisher's statement</b>	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Nucleic Acids Research following peer review. The definitive publisher-authenticated version, XRCC1 interacts with the p58 subunit of DNA Pol -primase and may coordinate DNA repair and replication during S phase, Nucleic Acids Research (2009) 37(10): 3177-3188, first published online 2009-03-21 is available online at <a href="http://dx.doi.org/10.1093/nar/gkp144">http://dx.doi.org/10.1093/nar/gkp144</a> .
<b>Publisher's version (DOI)</b>	10.1093/nar/gkp144

Downloaded 2026-05-01 23:51:04

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd\_oa)



© Some rights reserved. For more information

# XRCC1 interacts with the p58 subunit of DNA Pol $\alpha$ -primase and may coordinate DNA repair and replication during S phase.

Nicolas Lévy<sup>1, 5</sup>, Maren Oehlmann<sup>2</sup>, François Delalande<sup>3</sup>, Heinz Peter Nasheuer<sup>2</sup>, Alain Van Dorsselaer<sup>3</sup>, Valérie Schreiber<sup>1</sup>, Gilbert de Murcia<sup>1</sup>, Josiane Ménissier-de Murcia<sup>1,T</sup>, Domenico Maiorano<sup>4\*</sup> and Anne Bresson<sup>1\*</sup>

<sup>1</sup>: FRE 3211, Institut de Recherche de l'École de Biotechnologie de Strasbourg, CNRS/Université de Strasbourg, ESBS, Bld S. Brant, BP 10413 67412 Illkirch-Cedex, France

<sup>2</sup>: National University of Ireland, Galway, Department of Biochemistry, Cell Cycle Control Laboratory, Galway, Ireland

<sup>3</sup>: Laboratoire de Spectrométrie de Masse Bio-Organique, UMR 7512, CNRS/ ULP, ECPM 67087 Strasbourg, France

<sup>4</sup>: Surveillance et Stabilité du Génome, UPR 1142, CNRS, Institut de Génétique Humaine, 141, rue de la Cardonille 34396 Cedex 5 Montpellier, France

<sup>5</sup>: IGBMC, IGBS 1, Rue L. Fries 67404 Illkirch Cedex

\*: Corresponding authors: Dr. Anne Bresson, FRE 3211, Institut de Recherche de l'École de Biotechnologie de Strasbourg, CNRS/Université de Strasbourg, Ecole Supérieure de Biotechnologie de Strasbourg, Boulevard S. Brant, F-67412 Illkirch. France. Tel. +33 390 244 700, Fax. +33 390 244 686, e-mail: anne.bresson@esbs.u-strasbg.fr

Dr. Domenico Maiorano, Surveillance et Stabilité du Génome, UPR 1142, CNRS, Institut de Génétique Humaine, 141, rue de la Cardonille -34396 Cedex 5 Montpellier. France. Tel. +33 499 619 912; Fax: +33 499 619 901; E-mail: maiorano@igh.cnrs.fr

<sup>T</sup>: This paper is dedicated to the memory of our colleague Josiane Ménissier-de Murcia who passed away on July 15<sup>th</sup> 2007.

**Keywords:** XRCC1-BRCT1 / Pol  $\alpha$ -primase p58 subunit / poly(ADP-ribose) / Replication / Single-Strand Break Repair

## ABSTRACT

Repair of single stranded DNA breaks before DNA replication is critical in maintaining genomic stability, however, how cells deal with these lesions during S phase is not clear. Using combined approaches of proteomics and *in vitro* and *in vivo* protein-protein interaction we identified the p58 subunit of DNA Pol  $\alpha$ -primase as a new binding partner of XRCC1, a key protein of the single strand break repair complex. *In vitro* experiments reveal that the binding of poly(ADP-ribose) to p58 inhibits primase activity by competition with its DNA binding property. Overexpression of the XRCC1-BRCT1 domain in HeLa cells induces poly(ADP-ribose) synthesis, PARP-1 and XRCC1-BRCT1 poly(ADP-ribosylation) and a strong S phase delay in the presence of DNA damage. Addition of recombinant XRCC1-BRCT1 to *Xenopus* egg extracts slows down DNA synthesis and inhibits the binding of PCNA, but not MCM2 to alkylated-treated chromatin, thus indicating interference with the assembly of functional replication forks. Altogether these results suggest a critical role for XRCC1 in connecting the single strand break repair machinery with the replication fork to halt DNA synthesis in response to DNA damage.

## INTRODUCTION

The cellular response to DNA damage produced by environmental agents or generated by the cellular metabolism involves the coordinated activation of various enzymatic activities aimed at detecting, signaling and resolving faithfully genomic discontinuities. XRCC1 plays a crucial role in the coordination of two overlapping repair pathways, base excision repair (BER) and single strand break repair (SSBR), through the association with and stimulation of several key enzymes involved at different steps of these pathways (Reviewed in (1,2)). The two BRCT domains (BRCT1 and BRCT2) of XRCC1 mediate a network of protein-protein interactions with these repair factors. The BRCT1 domain is the most evolutionarily conserved and is required for survival after methylation damage (3,4). It interacts with PARP-1 and PARP-2, and contains a binding site for poly(ADP-ribose) (PAR) mediating the rapid recruitment of XRCC1 at site of DNA damage (5-9). The BRCT2 domain of XRCC1 binds to and stabilizes DNA ligase III (Lig III) (10).

Several observations suggest that the hypersensitivity of XRCC1-mutant cell lines to monofunctional alkylating agents results from the persistence of unrepaired SSBs that are encountered by the DNA replication fork during S-phase. The XRCC1 deficient EM9 cell line

1  
2 exhibits an increased doubling time and an elevated level of sister-chromatid exchange (SCE)  
3 (11,12). Kubota and Horiuchi found that a mutant in the BRCT1 domain of XRCC1 is defective  
4 in the restart of DNA replication following methyl-methane sulfonate (MMS) treatment, while  
5 this mutant is proficient in DNA repair (4). Recently, Lan et al showed that suppressing XRCC1  
6 expression by RNA interference decreased PCNA accumulation on SSBs induced by laser  
7 irradiation (13) and Fan et al reported a direct interaction between XRCC1 and PCNA *in vitro*  
8 and *in vivo* in S-phase (14). Altogether, these results further extended a possible link between the  
9 SSBR machinery and the replicative apparatus.

10  
11 The formation of functional DNA replication forks occurs by the sequential assembly of large  
12 multiprotein complexes at DNA replication origins (reviewed in (15,16). The Origin Recognition  
13 Complex (ORC1-6) together with the Cdc6 and Cdt1 proteins, catalyze the formation of pre-  
14 replicative complexes (pre-RCs), namely the assembly of the MCM2-7 helicase complex.  
15 Activation of pre-RCs during S phase allows the recruitment of additional replication factors to  
16 form pre-initiation complexes (pre-ICs) that can support DNA unwinding and recruit the DNA  
17 polymerases and other factors required to promote DNA synthesis. To begin DNA synthesis, an  
18 initial RNA primer is synthesized by the DNA primase, a heterodimer of two subunits, p48 and  
19 p58. This short RNA primer is then extended by DNA Pol  $\alpha$  and marks the formation of  
20 initiation complexes (ICs). Then replication factor C (RFC) binds to the primer template junction  
21 and catalyzes the loading of the ring-shaped replication factor PCNA that encircles DNA and  
22 associates with the replicative polymerases Pol  $\delta$  or Pol  $\epsilon$ , taking over DNA synthesis from Pol  $\alpha$   
23 (elongation step).  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39

40  
41 Here we show that the BRCT1 domain of XRCC1 specifically interacts *in vitro* and *in vivo* with  
42 the p58 subunit of DNA Pol  $\alpha$ -primase in HeLa cells. p58 interacts also with PAR resulting in  
43 the inhibition of the p48-p58 primase activity *in vitro*. Consistent with these findings, the  
44 expression of the BRCT1 domain of XRCC1 in HeLa cells or in *Xenopus* extracts interferes with  
45 ongoing DNA synthesis in the presence of DNA damage in a PAR-dependent manner. These  
46 results suggest that the BRCT1 domain of XRCC1 plays a central role in regulating DNA  
47 replication across SSBs during S phase.  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## MATERIALS AND METHODS

### Construction of XRCC1 and p58 Expression Vectors

From the human DNA primase p48-His-tagged-p58 and p58 C-terminus cloned in pET11 (17) we amplified by PCR the DNA sequence encoding p58 (amino acids 1 to 266) and cloned it in the *Nde*I and *Bam*HI restriction sites of the pET 15b vector (Novagen).

Vectors allowing the expression in mammalian cells of GST-tagged fragments of human hXRCC1 are described in (18). PCR amplified fragment encoding *Xenopus* xXRCC1-BRCT1 (aa 307 to 414) was subcloned into the *Eco*R I and *Xho* I of pGEX 4T vector allowing overproduction of the GST fused proteins in *Escherichia coli*.

### Antibodies

The following antibodies were used: Rat monoclonal anti-p58 antibody (19), mouse monoclonal anti-GST antibody (kindly given by M. Oulad, IGBMC, Illkirch), rabbit polyclonal anti-RPA antibody (D. Maiorano, IGH, Montpellier), rabbit polyclonal anti-ORC1 antibody (20), rabbit polyclonal anti-MCM2 antibody (AbCam), mouse monoclonal anti-PARP-1 (EGT69), rabbit polyclonal anti-GST, mouse monoclonal anti-PCNA antibody (PC10, Sigma-Aldrich), goat polyclonal anti-XRCC1 antibody (D-18 sc-5902, Santa Cruz Biotechnology Inc), rabbit polyclonal anti-XRCC1 (Alexis), rabbit polyclonal anti-PAR, and mouse monoclonal anti-BrdU antibodies (Becton Dickinson and Company), rabbit polyclonal anti-Phospho Chk1 (S345) antibody (Cell Signalling). Secondary antibodies, from Molecular Probes, are either goat anti-mouse, donkey anti rat or goat anti-rabbit antibodies, peroxidase-conjugated or alexa 488/alexa 568-conjugated.

### Protein expression, purification, GST pull-down assays and immunoprecipitation

Fusion proteins were expressed in *E. coli* (BRCT1: aa 282-428 or BRCT2: 538-633 domains) and purified using the affinity of either GST for glutathione-coupled beads (GE Healthcare, Amersham Biosciences) or His-tag for Ni<sup>2+</sup> ions immobilized on a silica-based resin (Protino<sup>R</sup> Ni, Machery-Nagel) as recommended by manufacturers.

GST pull-down and immunoprecipitation experiments were carried as described in (18). When indicated, cells were treated with aphidicolin (A, 5 µg/mL 16 h) followed or not by Hydroxyurea treatment (HU, 4 mM for 4 h) or by Methylmethane Sulfonate (MMS 2.5 mM for 30 min) with or without PARP inhibitor (Ku-0058948, 100nM).

### Immunofluorescence.

Cells ( $10^5$ ) grown on glass cover slips, in DMEM medium containing 10% Fetal Bovine Serum and 0.5% gentamicin were treated or not with Hydroxyurea (HU, 4 mM for 4 h at 37°C) and processed further as described in (18).

### Mass spectrometry

GST or GST-hXRCC1-BRCT1 (aa 282-428) produced in *E. coli*, fixed on glutathione-coupled beads were used to purify interacting proteins from HeLa cell extracts (18). Proteins were separated on SDS-PAGE gel, stained with SYPRO-ruby and gel slices containing protein bands of interest were excised and processed for mass spectrometry. In-gel digestion was performed with an automated protein digestion system, MassPREP Station (Waters, Milford, MA). The resulting peptide extracts were directly injected for nanoscale capillary LC-MS/MS (nano-LC-MS/MS) analysis (21). Mass data acquisitions were piloted by MassLynx software (Waters) using automatic switching between MS and MS/MS modes as described previously (22).

### PAR-binding, DNA-binding, Far-western blotting

Proteins were separated by SDS-PAGE (2 µg) or dot-blotted directly on membrane (1 or 2 µg, as indicated). Polyacrylamide gels were incubated 1h at RT in a 50 mM Tris pH8, 30% glycerol buffer, and proteins were transferred on nitrocellulose membranes for PAR and DNA binding or on PVDF membranes for far-western blotting. Proteins were then renatured overnight at 4°C in a 50 mM Tris pH8, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA, 0,3% Tween20, 5% non-fat dried milk buffer. *In vitro* PAR synthesis was performed as in (23). For PAR binding either anti-PAR immunostaining was performed or, as for DNA binding, membranes were incubated 1h at 4°C with <sup>32</sup>P radiolabeled PAR or DNA, washed 3 times in PBS and submitted to autoradiography. Far-western blotting was done as described in (18).

### Band shift assay

Electrophoretic mobility shift assays (EMSA) were carried out to analyze primase binding with PhiX174-ssDNA as previously described (24). Briefly, 2 µg of primase (p48/p58) were incubated with 70 ng PhiX174-ssDNA and increasing amounts (0, 63, 127 and 255 ng) of PAR in binding buffer (final concentration: 10 mM Tris/HCl pH 7.5 and 5 mM EDTA, 50 mM NaCl and 1 µg BSA) at 25°C for 30 min. The protein-DNA complexes were separated by 0.8% agarose gel electrophoresis with 60 mA for 4h. The DNA was then stained with ethidium bromide and ssDNA and protein-DNA complexes were determined using a fluorimager FLA-5100 and the software ImageGauge version 4.2.3 (Fuji Europe, Düsseldorf, Germany).

### Primase activity

One unit of DNA primase (p48-p58) was incubated with 0.1 mM (nucleotide concentration) oligo (dT)<sub>20</sub>, 500 μM, ATP and 10 μCi [ $\alpha$ -<sup>32</sup>P]ATP in a 10 mM Tris Ac pH 7.3, 10 mM MgAc, 1 mM DTT and 0.1 mg/mL BSA buffer. After 15 min at 37°C, the reactions were spotted on DE81 filters (Whatman). Filters were washed 4 times in 0,4 M ammonium bicarbonate, 1% PPI rinsed twice in H<sub>2</sub>O, dried, and submitted to scintillation counting in Ultima Gold scintillation liquid (Packard).

### Cell cycle experiments

HeLa cells (10<sup>6</sup>) grown in 10 cm Petri were transiently transfected with the pBC or pBC-hXRCC1-fragments vectors using JetPEI transfection reagent (Polyplus Transfection). When indicated, cells were incubated 30 min at 37°C with 1 mM MNU (Sigma), washed and grown in fresh culture medium for 20 h. Cells were pulse labelled 30 min at 37°C in DMEM medium supplemented with 5 μM BrdU (Sigma). After two PBS washes, cells were detached by trypsination, washed in PGE (PBS 1x, 1g/L glucose, 1 mM EDTA) buffer and fixed for 30 min on ice in EtOH 70% in PGE. Cells were centrifuged and incubated 4 h at 4°C in 5 mL PGE for rehydration. Cells were treated 15 min at RT in 2N HCl in PGE, collected by centrifugation, and resuspended in 2 mL neutralization PTTB buffer (PBS 1x, 0,5% Tween20, 0,5% BSA, 0,1M sodium tetraborate) and washed twice in PTB buffer (PBS 1x, 0,5% Tween20, 0,5% BSA). Cells were then incubated 1 h at room temperature in mouse monoclonal anti-BrdU antibodies (Becton Dickinson) diluted 1:3 and rabbit polyclonal anti-GST antibodies (Sigma) diluted 1:2000. After two washes in PTB buffer, cells were incubated 1 h at room temperature in Alexa 488 conjugated Goat anti-mouse secondary antibodies (Invitrogen) at 1:500 and PE conjugated donkey anti-rabbit secondary antibodies (Jackson Immuno Research) at 1:200. After two washes in PTB buffer cells were counterstained with 10 μg/mL 7-Aminoactinomycin-D (7-AAD, Sigma) in PGE. Flow cytometry analysis was performed using a FACS Calibur and the Cell Quest software (Becton Dickinson).

### Xenopus extracts and DNA replication assay

Sperm nucleus and egg extracts were prepared as described previously (25). Upon thawing, extracts were supplemented with cycloheximide (250 μg/ml) and an energy regeneration system (10 μg/ml creatine kinase, 10 mM creatine phosphate, 1 mM ATP, 1 mM MgCl<sub>2</sub>). MMS-treatment (either 50 or 75 mM depending upon egg extracts) of sperm nuclei was as previously described (26) When recombinant proteins were added, these were incubated in egg extracts 10

1  
2 min on ice before addition of sperm chromatin. To follow DNA replication by incorporation of  
3 radiolabelled nucleotide into newly replicated DNA, 1  $\mu$ l of  $\alpha$ -[<sup>32</sup>P] dATP or dCTP (3000  
4 Ci/mmol) was added to a standard reaction of 50  $\mu$ l and the amount of newly synthesized DNA  
5 was determined TCA precipitation on GF/C glass fibers filters followed by scintillation  
6 counting. Ku-0058948 was used as PARP inhibitor (27). Alkaline gel electrophoresis of DNAs  
7 and chromatin isolation were performed as previously described (28) and (29), respectively).  
8  
9  
10  
11  
12

## 13 14 15 16 17 **RESULTS**

### 18 19 20 21 **hXRCC1 interacts with the p58 subunit of DNA Pol $\alpha$ -primase**

22  
23 To identify new proteins interacting with the BRCT1 domain of hXRCC1, we used recombinant  
24 GST-tagged hXRCC1 fragment (aa 282 to 428) to pull down protein partners from HeLa cell  
25 extracts. Co-purified proteins were separated by SDS-PAGE and analyzed by mass  
26 spectrometry. The identification of proteins already known to interact with XRCC1 such as  
27 PARP-1, PCNA and DNA-PKcs validated our analysis (5,14,18). In addition, we identified the  
28 p58 subunit of the DNA Pol  $\alpha$ -primase as a novel hXRCC1-BRCT1-interacting protein (Figure  
29 1A).  
30  
31  
32  
33  
34  
35

36 To test whether endogenous p58 and XRCC1 coexist in a common protein complex,  
37 immunoprecipitation experiments were performed using extracts from HeLa cells treated or not  
38 with Aphidicolin (A), with Hydroxyurea (HU) or with both drugs to block DNA replication. As  
39 shown in Figure 1B, p58 was weakly but reproducibly co-immunoprecipitated with XRCC1,  
40 specifically after HU treatment. These results suggest a preferential interaction between XRCC1  
41 and DNA primase when DNA replication forks are stalled.  
42  
43  
44  
45  
46  
47

48 In order to map the p58 interaction domain(s) within XRCC1, GST fusion proteins that  
49 encompass truncated versions of human XRCC1 were generated (Figure 1C). These fusion  
50 proteins were expressed in HeLa cells and GST pull-down experiments were performed,  
51 followed by western blot analysis. The endogenous p58 subunit efficiently copurified with  
52 polypeptides carrying either the N-terminal part of XRCC1 (aa 1-170) or the BRCT1 domain but  
53 not the BRCT2 domain (Figure 1C).  
54  
55  
56  
57

58 To further characterize the interaction between XRCC1 and p58, we analyzed the localization of  
59 both endogenous proteins by immunofluorescence in asynchronous cells treated or not with HU.  
60 No or very few XRCC1 and p58 foci colocalized in untreated cells, whereas after HU treatment,

1  
2 p58 and XRCC1 foci number increased and significant colocalization was observed between the  
3 two proteins (Figure 1D).

4  
5 Taken together, these results suggest that hXRCC1 and DNA primase, via its p58 subunit, can  
6 associate *in vivo* in response to stalled replication forks.  
7  
8  
9

### 10 11 **In vitro association of XRCC1 with p58-Nter**

12  
13 Far western blot analyses were performed to assess whether the interaction between p58 and  
14 hXRCC1 was direct. The p48-His-tagged p58 complex and the N- (aa 1 to 266) or C-terminal  
15 (aa 267 to 509) part of p58 were independently expressed in *E. coli*, purified by affinity  
16 chromatography and separated by SDS-PAGE along with negative (tropomyosine, BSA) and  
17 positive (PARP-1) controls. Proteins transferred to PVDF membranes were renatured prior  
18 incubation with purified hXRCC1 protein and immunodetection with anti-XRCC1 antibody  
19 (Figure 2, right part). XRCC1 interacted only with p58 and its N-terminus fragment, neither with  
20 p48 or tropomyosine or BSA, demonstrating a direct contact between XRCC1 and the N-  
21 terminus domain of p58.  
22  
23  
24  
25  
26  
27  
28  
29  
30

### 31 32 **PAR binds p58 and strongly inhibits DNA primase activity of the p48-p58 complex**

33  
34 Since XRCC1-BRCT1 has strong affinity for PAR we then investigated whether p58 could also  
35 bind PAR. A search for putative PAR binding motifs in the sequence of p48 and p58 identified  
36 two potential PAR binding sites in p58, one in the N-terminal part (aa 101 to 111) and one in the  
37 C-terminal part (aa 286-296) of the protein. To determine whether these sites were functional,  
38 binding of DNA primase to PAR was first analyzed by dot blot. As expected, PAR bound to  
39 PARP-1, XRCC1 and the BRCT1 domain of XRCC1 but not to the BRCT2 domain of XRCC1  
40 or to GST. Under the same conditions, the p48-p58 complex interacted with PAR (Figure 3A).  
41 To identify precisely the domain of the DNA primase responsible for PAR binding, we separated  
42 the p48-p58 subunits along with the N-terminal (aa 1 to 266) and C-terminal (aa 266 to 509) part  
43 of p58 by SDS-PAGE and analyzed radiolabelled PAR binding on proteins blotted on  
44 membrane. Results showed that PAR only bound to the N-terminal fragment of p58 (Figure 3B).  
45 Next, we wanted to determine whether PAR binding could affect the ability of primase to bind  
46 DNA. Purified p48-p58 DNA primase (1 and 2  $\mu$ g) was dotted on nitrocellulose and incubated  
47 successively with oligo(dT)<sub>20</sub> and radioactively labelled PAR. PAR was able to bind to p48-p58  
48 in absence of preincubation with DNA whereas incubation with unlabelled oligo(dT)<sub>20</sub> prior to  
49 PAR addition dramatically decreased the efficiency of PAR binding (Figure 3C). When the  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2 membranes were first incubated with radioactively labeled DNA oligo(dT)<sub>20</sub> and then with  
3 increasing concentrations of unlabelled PAR, we observed a decrease in the intensity of the  
4 radioactive signal retained onto the membrane indicating that PAR can dislodge DNA from p48-  
5 p58 complex (Figure 3C). This observation was confirmed by band-shift assays performed with  
6 purified recombinant p48-p58 and single-stranded DNA. Adding increasing concentrations of  
7 PAR (PAR/DNA ratio: 0, 1 to 4) to the reaction strongly decreased the binding of p48-p58 to  
8 DNA (Figure 3D).

9  
10 To evaluate the effect of PAR on primase activity, we monitored the incorporation of  
11 radiolabeled ribonucleotide on an oligo(dT)<sub>20</sub> DNA substrate in the presence of increasing PAR  
12 concentrations. Low PAR/template DNA ratio had no effect on primase activity, whereas  
13 equimolar conditions of PAR and DNA in the reaction strongly inhibited the radioactive  
14 nucleotide incorporation (Figure 3E). Altogether these results indicate that PAR competes with  
15 DNA for p58 binding to inhibit DNA primase activity.

### 26 27 **XRCC1-BRCT1 stimulates PARP-1 activity and is poly(ADP-ribosyl)ated in response to** 28 **DNA damage**

29  
30  
31  
32 The fact that PAR affects primase activity and that hXRCC1-BRCT1 interacts with p58  
33 prompted us to examine whether hXRCC1-BRCT1 could be poly(ADP-ribosyl)ated *in vivo*.  
34 GST-tagged hXRCC1-BRCT1 overexpressed in undamaged HeLa cells was slightly poly(ADP-  
35 ribosyl)ated to some extent, in contrast to GST alone or GST-hXRCC1-BRCT2 which were not  
36 poly(ADP-rybosyl)ated (Figure 4, right panel compare lane 1 with lanes 4 and 7). Treatment of  
37 cells with 2.5 mM MMS for 30 min triggered PARP-1 activation and PAR synthesis, and cells  
38 overexpressing GST-hXRCC1-BRCT1 displayed high levels of automodified PARP-1  
39 suggesting that hXRCC1-BRCT1 overexpression stimulates PARP-1 activity (Figure 4, right  
40 panel lanes 2, 5 and 8). In addition, GST-hXRCC1-BRCT1 was poly(ADP-ribosyl)ated in  
41 MMS-treated cells and this modification decreased in the presence of the PARP inhibitor Ku-  
42 0058948 (Figure 4 right panel lane 2 and 3). The signals recognized by the PAR antibody with a  
43 molecular weight higher than 116 kDa, copurifying with GST-hXRCC1-BRCT1 correspond to  
44 automodified PARP-1 which strongly interacts with the BRCT1 domain of XRCC1 (Masson et  
45 al, 1998). The observation that GST-hXRCC1-BRCT1 is poly(ADP-ribosyl)ated *in vivo*  
46 confirmed previous *in vitro* data showing that this domain could be covalently poly(ADP-  
47 ribosyl)ated by PARP-1 (and PARP-2) in addition to its non-covalent binding to PAR (6). The  
48 increased PAR level observed in undamaged GST-hXRCC1-BRCT1 expressing cells was  
49 confirmed by immunofluorescence microscopy. PAR synthesis was detected in HeLa cells  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2 overexpressing GFP-hXRCC1-BRCT1 but not GFP-hXRCC1-BRCT2 (Supplementary figure 1).  
3  
4 Treatment of cells with hydrogen peroxide triggered higher amount of PAR produced in GFP-  
5 hXRCC1-BRCT1 expressing cells compared to untransfected or GFP-hXRCC1-BRCT2  
6  
7 expressing cells (Supplementary Figure 1). In addition, some PAR foci colocalized with GFP-  
8 hBRCT1 but not with GFP-hBRCT2.  
9

10  
11 Altogether, these results reveal that the overexpression of hXRCC1-BRCT1 domain in HeLa  
12 cells stimulates PAR synthesis in damaged cells which leads to its poly(ADP-ribosyl)ation.  
13  
14

### 15 16 **XRCC1-BRCT1 overexpressing cells accumulate in S-phase following DNA damage**

  
17

18  
19 The poly(ADP-ribosyl)ation of hXRCC1-BRCT1 in response to DNA damage, the interaction of  
20 p58 with PAR and hXRCC1-BRCT1 and the inhibition of DNA primase activity by PAR let us  
21  
22 hypothesized that the overexpression of the BRCT1 domain of XRCC1 would have the potential  
23  
24 to halt DNA replication of damaged DNA.  
25

26  
27 To test this possibility, truncated fragments of hXRCC1 fused to GST were overexpressed in  
28 HeLa cells and the progression through the cell cycle was analyzed by flow cytometry (FACS).  
29 The cell cycle of untreated transfected cells was not affected by the overexpression of any of the  
30 hXRCC1 fusion proteins (Figure 5A). In contrast, 20 h following treatment with the alkylating  
31 agent methyl nitrosourea (MNU), cells expressing hXRCC1 fragments containing the BRCT1  
32 domain (aa 170-428, aa 282-428 and aa 314-428) showed a strong accumulation in S phase  
33 leading to a decreased G2/M phase (Figure 5A). Quantification of the DNA synthesis was  
34 performed by monitoring BrdU incorporation in cells overexpressing either the hBRCT1 (aa  
35 282-428) fused to GST or GST alone, 20 h after treatment with 1 mM MNU (Figure 5B). MNU  
36 treatment lead to an increased number of BrdU positive cells that was higher for GST-hXRCC1-  
37 BRCT1 than GST overexpression (25 % versus 15.5%, respectively, Figure 5B). However, HeLa  
38 cells expressing GST were evenly distributed throughout the S phase whereas GST-hXRCC1-  
39 BRCT1-expressing cells were enriched in the early S phase just after the G1 exit.  
40  
41

42  
43 Altogether, these results indicate that the overexpression of the BRCT1 domain of hXRCC1  
44 leads to a strong accumulation of MMS-treated human cells in early S-phase. This effect is  
45 specific for the BRCT1 domain, since over-expression of GST-hBRCT2 had no particular effect  
46 on cell cycle progression whether the cells were treated with alkylating agents (Figure 5A and  
47 data not shown).  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## XRCC1-BRCT1 slows down DNA synthesis in *Xenopus* egg extracts

To gain further insight into the biological significance of the interaction between XRCC1, PAR and the p58 subunit of Pol  $\alpha$ -primase during the replication of damaged DNA, we used *Xenopus laevis* egg extracts. The cell-free aspect of this system allows the detailed analysis at the molecular level of the different steps of DNA synthesis and its biochemical manipulation, by the addition of either purified proteins to assess for a dominant effect or pharmacological drugs to inhibit specific activities. Introduction of *Xenopus* sperm chromatin in such extracts results in the assembly of a nuclear membrane around DNA and the execution of a single complete round of semi conservative replication (30). To this end, sperm chromatin treated or not with MMS (see Materials and Methods) was introduced into egg extracts synchronized in early S phase and DNA replication was monitored by following the incorporation of a radiolabeled nucleotide precursor. Either GST or GST-xXRCC1-BRCT1 (aa 307 to 414 from *Xenopus laevis* XRCC1) were added to egg extracts prior to the initiation of DNA synthesis.

As shown in Figure 6A, replication of sperm chromatin treated with low concentrations of MMS was slightly impeded in extracts supplemented with GST as control (about 85% of replication compared to the 100% in the untreated reaction), confirming that replication of alkylated DNA is slowed down in this system (31). Consistent with what was observed in HeLa cells (Figure 5), replication of MMS-treated chromatin was strongly slowed down when egg extracts were supplemented with GST-xXRCC1-BRCT1 (over 40% reduction of replication, Figure 6A). To further characterize the defect in DNA synthesis induced by the xXRCC1-BRCT1 domain, replication intermediates were analyzed by alkaline gel electrophoresis. In this assay, nascent DNA is detected as a smear corresponding to growing DNA chains, while fully replicated DNA is visible as high molecular weight species. As expected, nascent DNA accumulated in the presence of MMS compared to the mocked-treated reaction (Figure 6B, compare right to left panel), which is due to a delay in ongoing DNA synthesis. Upon MMS treatment, the addition of GST-xXRCC1-BRCT1 resulted in a much stronger accumulation of nascent DNA compared to the GST control (Figure 6B, right panel). After 100 min of incubation in the presence of GST-XRCC1-BRCT1, very little fully replicated DNA was synthesized, while by this time the replication of mock-treated chromatin was complete. Moreover, the abundance of early replication intermediates (within 40 minutes) decreased in the presence of GST-xXRCC1-BRCT1 suggesting inhibition of both the initiation and elongation steps of DNA synthesis.

We examined whether PAR production could be observed on MMS-treated sperm chromatin in *Xenopus* extracts supplemented with GST-xXRCC1-BRCT1. Indeed, PAR production, which was detectable in nuclei treated with MMS, was dramatically increased by the addition of GST-

1  
2 xXRCC1-BRCT1 (data not shown) as observed in HeLa cells. Owing to the inhibitory effect of  
3 PAR on primase activity, we hypothesized that the massive PAR production caused by the  
4 presence of GST-xXRCC1-BRCT1 could be responsible for the inhibition of replication and that  
5 this effect could be reversed by addition of a PARP inhibitor. Results shown in Figure 6C  
6 confirmed this hypothesis, since preincubation of *Xenopus* extracts with the PARP inhibitor  
7 rescued the DNA synthesis delay induced by the addition of GST-xXRCC1-BRCT1, whereas  
8 this inhibitor had no effect on replication of MMS-treated chromatin in the presence of GST  
9 (Supplementary Figure 2A). Taken together, these results confirm that the BRCT1 domain of  
10 XRCC1 inhibits replication of damaged DNA in *Xenopus* egg extracts.  
11  
12  
13  
14  
15  
16  
17  
18  
19

### 20 **XRCC1-BRCT1 binds to chromatin and interferes with formation of functional replication** 21 **forks** 22

23  
24  
25 To characterize in more detail the DNA synthesis defect induced by xXRCC1-BRCT1 in the  
26 presence of DNA damage, we analyzed the recruitment to chromatin of replication factors  
27 specific of the different steps of DNA synthesis. Hence, sperm chromatin was exposed to MMS  
28 and incubated in egg extracts supplemented with the indicated proteins, as described in Figure  
29 6A. Chromatin fractions were analyzed by western blot for the binding of the indicated proteins.  
30 The GST-xXRCC1-BRCT1 domain bound *Xenopus* chromatin, and its chromatin association  
31 was enhanced by MMS treatment (Figure 6D). Addition of the xXRCC1-BRCT1 domain did not  
32 induce phosphorylation of the Chk1 protein kinase in the absence of MMS, demonstrating that  
33 the inhibition of DNA synthesis observed when xXRCC1-BRCT1 is added to MMS-treated  
34 chromatin is not due to a direct activation of the DNA damage checkpoint (Supplementary  
35 Figure 2B). The chromatin binding of ORC1, a subunit of the origin recognition complex  
36 essential for the assembly of preRCs, and that of RPA32, a component of pre-ICs that binds to  
37 single stranded DNA at replication forks, were not significantly affected by the addition of GST-  
38 xXRCC1-BRCT1, indicating that pre-RCs and pre-ICs assembled normally. Consistent with this  
39 conclusion, the chromatin binding of the pre-RC component MCM2 was not affected but, even it  
40 increased following addition of GST-xXRCC1-BRCT1. In contrast, PCNA binding to MMS-  
41 treated sperm chromatin was abolished in the presence of GST-xXRCC1-BRCT1 (Figure 6D),  
42 while this inhibition was significantly rescued in the presence of PARP inhibitor (Supplementary  
43 Figure 2C).  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58

59 Since PCNA requires the activity of DNA Pol  $\alpha$ -primase to bind to chromatin and stimulates  
60 replication elongation (32) (33), these results strongly argue that the BRCT1 domain of  
xXRCC1, by interfering with the activity of DNA Pol  $\alpha$ -primase, regulates the progression of

1  
2 replication forks through damaged DNA.  
3  
4  
5  
6

## 7 8 **DISCUSSION** 9

10  
11 Several reports put forward the idea that XRCC1 could play a role in the control of replication  
12 fork progression when DNA is damaged. The first indication came from the observation that  
13 XRCC1 deficient EM9 cells display high levels of SCEs, reflecting the accumulation of  
14 unrepaired SSB converted to DBS at collapsed replication forks (2). Kubota and Horiuchi  
15 showed that complementation of EM9 cells with XRCC1 point mutated in the BRCT1 domain  
16 could not restore nascent DNA replication after MMS treatment (4). While the BRCT2 domain  
17 of XRCC1 is only required for BER/SSBR during G1, the BRCT1 domain was shown to be  
18 critical for efficient repair during G1 but also S/G2 phase of the cell cycle (3). More recently,  
19 Brem and Hall found that lowering XRCC1 levels by RNA interference led to a significant delay  
20 in S-phase progression after exposure to MMS (34). XRCC1 was shown to interact with PCNA  
21 and both proteins displayed colocalization during S phase of undamaged cells (14). In addition,  
22 Parlanti et al demonstrated the existence of a multiprotein complex containing the DNA  
23 replicative polymerases Pol  $\alpha$ ,  $\delta$ ,  $\epsilon$ , the replication protein MCM7, BER/SSBR components  
24 including XRCC1 and Pol  $\beta$  and the cell cycle regulatory protein cyclin A (35). They proposed  
25 that XRCC1 could act as an early effector of cellular response to DNA breaks at stalled  
26 replication. All these studies clearly designed XRCC1 as a critical factor acting when replication  
27 forks encounter damaged DNA. The idea most commonly advanced was that BER/SSBR  
28 machineries are associated to the replication machinery in order to coordinate the repair of DNA  
29 lesions with replication fork progression, thus avoiding the conversion of unrepaired damaged  
30 bases or single strand breaks to mutations or highly toxic DSBs during replication. However, the  
31 precise role of XRCC1 in this process remained an open question  
32

33  
34 In this study, we identified the p58 subunit of the Pol  $\alpha$ -primase complex as a novel important  
35 element that links XRCC1 to the replication apparatus. We demonstrated an interaction between  
36 the BRCT1 domain of XRCC1 and the N-terminal part of the p58 and observed colocalization  
37 between XRCC1 and p58 in damaged cell nuclei. In addition, we found that the N-terminal  
38 domain of p58 bound PAR, leading to inhibition of primase activity. XRCC1-BRCT1 domain  
39 overexpressed in HeLa cells is poly(ADP-ribosyl)ated following DNA damage and led to the  
40 accumulation of MNU-treated HeLa cells in early S-phase. The inhibition of replication of  
41 damaged DNA was also observed in *Xenopus* egg extracts, suggesting that this regulation is  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2 conserved in vertebrates. Looking more deeply into the molecular mechanism, we found that in  
3 the presence of DNA damage, the BRCT1 domain of *Xenopus* XRCC1 did not interfere with the  
4 formation of both pre-RCs and pre-ICs, but strongly inhibited the association of PCNA with  
5 replicating chromatin. Thus, our results suggest that overexpression of XRCC1-BRCT1 could  
6 interfere either with SSBR, as dominant negative factor, or with the establishment of functional  
7 replication forks and/or ongoing DNA synthesis. But the observed interaction of XRCC1-  
8 BRCT1 with p58, the distribution of cells in S phase in HeLa XRCC1-BRCT1 and the inhibition  
9 of chromatin association of PCNA with MMS-treated chromatin led us to preferentially propose  
10 a role for XRCC1 in the coordination of DNA repair and replication during S phase.  
11

12 The inhibition of p58 primase activity by PAR in the presence of damaged DNA is strikingly  
13 reminiscent of the mechanism recently described in *Bacillus subtilis* by which guanosine penta-  
14 and tetraphosphate ((p)ppGpp) synthesized by RelA in response to nutritional stress, directly  
15 inhibits primase activity to stop ongoing DNA replication (36). It was proposed that this  
16 regulation might avoid replication fork collapse during nutrient deprivation and therefore  
17 maintain genome integrity. In eukaryotes, PAR may play a similar role in response to DNA  
18 damage during S phase. The implication of PARP-1 and poly(ADP-ribosyl)ation in the  
19 replication arrest in response to DNA damage has been previously documented. PARP-1 activity  
20 was shown to inhibit the replicative polymerase activities of DNA Pol  $\alpha$  and  $\delta$  and the DNA  
21 repair polymerase activity of Pol  $\beta$  *in vitro* (37) (38). We and others previously reported the  
22 physical interaction between PARP-1 and the 180 kDa catalytic subunit of DNA Pol  $\alpha$ -primase,  
23 as well as the impaired S-phase DNA synthesis in PARP-1-deficient or depleted mouse  
24 embryonic fibroblasts (39) (40). In contrast, our experiments presented here reveal that HeLa  
25 cells overexpressing GST-hXRCC1-BRCT1 displayed an increased PAR synthesis under  
26 damage conditions and cells accumulated in early S phase. In addition, the delayed replication of  
27 MMS-treated chromatin from *Xenopus* egg extracts upon addition of GST-xXRCC1-BRCT1 was  
28 alleviated in the presence of a PARP inhibitor, thus highlighting the role of PAR in the  
29 replication arrest of damaged chromatin. The inhibition of primase activity *in vitro* by PAR that  
30 competes with template DNA points out to a critical step subjected to regulation by poly(ADP-  
31 ribosyl)ation. Our results shed light into previous observations by Yoshihara et al (38) who  
32 reported that PARP-1, in the presence of NAD<sup>+</sup>, could inhibit, *in vitro*, the DNA Pol  $\alpha$  and the  
33 primase activities, which are restored by a PARP inhibitor, suggesting that both the primase and  
34 polymerase are directly controlled by PAR. Very recently, PARP-1 was also shown to slow  
35 down fork progression in response to damage induced by the topoisomerase I inhibitor  
36 camptothecin (41).  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2 This points out the need of an appropriate PAR bearing protein, such as XRCC1 through its  
3 BRCT1 domain, and suggests that poly(ADP-ribosyl)ated XRCC1 could be a factor that targets  
4 p58 to inhibit replication on a damaged template.  
5  
6

7 Interaction between XRCC1 and primase could also help to stabilize stalled replication forks  
8 preventing them from collapsing. Subsequently, degradation of PAR could help to stabilize the  
9 replication machinery and promotes its reactivation. This hypothesis refers to the work of Maruta  
10 et al who proposed that PAR degradation by PARG could be a source of ATP required for  
11 replisome stability (42). If replication fork arrest fails when a SSB is present and leads to the  
12 generation of a DSB, then the activation of double-strand end-joining repair will be turned on by  
13 another function of XRCC1, through its interaction and stimulation of the NHEJ factor DNA-PK  
14 (18). In a recent paper, Brem et al showed that unresolved BER intermediates give rise to lesions  
15 activating both ATM and ATR and leading to S-phase delay. This delay is required to repair  
16 damages through a recombinational repair pathway involving Rad51 (43).  
17  
18

19 These findings raise the question about the relevance of the XRCC1-PAR-p58 interaction? The  
20 more likely hypothesis is that inhibiting primase activity by PAR, which is facilitated by the  
21 interactions of XRCC1 with p58 and PARP-1, could give cells more time to repair DNA lesions  
22 prior to resume the replication process and thus avoid conversion of SSBs into DSBs. A role for  
23 DNA primase to delay the progression of replication when DNA needs first to be mended, is  
24 consistent with its role as a molecular brake in DNA replication when leading strand synthesis is  
25 prevented from outspacing lagging strand synthesis, as reported for bacteria and T7  
26 bacteriophage DNA replication (44,45). As the Pol  $\alpha$ -primase complex is required for both the  
27 initiation of DNA replication and the lagging strand DNA synthesis, it is therefore a likely target  
28 for coupling DNA replication to the DNA damage response (46).  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42

43 We propose that XRCC1 plays a central role in the coordination of DNA repair and replication  
44 during S phase. In response to DNA damage, poly(ADP-ribosyl)ated XRCC1 slows down  
45 replication via its functional interaction with the p58 subunit of the Pol  $\alpha$ -primase complex in  
46 order to allow damage repair to take place or acting, if necessary, as a molecular switch between  
47 SSBR and DSBR.  
48  
49  
50  
51  
52  
53

#### 54 **ACKNOWLEDGEMENTS**

55  
56  
57

58 The authors wished to thank all the members of the team Poly(ADP-ribosyl)ation et intégrité du  
59 génome, especially F. Dantzer, for fruitful discussions. We are especially indebted to M. Mechali  
60 for getting us in touch with D. Maiorano and allowing N. Levy to perform experiments in  
Montpellier's laboratory. This work was supported by funds from Centre National de la

1  
2 Recherche Scientifique, Association pour la Recherche contre le Cancer, Electricité de France,  
3 Ligue contre le Cancer Comité du Bas-Rhin, Commissariat à l'Energie Atomique and Agence  
4 Nationale pour la Recherche and by a grant of Health Research Board, Ireland and INTAS  
5  
6 (Brussels, Belgium).  
7  
8  
9

## 10 REFERENCES

- 11 1. Thompson, L.H. and West, M.G. (2000) XRCC1 keeps DNA from getting stranded. *Mutation research*, **459**, 1-18.
- 12 2. Caldecott, K.W. (2003) XRCC1 and DNA strand break repair. *DNA Repair (Amst)*, **2**,  
13 955-969.
- 14 3. Taylor, R.M., Thistlethwaite, A. and Caldecott, K.W. (2002) Central role for the XRCC1  
15 BRCT I domain in mammalian DNA single-strand break repair. *Mol Cell Biol*, **22**, 2556-  
16 2563.
- 17 4. Kubota, Y. and Horiuchi, S. (2003) Independent roles of XRCC1's two BRCT motifs in  
18 recovery from methylation damage. *DNA Repair (Amst)*, **2**, 407-415.
- 19 5. Masson, M., Niedergang, C., Schreiber, V., Muller, S., Menissier-de Murcia, J. and de  
20 Murcia, G. (1998) XRCC1 is specifically associated with poly(ADP-ribose) polymerase  
21 and negatively regulates its activity following DNA damage. *Mol Cell Biol*, **18**, 3563-  
22 3571.
- 23 6. Schreiber, V., Ame, J.C., Dolle, P., Schultz, I., Rinaldi, B., Fraulob, V., Menissier-de  
24 Murcia, J. and de Murcia, G. (2002) Poly(ADP-ribose) polymerase-2 (PARP-2) is  
25 required for efficient base excision DNA repair in association with PARP-1 and XRCC1.  
26 *J Biol Chem*, **277**, 23028-23036.
- 27 7. El-Khamisy, S.F., Masutani, M., Suzuki, H. and Caldecott, K.W. (2003) A requirement  
28 for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative  
29 DNA damage. *Nucleic Acids Res*, **31**, 5526-5533.
- 30 8. Okano, S., Lan, L., Caldecott, K.W., Mori, T. and Yasui, A. (2003) Spatial and temporal  
31 cellular responses to single-strand breaks in human cells. *Mol Cell Biol*, **23**, 3974-3981.
- 32 9. Mortusewicz, O., Ame, J.C., Schreiber, V. and Leonhardt, H. (2007) Feedback-regulated  
33 poly(ADP-ribosylation) by PARP-1 is required for rapid response to DNA damage in  
34 living cells. *Nucleic Acids Res*.
- 35 10. Taylor, R.M., Wickstead, B., Cronin, S. and Caldecott, K.W. (1998) Role of a BRCT  
36 domain in the interaction of DNA ligase III-alpha with the DNA repair protein XRCC1.  
37 *Curr Biol*, **8**, 877-880.
- 38 11. Dillehay, L.E., Thompson, L.H., Minkler, J.L. and Carrano, A.V. (1983) The relationship  
39 between sister-chromatid exchange and perturbations in DNA replication in mutant EM9  
40 and normal CHO cells. *Mutation research*, **109**, 283-296.
- 41 12. Thompson, L.H., Brookman, K.W., Jones, N.J., Allen, S.A. and Carrano, A.V. (1990)  
42 Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand  
43 break repair and sister chromatid exchange. *Mol Cell Biol*, **10**, 6160-6171.
- 44 13. Lan, L., Nakajima, S., Oohata, Y., Takao, M., Okano, S., Masutani, M., Wilson, S.H. and  
45 Yasui, A. (2004) In situ analysis of repair processes for oxidative DNA damage in  
46 mammalian cells. *Proceedings of the National Academy of Sciences of the United States*  
47 *of America*, **101**, 13738-13743.
- 48 14. Fan, J., Otterlei, M., Wong, H.K., Tomkinson, A.E. and Wilson, D.M., 3rd. (2004)  
49 XRCC1 co-localizes and physically interacts with PCNA. *Nucleic Acids Res*, **32**, 2193-  
50 2201.

15. Maiorano, D., Lutzmann, M. and Mechali, M. (2006) MCM proteins and DNA replication. *Curr Opin Cell Biol*, **18**, 130-136.
16. Sclafani, R.A. and Holzen, T.M. (2007) Cell Cycle Regulation of DNA Replication. *Annu Rev Genet*.
17. Schneider, A., Smith, R.W., Kautz, A.R., Weisshart, K., Grosse, F. and Nasheuer, H.P. (1998) Primase activity of human DNA polymerase alpha-primase. Divalent cations stabilize the enzyme activity of the p48 subunit. *J Biol Chem*, **273**, 21608-21615.
18. Levy, N., Martz, A., Bresson, A., Spenlehauer, C., de Murcia, G. and Menissier-de Murcia, J. (2006) XRCC1 is phosphorylated by DNA-dependent protein kinase in response to DNA damage. *Nucleic Acids Res*, **34**, 32-41.
19. Weisshart, K., Forster, H., Kremmer, E., Schlott, B., Grosse, F. and Nasheuer, H.P. (2000) Protein-protein interactions of the primase subunits p58 and p48 with simian virus 40 T antigen are required for efficient primer synthesis in a cell-free system. *J Biol Chem*, **275**, 17328-17337.
20. Rowles, A., Chong, J.P., Brown, L., Howell, M., Evan, G.I. and Blow, J.J. (1996) Interaction between the origin recognition complex and the replication licensing system in *Xenopus*. *Cell*, **87**, 287-296.
21. Brizard, J.P., Carapito, C., Delalande, F., Van Dorsselaer, A. and Brugidou, C. (2006) Proteome analysis of plant-virus interactome: comprehensive data for virus multiplication inside their hosts. *Mol Cell Proteomics*, **5**, 2279-2297.
22. Richert, S., Luche, S., Chevallet, M., Van Dorsselaer, A., Leize-Wagner, E. and Rabilloud, T. (2004) About the mechanism of interference of silver staining with peptide mass spectrometry. *Proteomics*, **4**, 909-916.
23. Dantzer, F., Giraud-Panis, M.J., Jaco, I., Ame, J.C., Schultz, I., Blasco, M., Koering, C.E., Gilson, E., Menissier-de Murcia, J., de Murcia, G. *et al.* (2004) Functional interaction between poly(ADP-Ribose) polymerase 2 (PARP-2) and TRF2: PARP activity negatively regulates TRF2. *Mol Cell Biol*, **24**, 1595-1607.
24. Grosse, F., Nasheuer, H.P., Scholtissek, S. and Schomburg, U. (1986) Lactate dehydrogenase and glyceraldehyde-phosphate dehydrogenase are single-stranded DNA-binding proteins that affect the DNA-polymerase-alpha-primase complex. *European journal of biochemistry / FEBS*, **160**, 459-467.
25. Menut, S., Lemaitre, J.M., Hair, A. and Méchali, M. (1988) DNA replication and chromatin assembly using *Xenopus* egg extracts. In *Advances in Molecular Biology: A comparative Methods Approach to the Study of Oocytes and Embryos*, Oxford University Press, Ed J.D. Richter.
26. Stokes, M.P. and Michael, W.M. (2003) DNA damage-induced replication arrest in *Xenopus* egg extracts. *J Cell Biol*, **163**, 245-255.
27. Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C. *et al.* (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*, **434**, 917-921.
28. Maiorano, D., Cuvier, O., Danis, E. and Mechali, M. (2005) MCM8 is an MCM2-7-related protein that functions as a DNA helicase during replication elongation and not initiation. *Cell*, **120**, 315-328.
29. Maiorano, D., Lemaitre, J.M. and Mechali, M. (2000) Stepwise regulated chromatin assembly of MCM2-7 proteins. *J Biol Chem*, **275**, 8426-8431.
30. Blow, J.J. and Laskey, R.A. (1986) Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell*, **47**, 577-587.
31. Stokes, M.P., Van Hatten, R., Lindsay, H.D. and Michael, W.M. (2002) DNA replication is required for the checkpoint response to damaged DNA in *Xenopus* egg extracts. *J Cell Biol*, **158**, 863-872.
32. Garg, P. and Burgers, P.M. (2005) DNA polymerases that propagate the eukaryotic DNA replication fork. *Critical reviews in biochemistry and molecular biology*, **40**, 115-128.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60
33. Philpott, A. and Yew, P.R. (2008) The *Xenopus* cell cycle: an overview. *Molecular biotechnology*, **39**, 9-19.
34. Brem, R. and Hall, J. (2005) XRCC1 is required for DNA single-strand break repair in human cells. *Nucleic Acids Res*, **33**, 2512-2520.
35. Parlanti, E., Locatelli, G., Maga, G. and Dogliotti, E. (2007) Human base excision repair complex is physically associated to DNA replication and cell cycle regulatory proteins. *Nucleic Acids Res*, **35**, 1569-1577.
36. Wang, J.D., Sanders, G.M. and Grossman, A.D. (2007) Nutritional control of elongation of DNA replication by (p)ppGpp. *Cell*, **128**, 865-875.
37. Eki, T. (1994) Poly (ADP-ribose) polymerase inhibits DNA replication by human replicative DNA polymerase alpha, delta and epsilon in vitro. *FEBS letters*, **356**, 261-266.
38. Yoshihara, K., Tanaka, Y., Itaya, A., Kamiya, T., Hironaka, T., Minaga, T. and Koide, S.S. (1989) I. ADP-ribose transfer reactions. Mechanisms and biological significance. Jacobson, M.K. and Jacobson, E.L. Editors. Springer-Verlag. (1989) In vitro evidence for Poly(ADP-ribosylation) of DNA polymerase-primase and phosphorylation of poly(ADP-ribose) synthetase by protein kinase C. 39-46.
39. Dantzer, F., Nasheuer, H.P., Vonesch, J.L., de Murcia, G. and Menissier-de Murcia, J. (1998) Functional association of poly(ADP-ribose) polymerase with DNA polymerase alpha-primase complex: a link between DNA strand break detection and DNA replication. *Nucleic Acids Res*, **26**, 1891-1898.
40. Simbulan-Rosenthal, C.M., Rosenthal, D.S., Iyer, S., Boulares, H. and Smulson, M.E. (1999) Involvement of PARP and poly(ADP-ribosylation) in the early stages of apoptosis and DNA replication. *Molecular and cellular biochemistry*, **193**, 137-148.
41. Sugimura, K., Takebayashi, S., Taguchi, H., Takeda, S. and Okumura, K. (2008) PARP-1 ensures regulation of replication fork progression by homologous recombination on damaged DNA. *J Cell Biol*, **183**, 1203-1212.
42. Maruta, H., Okita, N., Takasawa, R., Uchiumi, F., Hatano, T. and Tanuma, S. (2007) The involvement of ATP produced via (ADP-Ribose)<sub>n</sub> in the maintenance of DNA replication apparatus during DNA repair. *Biological & pharmaceutical bulletin*, **30**, 447-450.
43. Brem, R., Fernet, M., Chapot, B. and Hall, J. (2008) The methyl methanesulfonate induced S-phase delay in XRCC1-deficient cells requires ATM and ATR. *DNA Repair (Amst)*.
44. Lee, J.B., Hite, R.K., Hamdan, S.M., Xie, X.S., Richardson, C.C. and van Oijen, A.M. (2006) DNA primase acts as a molecular brake in DNA replication. *Nature*, **439**, 621-624.
45. Heller, R.C. and Marians, K.J. (2006) Replication fork reactivation downstream of a blocked nascent leading strand. *Nature*, **439**, 557-562.
46. Foiani, M., Lucchini, G. and Plevani, P. (1997) The DNA polymerase alpha-primase complex couples DNA replication, cell-cycle progression and DNA-damage response. *Trends Biochem Sci*, **22**, 424-427.

## LEGENDS TO FIGURES

**Figure 1.** Interaction between XRCC1 and p58 subunit of Pol  $\alpha$ -primase. **(A)** Identification of proteins interacting with GST-XRCC1-BRCT1 by mass spectrometry. Sypro ruby stained gel after GST pull-down of HeLa cell extracts expressing either GST or GST-hXRCC1-BRCT1 fused proteins. **(B)** Identification of XRCC1 associated p58 by immunoprecipitation of XRCC1 from extracts of HeLa cells treated or not with aphidicolin (A: 5  $\mu$ g/mL, 16 h) and with or without HU (HU: 4mM, 4 h). Copurifying p58 and immunoprecipitated XRCC1 were identified by western blot. Inputs represent 5% of the total proteins used in immunoprecipitation. HU: hydroxyurea, pi: pre immune serum. **(C)** GST pull-down of p58 with different GST-fused XRCC1 domains overexpressed in HeLa cells. A schematic drawing of XRCC1 domain structure is shown. **(D)** Indirect immunofluorescence microscopy showing colocalisation of XRCC1 and p58 in HeLa cells treated (+HU) or not (-HU) with 4 mM HU for 4 h.

**Figure 2.** *In vitro* association of XRCC1 with the N-terminal domain of p58 assessed by Far-western blot. Purified p48-His-tagged-p58, His-tagged p58 N-terminal (Nter) or C-terminal (Cter) domains expressed in *E. coli* were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Tropomyosin, BSA and PARP-1 were also included as internal negative and positive controls respectively. The membrane was stained with amido black (left panel) or incubated with purified XRCC1 and revealed with an anti-XRCC1 antibody (right panel). p58 domains were determined by partial tryptic digests and peptide analysis (Schlott and Nasheuer, unpublished data).

**Figure 3.** The p58 subunit of DNA primase binds to PAR leading to its inactivation. **(A)** The indicated purified proteins (1  $\mu$ g each) were dot-blotted onto nitrocellulose and incubated with PAR. Bound PAR was immunodetected with anti-PAR antibody. Loading of proteins was quantified on a Coomassie stained SDS-polyacrylamide gel. **(B)** The indicated purified proteins (2  $\mu$ g) were separated on SDS-polyacrylamide gel, transferred on nitrocellulose membranes and incubated with  $^{32}$ P-labelled PAR. Visualisation of the loaded proteins was performed by Ponceau S staining of the membrane. **(C)** Increasing amounts of purified p48-p58 (1 and 2  $\mu$ g) were dot-blotted on nitrocellulose membrane and a first overlay (buffer, radioactively labelled or unlabelled oligo(dT)<sub>20</sub>, as indicated) was followed by a second incubation (radioactively labelled or unlabelled PAR or buffer) to analyze the competition between PAR and DNA for p58 binding. **(D)** Band shift assay to analyze complex formation of p48-p58 and ssDNA (ssDNA:

1  
2 FX174 single-stranded DNA). Two  $\mu\text{g}$  of purified p48-p58 was incubated with ssDNA (70ng)  
3 and PAR was added in increasing concentration to displace the p48-p58-ssDNA complex. PAR  
4 to DNA ratio varied from 0, 1 to 4 excess of PAR. (E) Addition of increasing amounts of PAR to  
5 primase assay (ratio 0, 1/10, 1:1) significantly inhibited primase activity.  
6  
7  
8  
9

10  
11 **Figure 4.** GST-XRCC1-BRCT1 overexpressed in HeLa cells triggers PAR synthesis and is  
12 poly(ADPribosyl)ated. HeLa cells overexpressing GST-hXRCC1-BRCT1 (aa 282-428, lanes 1,  
13 2, 3), GST-hXRCC1-BRCT2 (aa 427-633, lanes 4, 5, 6) or GST (lanes 7, 8) were either  
14 untreated (lanes 1, 4, 7) or treated with 2.5 mM MMS for 30 min (lanes 2, 5, 8). Where  
15 indicated, cells were preincubated 2 hrs with 100nM of the PARP inhibitor Ku-0058948 (lanes 3  
16 and 6). Proteins were analysed by GST-pull down and Western blot (right panel) using  
17 successively the anti-PAR, anti-PARP-1 and anti-GST antibodies. Left panel: inputs  
18 corresponding to 2% of each lysates.  
19  
20  
21  
22  
23  
24  
25  
26

27 **Figure 5.** GST-hXRCC1-BRCT1 overexpressing cells accumulate in S-phase following DNA  
28 damage. (A) Cell cycle analysis (right) of HeLa cells overexpressing GST-tagged fragments of  
29 hXRCC1 (schematically represented on the right), treated by 1 mM MNU and analysed 20 hrs  
30 after (B) Flow cytometry profiles of BrdU incorporation (DNA synthesis) versus DNA content  
31 (7AAD fluorescence) of HeLa cells overexpressing either GST alone or GST-hXRCC1-BRCT1  
32 20 hrs after treatment or not with 1 mM MNU.  
33  
34  
35  
36  
37  
38

39 **Figure 6.** xXRCC1-BRCT1 inhibits the initiation of DNA synthesis in *Xenopus* egg extracts. (A)  
40 Kinetics of replication of either mock- or MMS-treated sperm chromatin after addition of either  
41 purified GST or GST-xXRCC1-BRCT1 to egg extracts (B) Size of replication products  
42 synthesized in (A) at different times as determined by alkaline gel electrophoresis and  
43 autoradiography (C) Replication reactions were performed as in A) in the presence of MMS or  
44 MMS and PARP inhibitor Ku-0058948 (green curve). (D) Western blot of chromatin-associated  
45 proteins after 60 min incubation in *Xenopus* extracts treated or not with MMS, in the presence of  
46 either GST or GST-xBRCT1.  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

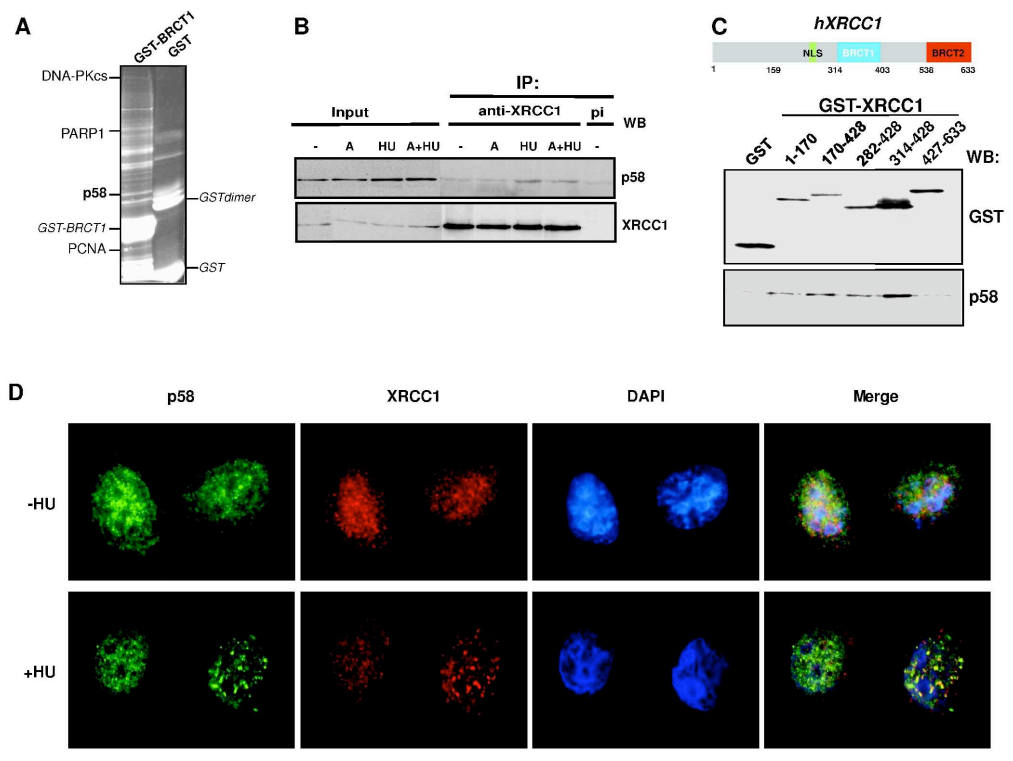


Figure 1

Figure 1  
175x155mm (600 x 600 DPI)

iew

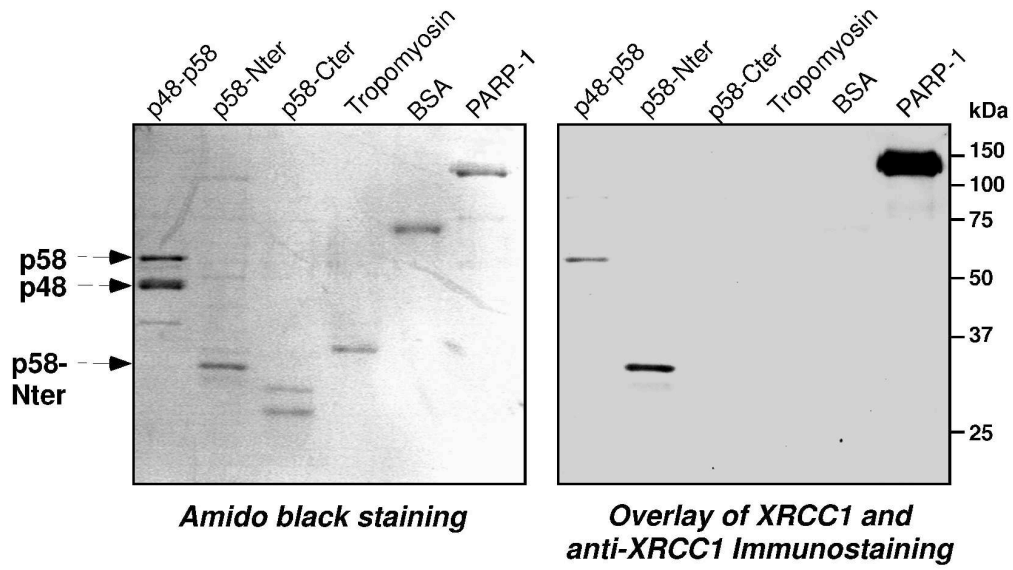


Figure 2

Figure 2  
139x123mm (600 x 600 DPI)

ew

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

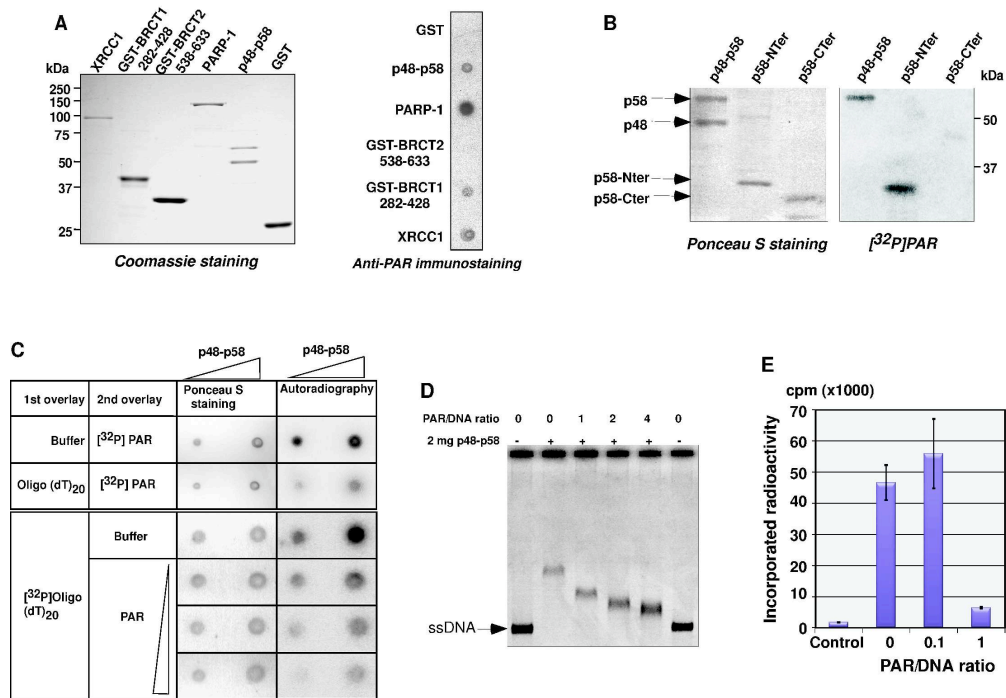


Figure 3

Figure 3  
187x160mm (600 x 600 DPI)

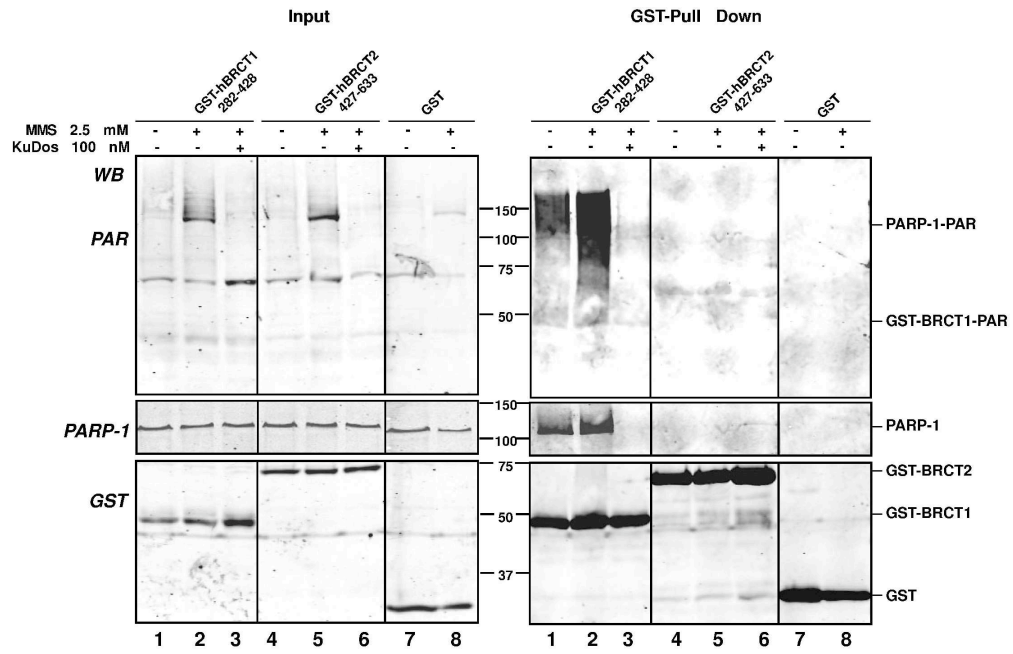


Figure 4

Figure 4  
188x151mm (600 x 600 DPI)

view

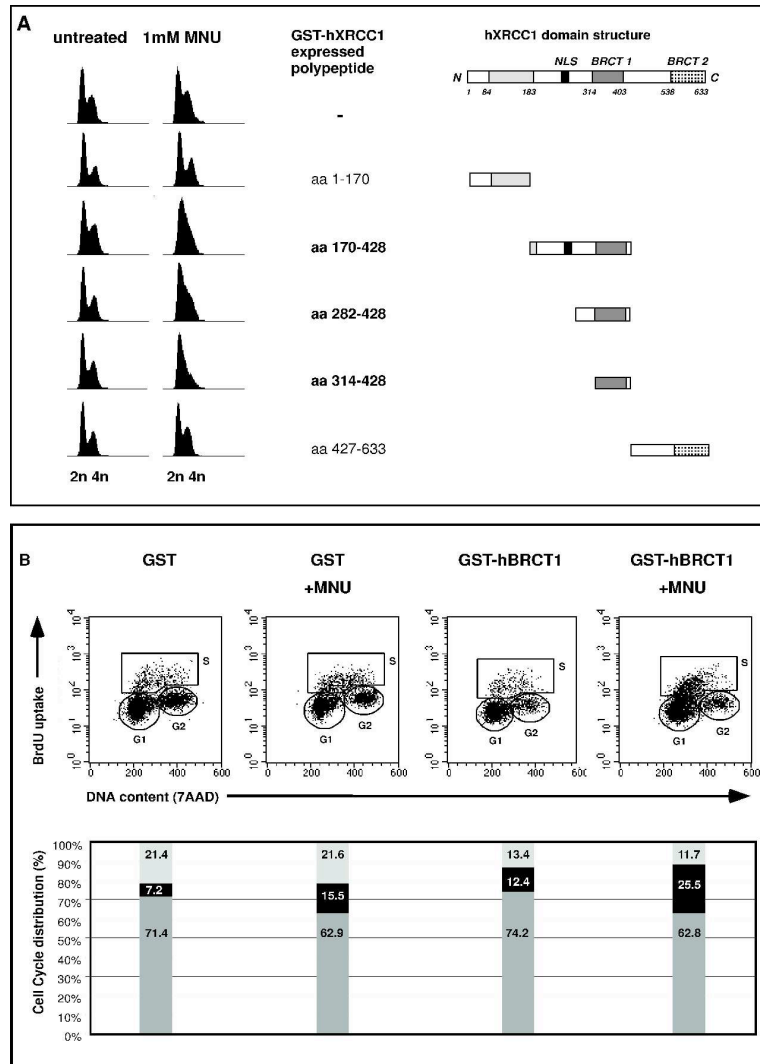


Figure 5

Figure 5  
185x256mm (600 x 600 DPI)

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

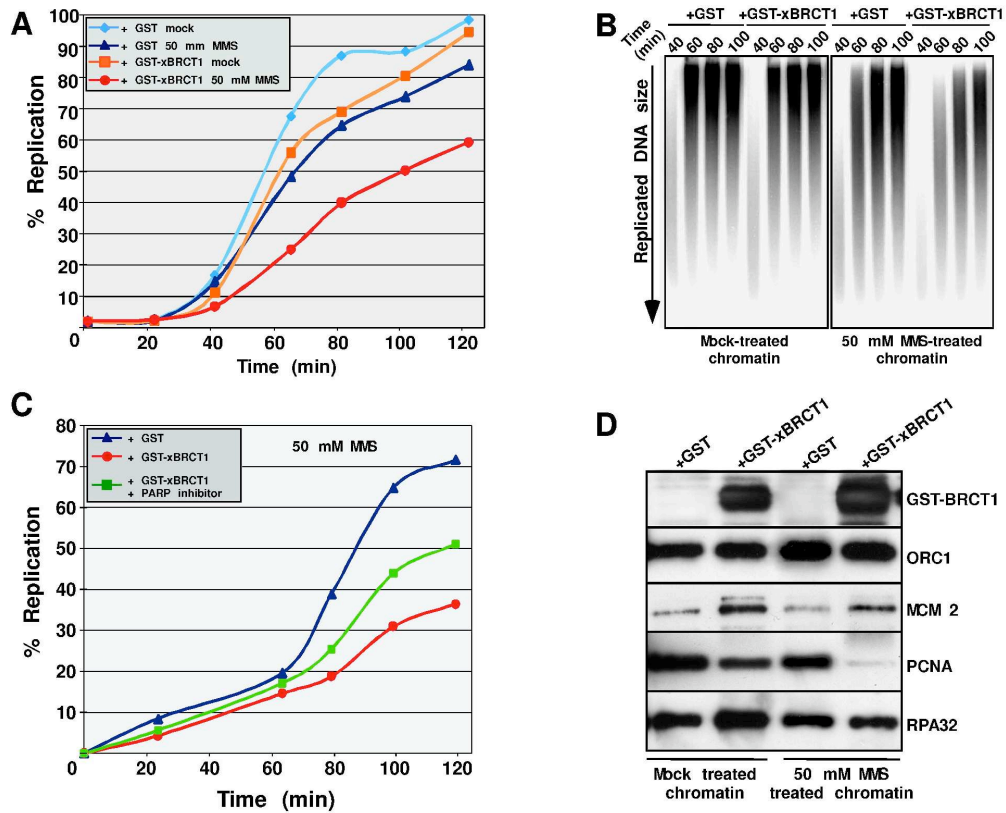


Figure 6

Figure 6  
162x166mm (600 x 600 DPI)

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60