Supplementary Information

hPrimpol1/CCDC111 is a human DNA primase-polymerase required for the maintenance of genome integrity

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Supplementary Materials and Methods

Antibodies

Polyclonal anti-hPrimpol1 or anti-RPA2 antibody was generated by immunizing Rabbits with MBP-hPrimpol1 or MBP-RPA2 fusion proteins expressed and purified from *E. Coli*. Antisera were affinity-purified using AminoLink plus Immobilization and purification kit (Pierce). Monoclonal anti-RPA2 antibody was purchased from EMD Chemicals. Antibodies specifically recognizing the γH2AX, 53BP1, pCHK1, and RPA1 were described previously [1-3]. Anti-Myc (9E10) and anti-GST antibodies were purchased from Covance and Santa Cruz, respectively. Anti-GAPDH and anti-Flag (M2) antibodies were purchased from Millipore and Sigma, respectively. Anti-ATM-pS1981 and anti-CHK2-pT68 antibodies were purchased from Rockland Immunochemicals and Cell Signaling Technology, respectively. CtIP antibody was purchased from Belthyl Laboratories.

Constructs

All cDNAs were subcloned into pDONR201 (Invitrogen) as entry clones and were subsequently transferred to gateway-compatible destination vectors for the expression of N- or C-terminal-tagged fusion protein. All point or deletion mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene) and verified by sequencing.

Cell culture, transfection and shRNAs

293T and HeLa Cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Sf9 insect cells were maintained in Grace's medium supplemented with 2% fetal bovine serum. Human cell lines were maintained in 37°C incubator with 5% CO_2, whereas insect cells were maintained at 27°C. Cell transfection was performed using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol. Lentiviral nonsilencing control shRNA and shRNA target sets were purchased from Open Biosystems. The hPrimpol1 targeting sequences are: #1, 5'-CCCATAAGAGTAATAATAT-3'; #2, 5'-CCAGAAGAATTACTGGTTT-3'. The CtIP targeting sequence is: 5'-CGGCAGCAGAATCTTAAACTT-3'. The nonsilencing control sequence is: 5'-CCCATAAGAGTAATAATAT-3'. The shRNA-resistant wild-type and mutant hPrimpol1 constructs were generated by changing six nucleotides in the shRNA#1 targeting region (C1275T, T1278C, G1281A, T1284C, T1287C, and T1290C substitutions). The shRNAs were packaged into lentiviruses by cotransfecting with packaging plasmids pMD2G and pSPAX2 (kindly provided by Songyang Zhou, Baylor College of Medicine) into 293T cells. Forty-eight hours after transfection, the supernatant was collected for infection of HeLa cells. Infection was repeated twice with an interval of 24 hr to achieve maximal infection efficiency. Infected cells were selected with media containing puromycin (2 μg/ml).

The establishment of stable cell lines and affinity purification of S-Flag-SBP(SFB)-tagged protein complexes

293T cells were transfected with plasmids encoding SFB-tagged proteins. Cell lines stably expressing tagged proteins were selected by culturing in medium containing

puromycin (2 μg/ml) and confirmed by immunoblotting and immunostaining. For affinity purification, 293T cells stably expressing tagged proteins were lysed with NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) for 20 min. Crude lysates were removed by centrifugation at 14,000 rpm at 4°C for 10 min, and the pellet was sonicated for 40 s in high-salt solution (20 mM HEPES [pH 7.8], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitor) to extract chromatin-bound protein fractions. The supernatants were cleared at 14,000 rpm to remove debris and then incubated with streptavidin-conjugated beads (Amersham Biosciences) for 2 hr at 4°C. The beads were washed three times with NETN buffer, and then bead-bound proteins were eluted with NETN buffer containing 1 mg/ml biotin (Sigma). The elutes were incubated with S protein beads (Novagen) for 2 hr at 4°C. The beads were again washed three times with NETN buffer and subjected to SDS-PAGE. Protein bands were excised and digested, and the peptides were analyzed by mass spectrometry.

Co-immunoprecipitation and western blotting

For whole-cell extracts, the cells were solubilized in NETN lysis buffer supplemented with 50 U/μl benzonase (Novagen), protease inhibitors and phosphatase inhibitors. After removal of cell debris by centrifugation, the soluble fractions were collected. For Flag immunoprecipitations, a 0.8-ml aliquot of lysate was incubated with 1 μg of the Flag monoclonal antibody and 25 μl of a 1:1 slurry of Protein A Sepharose for 2 hours at 4°C. For endogenous immunoprecipitations, 1 mg of the whole-cell extract was incubated with 25 μl of a 1:1 slurry of Protein A Sepharose coupled with 2 μl indicated antibodies for 2 hr at 4°C. The Sepharose beads were washed three times with NTEN buffer, boiled in 2

X SDS loading buffer, and resolved on SDS-PAGE. Membranes were blocked in 5% milk in TBST buffer and then probed with antibodies as indicated.

Immunofluorescence staining

Indirect immunofluorescence was carried out as described[1, 2]. HeLa cells cultured on coverslips were treated with HU (2 mM) for 16 hr, or IR (10 Gy) for 3 hr, respectively. Cells were then washed with PBS, pre-extracted with buffer containing 0.5% Triton X-100 for 5 min and fixed with 3% paraformaldehyde for 10 min at room temperature. Cells were incubated in primary antibody for 30 min at room temperature. Following three 5-min washed with PBS, secondary antibody was added at room temperature for 30 min. Cells were then stained with DAPI to visualize nuclear DNA. The coverslips were mounted onto glass slides with anti-fade solution and visualized using a Nikon ECLIPSE i80 fluorescence microscope with a Nikon Plan Fluor 60 X oil objective lens.

Protein purification

Full-length RPA1 was cloned into MBP-His-tagged vector for the expression of MBP-His-tagged-RPA1 protein in insect cells. Transposition occurred in DH10Bac-competent cells, and correct bacmids confirmed by PCR were transfected into Sf9 cells for baculovirus production. After viral amplification, Sf9 cells were infected with baculovirus stocks expressing MBP-RPA1-His for 48 hr. Cells were harvested and washed with 1 X PBS and resuspended in lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 1% Triton X-100, and 1 μg/mL each of leupeptin, aprotinin and pepstatin). The extract was centrifuged at 18000 rpm for 40 min. The supernatant was collected and loaded onto

a pre-equilibrated Ni-NTA agarose and washed with lysis buffer plus 20 mM imidazole and protease inhibitor. The bound protein was then eluted with lysis buffer containing 200 mM imidazole and protease inhibitor. Peak fractions were pooled and incubated with Amylose resins for 2 hr at 4°C. After washing the beads with 100 ml of washing buffer (20 mM Tris-HCl, 500 mM NaCl, 0.5% NP-40, 1 mM DTT, and 1 μg/ml each of leupeptin, aprotinin and pepstatin), the bound protein was used for pull-down assays. Full-length RPA2 and RPA3 were cloned into pDONR201 as entry clones and were then transferred to destination vector for the expression of MBP-tagged fusion protein in *E. Coli*. Cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 1% Triton X-100, and 1 μg/mL each of leupeptin, aprotinin and pepstatin). After sonicating, the extract was centrifuged at 18000 rpm for 40 min. The supernatant was collected and incubated with Amylose resins for 2 hr at 4°C. After washing the beads with washing buffer (20 mM Tris-HCl, 500 mM NaCl, 0.5% NP-40, 1 mM DTT, and 1 μg/ml each of leupeptin, aprotinin and pepstatin), the bound protein was used for pull-down assays. hPrimpol1 wild-type and mutants were expressed in *E.coli* using PGEX-6P-1 vector containing a glutathione S-transferase (GST) tag. Cells were grown at 37ºC until log phase, and were induced with 0.2 mM IPTG at 17ºC for 16 hr. Cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 1 % Triton X-100, 1 mM DTT, and 1 μg/ml each of leupeptin, aprotinin and pepstatin) and then sonicated. The extract was centrifuged at 18000 rpm for 40 min. The supernatant was collected and incubated with glutathione-Sepharose resin for 4 hr at 4°C. After washing the beads with washing buffer (20 mM Tris-HCl, 500 mM NaCl, 0.5 % NP-40, 1 mM DTT, and 1 μg/ml each of leupeptin, aprotinin and pepstatin), the bound proteins were then washing with

PreScission Cleavage buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, and 1 μg/ml each of leupeptin, aprotinin and pepstatin) and incubated with GST-tagged PreScission enzyme overnight at 4°C. The released protein was loaded on Pre-equilibrated 5-ml Hitrap Q column (GE Healthcare), which was washed with 10 ml A buffer (50 mM Tris-HCl, PH 8.0, 1 mM DTT) and then eluted with a 100 ml gradient of 0-400 mM NaCl in A buffer. Peak fractions were pooled and concentrated with a 30-kDa Amicon Ultra centrifugal filter device (Millipore).

Cell survival assays

Control shRNA- and hPrimpol1 shRNA-expressing HeLa cells (1 X 10^3) were seeded onto 60 mm dishes in triplicates. 72 hr after seeding, cells were treated with HU or IR at indicated concentrations. Medium was replaced 24 hr later and cells were then incubated for 14 days. Resulting colonies were fixed and stained with Coomassie blue. Numbers of colonies were counted using a Gel Doc with Quantity One software (Bio-Rad).

Replication Labeling and DNA Fiber Spreads

Exponentially growing cells were labeled with 50 μM IdU for 20 min. Cells were then treated with 2 mM hydroxyurea for 2 hr and incubated in 50 mM CIdU for 20 min after washout of the drug. DNA spreads were prepared as previously described [4] with some modifications. Briefly, cells were trypsinized and resuspended in PBS at 1 X 10^6 cells per milliliter. Two milliliters of this suspension were spotted onto a clean glass slide and lysed with eight milliliters of spreading buffer (0.5% SDS in 200 mM Tris-HCl at pH 7.4, 50 mM EDTA). After 6 min, the slides were tilted at 15°C to horizontal, allowing the

DNA to spread. Slides were air-dried, fixed in methanol and acetic acid (3:1) for 3 min, and refrigerated overnight before immunolabeling. DNA was denatured with 2.5 M HCl for 45 min at room temperature. Glass slides were rinsed three times in PBS and incubated in blocking buffer (PBS + 0.1% Triton X-100 + 5% BSA) for 30 min at room temperature. The slides were then incubated for 1 hr in rat anti-BrdU antibody (Abcam, ab6326) diluted 1:1000 in blocking buffer. After washing with blocking buffer containing 500 mM NaCl, the slides were incubated for 2 hr in donkey anti-rat Alexa 488 antibody (1:1000, invitrogen). The slides were washed with blocking buffer and then incubated for 2 hr with mouse anti-Brdu antibody diluted in blocking buffe (1:100, Roche). Following an additional wash with blocking buffer containing 500 mM NaCl, the slides were stained for 2 hr with Rhodamine conjugated anti-mouse lgG (1:500, Jackson immunoresearch). Fibers were then analyzed using a Nikon ECLIPSE i80 microscope. The lengths of DNA tracts corresponding to IdU and CIdU labeling were measured using NIS-Elements software; Statistics were performed using a two-tailed unpaired t-test.

Lentivirus Packaging and Infection

Tet-On inducible SFB-tagged lentiviral vector and packaging plasmids (pMD2G and pSPAX2) were kindly provided by Professor Songyang Zhou (Baylor College of Medicine). hPrimpol1 entry constructs were transferred into the Gateway-compatible SFB-tagged lentiviral vector. Virus supernatant was collected 48 hr after the co-transfection of lentiviral vectors and packaging plasmids (pMD2G and pSPAX2) into 293T cells. Cells were infected with viral supernatants with the addition of 8 μg/ml polybrene (Sigma), and stable pools were selected with medium containing 500 μg/ml

G418 (Calbiochem). The expression of the indicated genes in the stable pools was induced by the addition of 1 μg/ml doxycycline (Sigma) for 24 hr for the experiments presented in this report.

Detection of chromosome aberrations

hPrimpol1-depleted HeLa cells were cultured in colcemid (1 μg/ml) for 4 hr and were then harvested and treated with hypotonic solution (75 mM KCl) for 15 min at 37°C. Cells were fixed with methanol/acetic acid (3:1) (vol/vol) for 20 min. The cell suspension was dropped onto ice-cold wet glass slides and air dried. The cells were stained with 5% Giemsa solution for 5 min and examined by light microscopy. Chromosome aberrations were scored in 50 metaphases per sample.

Supplementary References

1. Liu T, Ghosal G, Yuan J, Chen J, Huang J (2010) FAN1 acts with FANCI-FANCD2 to promote DNA interstrand cross-link repair. *Science* **329:** 693-696
2. Wan L, Han J, Liu T, Dong S, Xie F, Chen H, Huang J (2013) Scaffolding protein SPIDR/KIAA0146 connects the Bloom syndrome helicase with homologous recombination repair. *Proc Natl Acad Sci U S A*
3. Huang J, Huen MS, Kim H, Leung CC, Glover JN, Yu X, Chen J (2009) RAD18 transmits DNA damage signalling to elicit homologous recombination repair. *Nat Cell Biol* **11:** 592-603
4. Jackson DA, Pombo A (1998) Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J Cell Biol* **140:** 1285-1295

Supplementary Figure Legends

Figure S1. (**A-B**) Characterization of hPrimpol1 antibody. HeLa cells infected with the indicated shRNA were lysed in the presence of benzonase. Cell lysates were incubated with protein A agarose beads conjugated with indicated antibodies and Western blot analysis was carried out with affinity-purified anti-hPrimpol1 (**A**), or preserum (**B**). The anti-hPrimpol1 antibody recognized a doublet by Western blot, which may suggest that hPrimpol1 is post-translationally modified. Asterisk indicates a non-specific band. (**C**) CtIP depletion abolished IR-induced recruitment of RPA and the downstream hPrimpol1 to DNA damage sites. SFB-tagged hPrimpol1 was expressed in CtIP-depleted HeLa cells. Foci assembled by this fusion protein and by RPA2 following exposure to IR (10 Gy) followed by recovery for 2 hr were detected by immunofluorescence using anti-Flag and anti-RPA2 antibodies, respectively. A merged image shows colocalization. Scale bar, 10 μm. (**D**) Knockdown efficiency was confirmed by immunoblotting.

Figure S2. RPA1 associates with hPrimpol1 through its DBD-C domain. (**A**) Schematic representation of full length RPA1 and the mutants used in this study. Their ability to bind to hPrimpol1 is indicated. (**B**) RPA1 with DBD-C domain deletion could not bind to hPrimpol1. 293T cells were transfected with plasmids encoding SFB-tagged wild-type or mutant RPA1 together with plasmid encoding Myc-tagged hPrimpol1. Cell lysates were immunoprecipitated with anti-Flag antibody and Western blot analysis was performed with indicated antibodies. (**C**) The DBD-C domain of RPA1 is not required for its foci formation. 293T cells were transfected with plasmids encoding SFB-tagged wild-type or

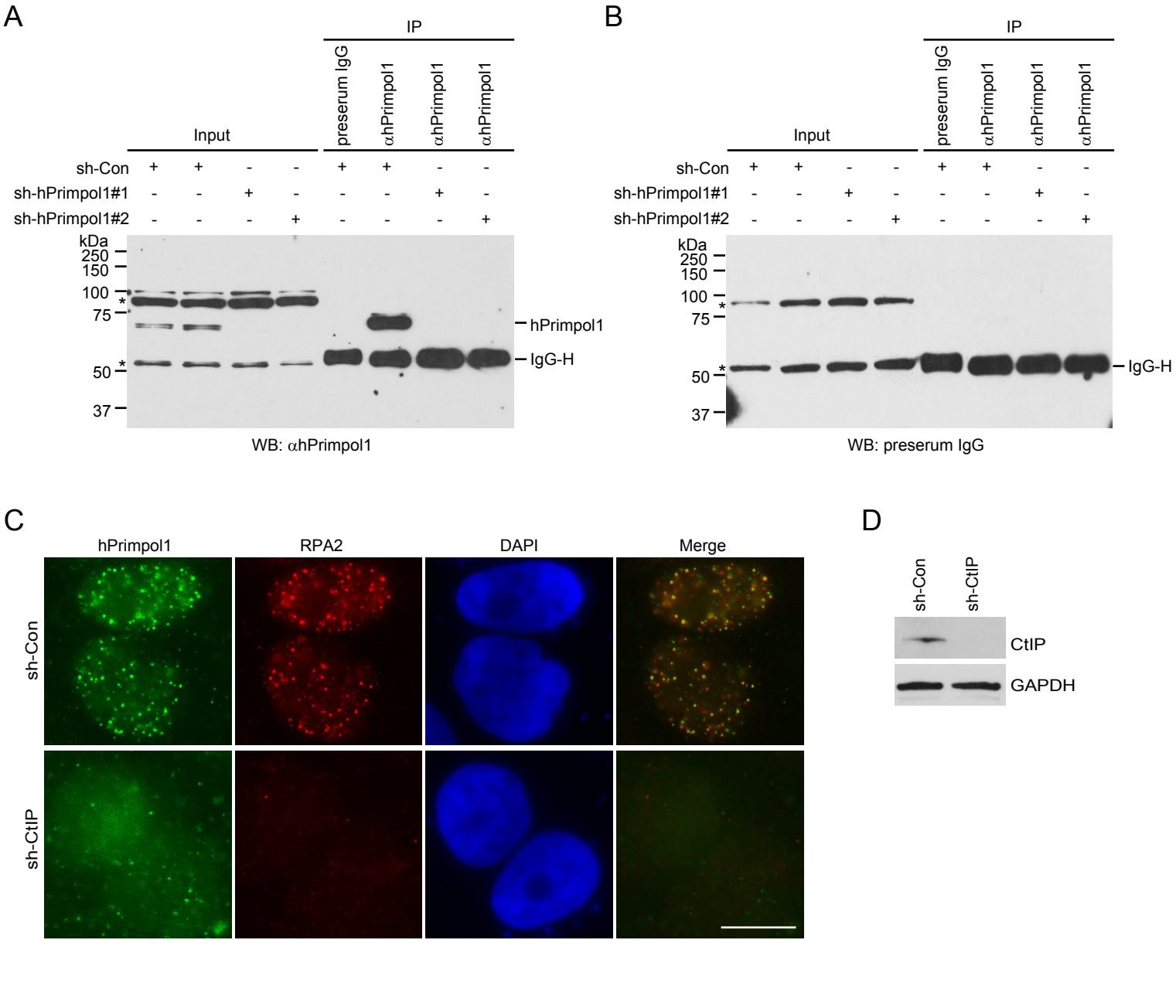
mutant RPA1. Immunostaining experiments were performed 16 hours after HU treatment using indicated antibodies. Scale bar, 10 μm.

Figure S3. (**A-B**) pATM and 53BP1foci are greatly increased in hPrimpol1-depleted HeLa cells. HeLa cells were infected with lentiviruses carrying non-target control or hPrimpol1 shRNAs. 72 hr later, cells were subjected to immunostaining using indicated antibodies (**A**). Scale bar, 10 μm. The quantification of foci-positive cells was performed by counting a total of 200 cells per sample (**B**). Error bars are s.d.; n=3. (**C**) CHK2 kinase was activated in HeLa cells depleted of hPrimpol1. hPrimpol1-depleted cells were collected and subjected to immunoblotting with indicated antibodies. (**D**) IdU tract length distributions from DNA fibers from control and hPrimpol1-depleted HeLa cells in the presence or absence of HU. (**E**) Quantification of the percentage of newly fired origins after the removal of HU was determined by counting CIdU-containing tracts. Error bars are s.d.; n=3.

Figure S4. Full gel scans of key blots in this study.

Supplementary Table S1. DNA fiber spread data analysis information. The lengths of DNA tracts corresponding to IdU and CIdU labeling were measured using NIS-Elements software; Statistics were performed using a two-tailed unpaired *t*-test.

Figure S1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αhPrimpol1
αhPrimpol1
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sh-hPrimpol1#2 - - - + - - - +
kDa
250
150
100
75
50
37
*
*
hPrimpol1
IgG-H
WB: αhPrimpol1
B
IP
Input
preserum IgG
αhPrimpol1
αhPrimpol1
αhPrimpol1
sh-Con + + - - + + - -
sh-hPrimpol1#1 - - + - - - + -
sh-hPrimpol1#2 - - - + - - - +
kDa
250
150
100
75
50
37
*
*
IgG-H
WB: preserum IgG
C
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RPA2
DAPI
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sh-CtIP
D
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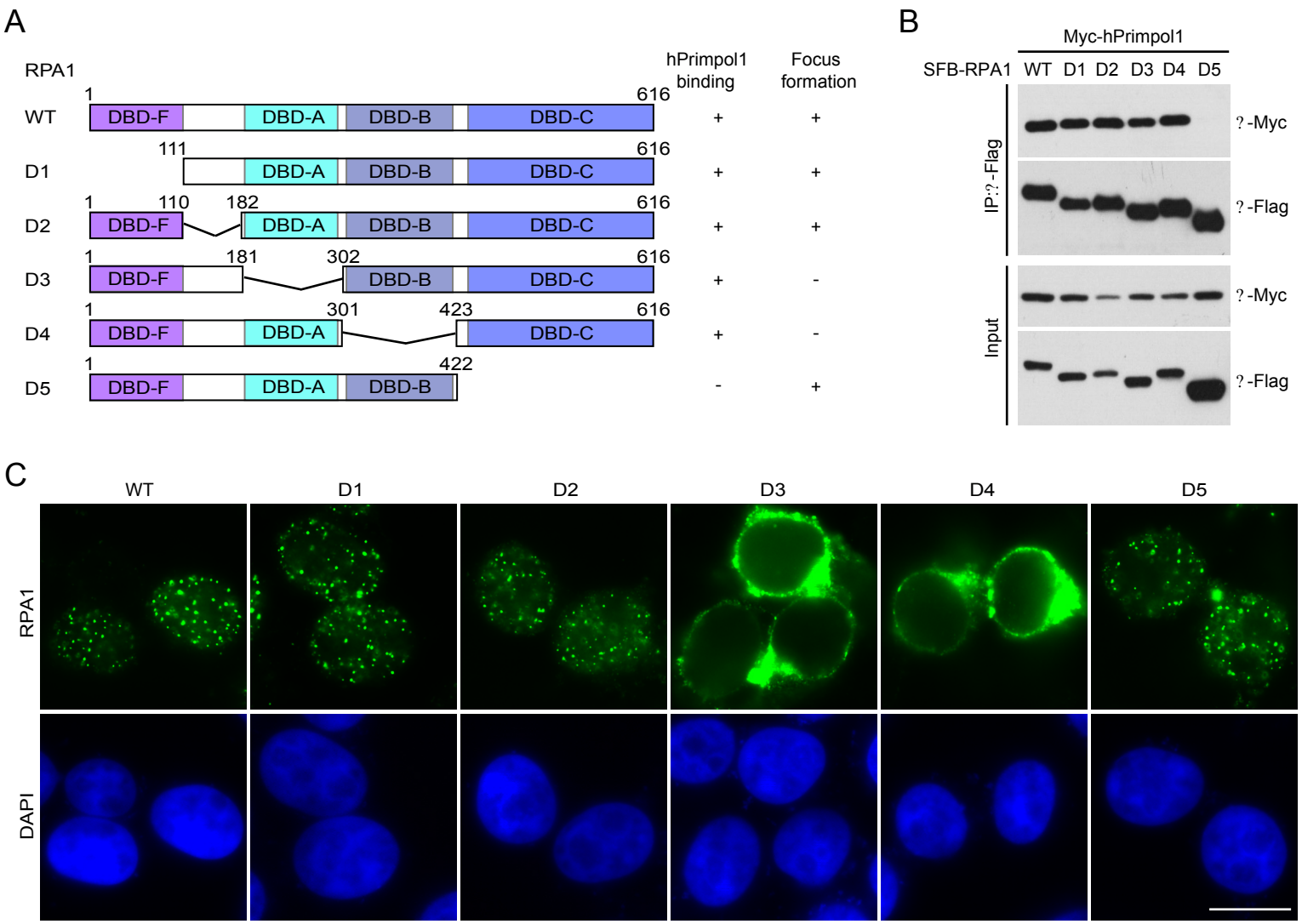
sh-CtIP
CtIP
GAPDH

Figure S2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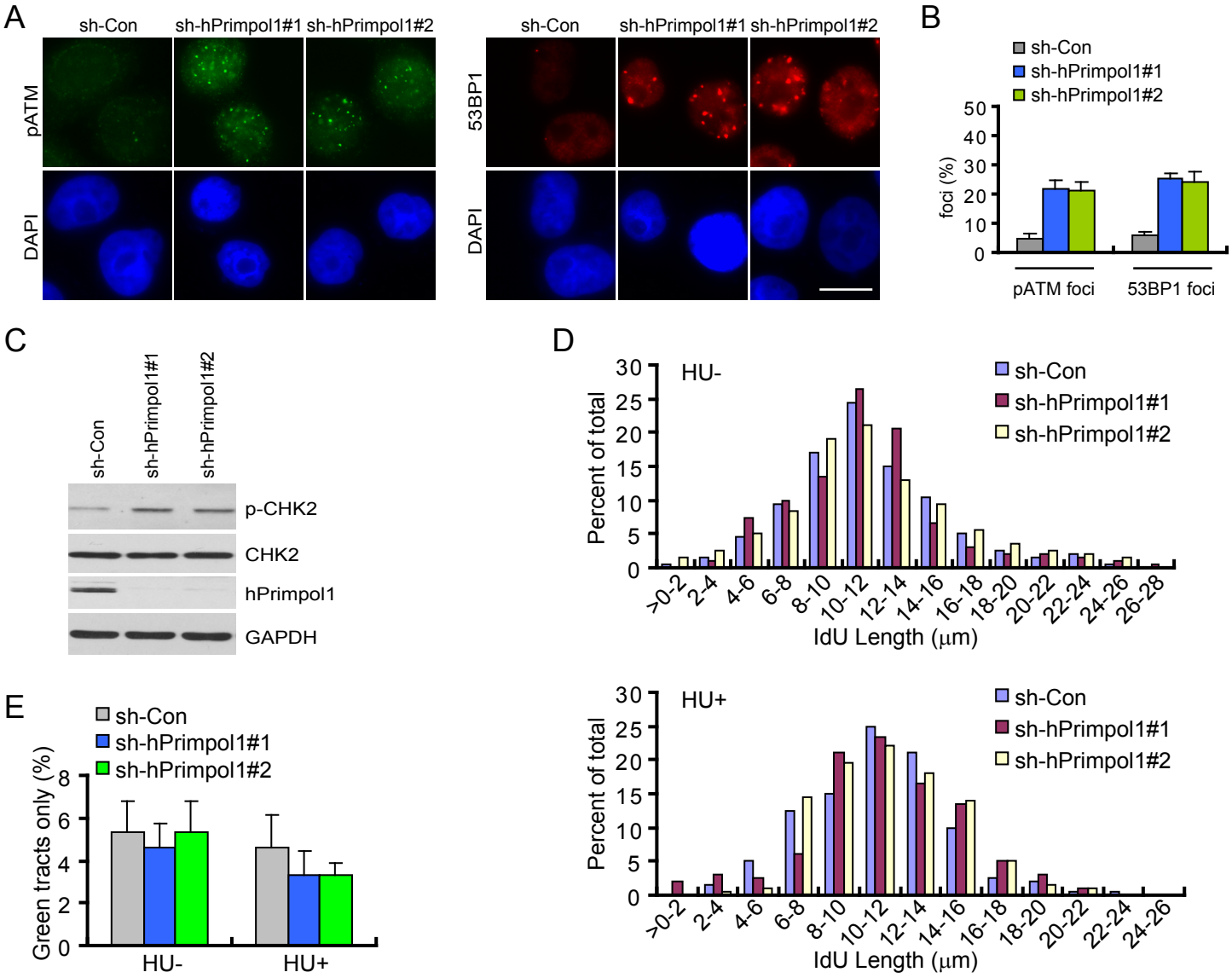
RPA1
hPrimpol1 binding
Focus formation
WT
D1
D2
D3
D4
D5
DBD-F
DBD-A
DBD-B
DBD-C
1
111
110
182
181
302
301
423
422
616
+
-
B
Myc-hPrimpol1
SFB-RPA1
WT D1 D2 D3 D4 D5
IP:?-Flag
Input
?-Myc
?-Flag
C
WT
D1
D2
D3
D4
D5
RPA1
DAPI

Figure S3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foci (%)
pATM foci
53BP1 foci
C
sh-Con
sh-hPrimpol1#1
sh-hPrimpol1#2
p-CHK2
CHK2
hPrimpol1
GAPDH
D
HU-
HU+
Percent of total
IdU Length (μm)
E
Green tracts only (%)
HU-
HU+

Figure S4

Figure1E

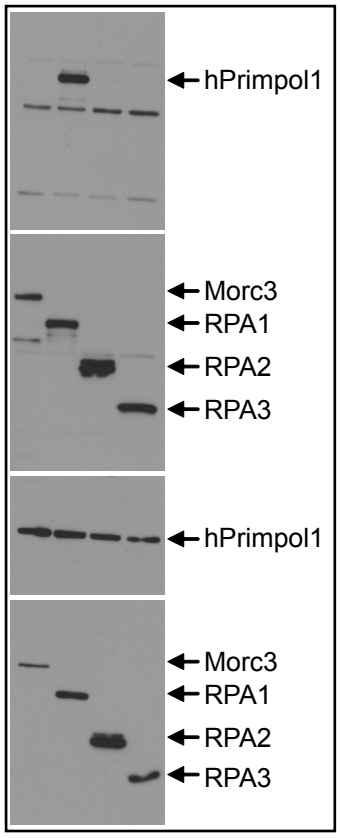

Figure1F

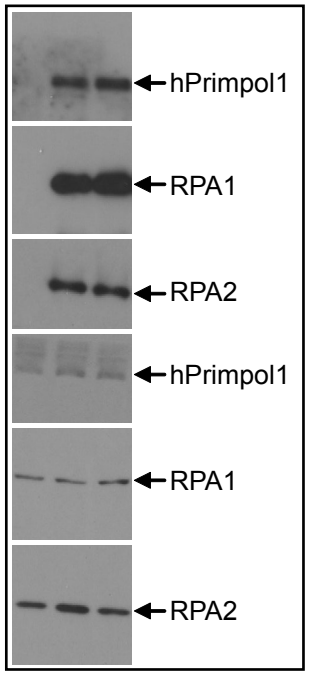

Figure1G

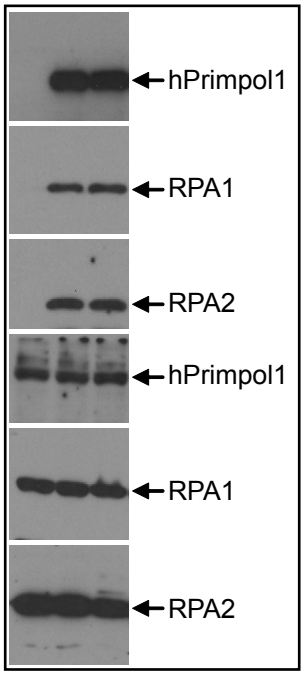

Figure1H

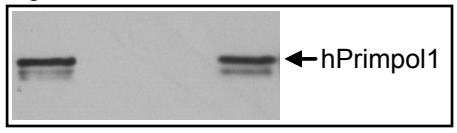

Figure2B

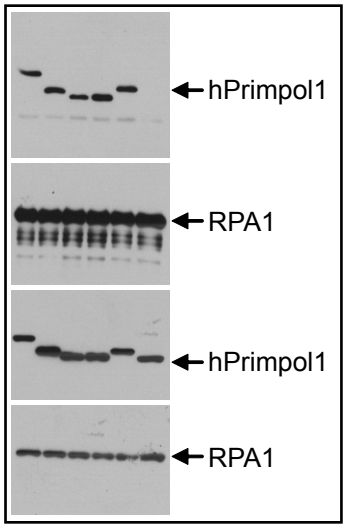

Figure2F

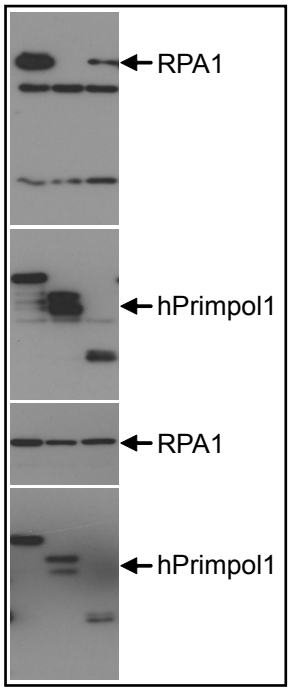

Figure3B

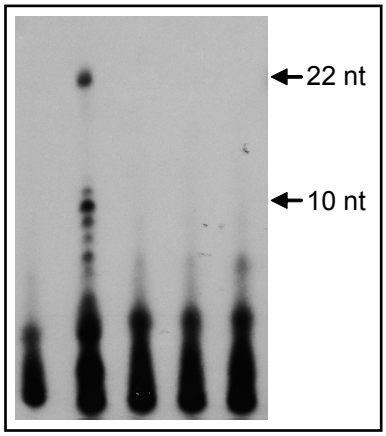

Figure3D

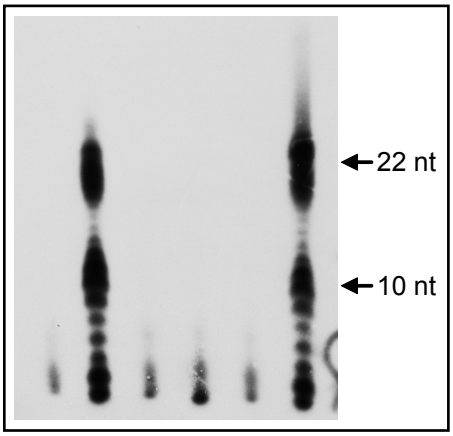

Figure4I

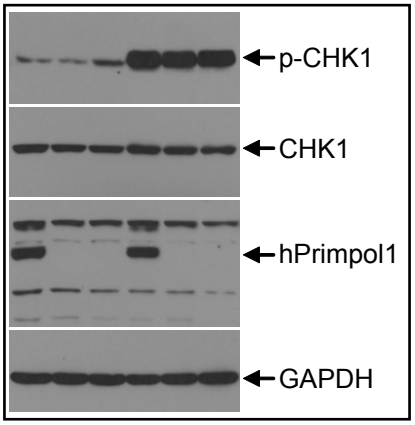

Figure5A

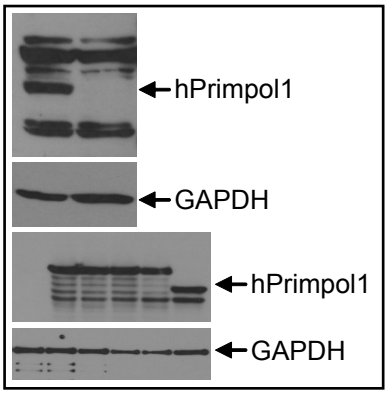

Table S1

Cell line	Treatment	Fiber analyzed	#fibers	#experiments	Mean (μM)	SEM	*p-value (two-tailed)
1.sh-Con	medium	IdU	189	3	11.47	0.2958	
2.sh-hPrimpol1#1	medium	IdU	191	3	11.49	0.3052	0.9670 vs. 1
3.sh-hPrimpol1#2	medium	IdU	190	3	11.74	0.3487	0.5952 vs 1
4.sh-Con	medium	CldU	200	3	10.76	0.2972	
5.sh-hPrimpol1#1	medium	CldU	200	3	10.59	0.3099	0.6933 vs 4
6.sh-hPrimpol1#2	medium	CldU	200	3	10.95	0.3166	0.6636 vs 4
7.sh-Con	HU	IdU	191	3	11.07	0.2500	
8.sh-hPrimpol1#1	HU	IdU	194	3	11.33	0.2735	0.2724 vs 7
9.sh-hPrimpol1#2	HU	IdU	194	3	11.45	0.2396	0.2805 vs 7
10.sh-Con	HU	CldU	200	3	3.09	0.1046	
11.sh-hPrimpol1#1	HU	CldU	200	3	1.51	0.0793	<0.0001 vs 10
12.sh-hPrimpol1#2	HU	CldU	200	3	1.60	0.0883	<0.0001 vs 10
13.Vector / sh-hPrimpol1#1	HU	CldU	200	3	1.44	0.0796	<0.0001 vs 14
14.WT / sh-hPrimpol1#1	HU	CldU	200	3	3.22	0.1243	
15.D114A / sh-hPrimpol1#1	HU	CldU	200	3	1.36	0.0612	<0.0001 vs 14
16.H169N / sh-hPrimpol1#1	HU	CldU	200	3	1.47	0.0761	<0.0001 vs 14
17.H426D / sh-hPrimpol1#1	HU	CldU	200	3	1.52	0.0790	<0.0001 vs 14
18.D5 / sh-hPrimpol1#1	HU	CldU	200	3	1.58	0.0703	<0.0001 vs 14