Supplementary Information

for

Human RECQ1 promotes restart replication forks reversed by DNA topoisomerase I inhibition.

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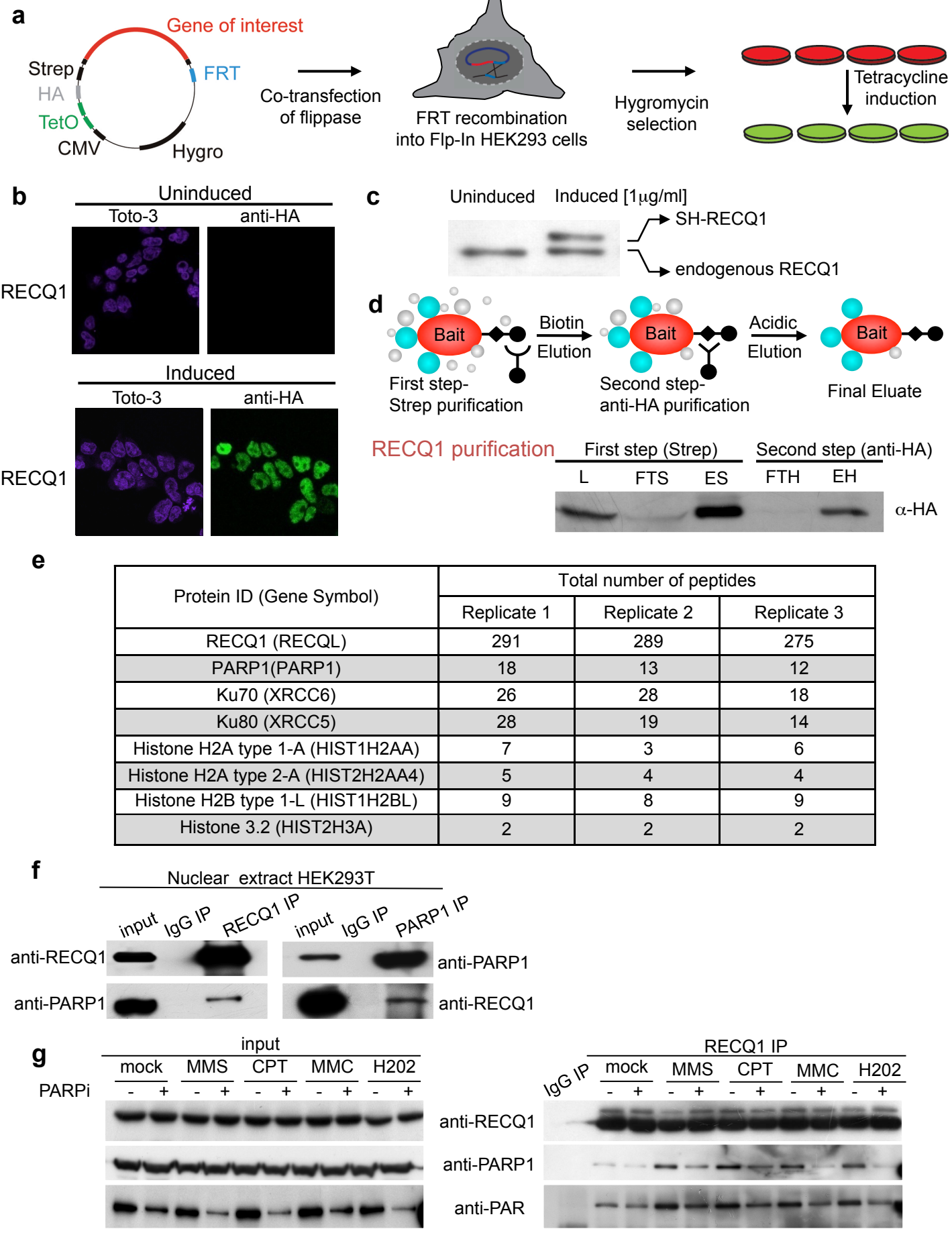
List of Supplementary Information

Supplementary Figure 1

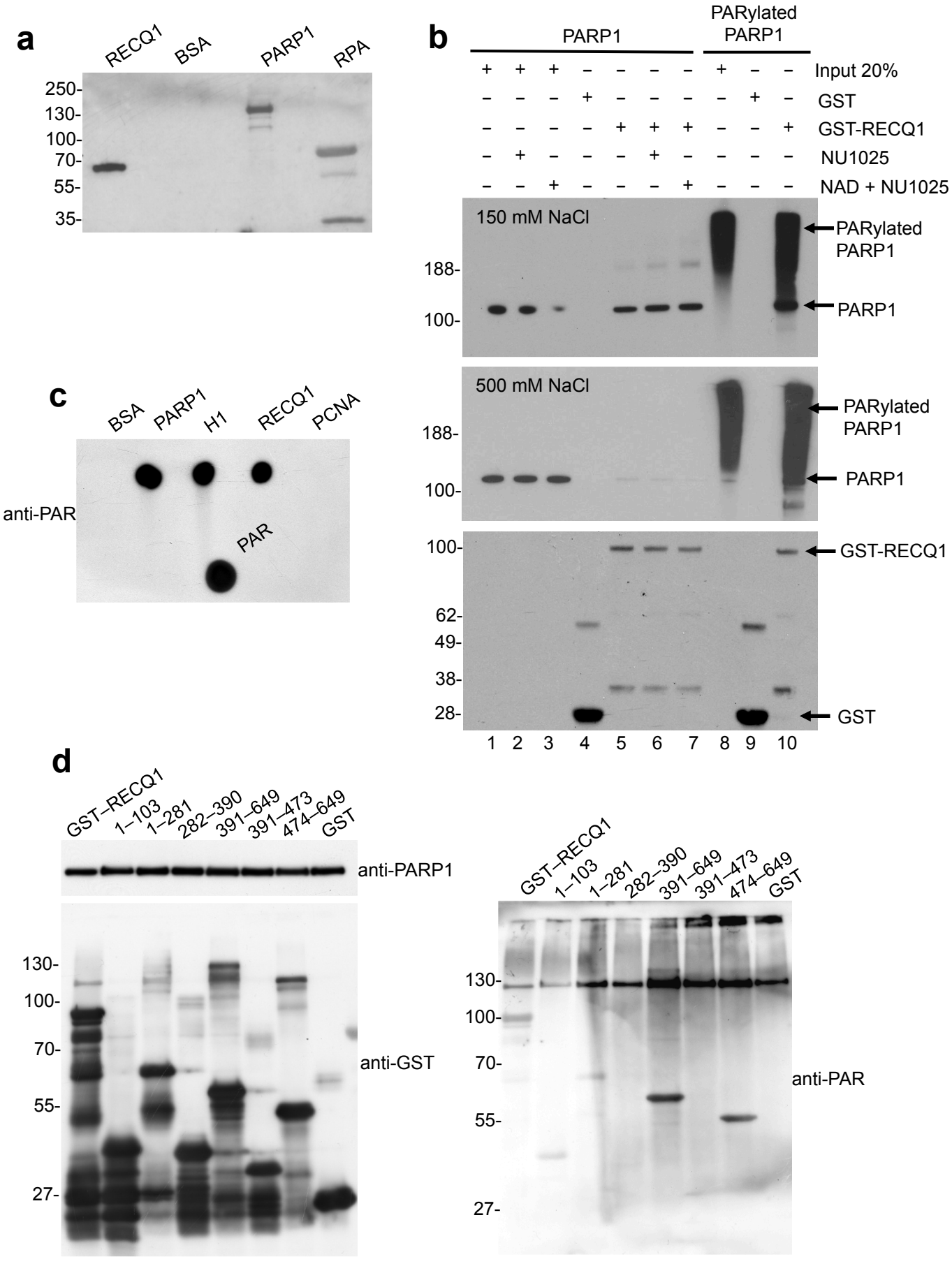


e

Protein ID (Gene Symbol)	Total number of peptides		
	Replicate 1	Replicate 2	Replicate 3
RECQ1 (RECQL)	291	289	275
PARP1(PARP1)	18	13	12
Ku70 (XRCC6)	26	28	18
Ku80 (XRCC5)	28	19	14
Histone H2A type 1-A (HIST1H2AA)	7	3	6
Histone H2A type 2-A (HIST2H2AA4)	5	4	4
Histone H2B type 1-L (HIST1H2BL)	9	8	9
Histone 3.2 (HIST2H3A)	2	2	2

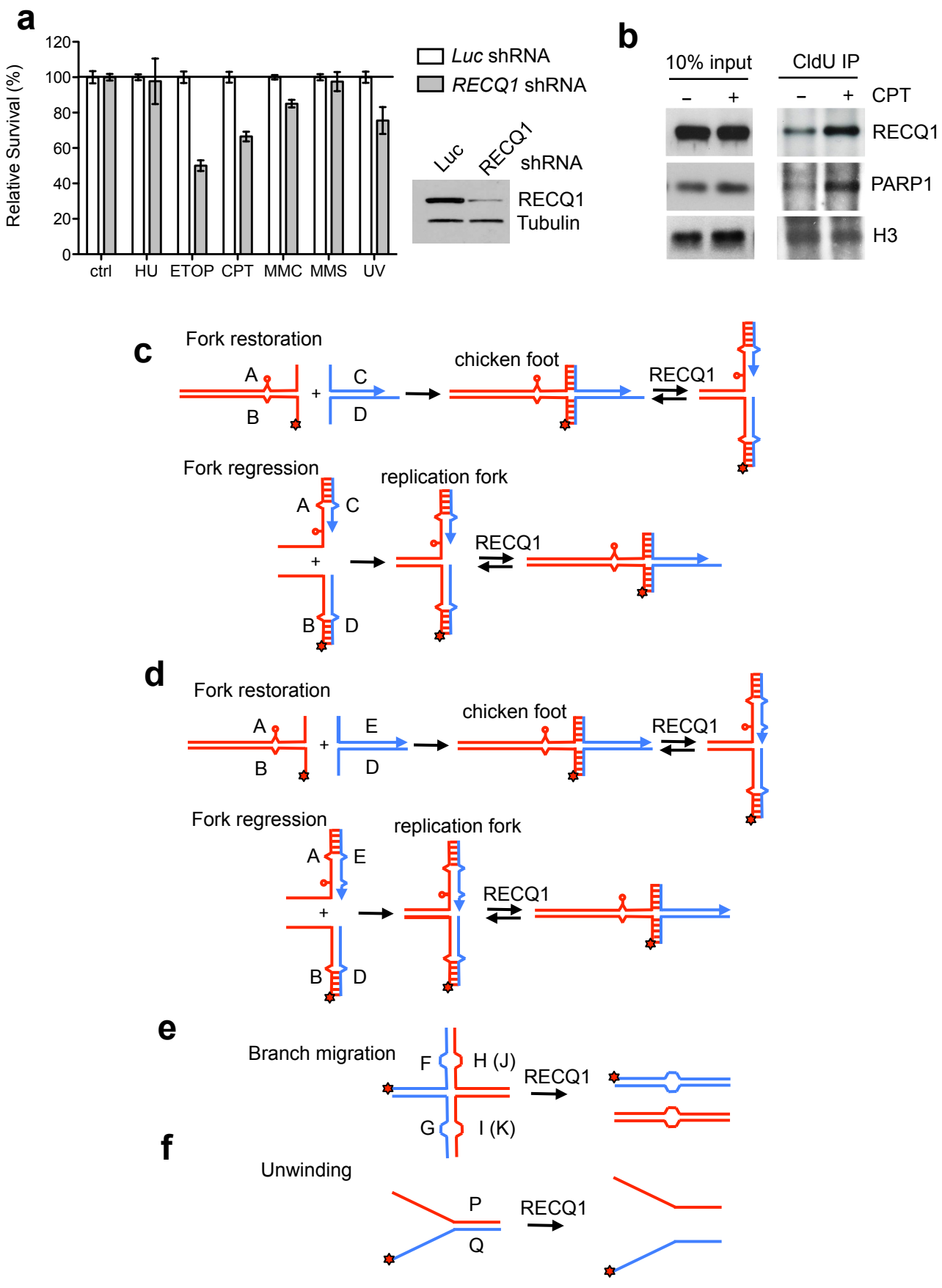
Supplementary Figure 1. Analysis of the human RECQ1 helicase interactome. (**a**) Schematic representation of the procedure followed for the generation of the tetracycline-inducible cell lines expressing a double-tagged version of human RECQ1. Isogenic cell lines were generated using Flp-recombinase-mediated integration into a single FRT site in Flp-in 293T-Rex cells that contain the genomic Flp-In site and a tet repressor (Invitrogen). The expression levels of the bait protein can be easily adjusted by tetracycline to levels that are comparable with corresponding endogenous protein levels. (**b**) Isogenic bait protein expression in the presence and absence of 1µg/ml tetracycline was visualized by indirect fluorescence microscopy with an anti-HA antibody. (**c**) Bait protein expression monitored by immunoblotting using an anti-HA antibody. (**d**) Schematic representation of the procedure for SH-tagged RECQ1 purification. The protein complexes containing RECQ1 were isolated using a small double-affinity tag (SH-tag) consisting of a streptavidin-binding peptide and a hemagglutinin (HA) epitope tag. Western blot analysis of the SH-purification steps monitored using the anti-HA antibody. L: lysate; FTS: flow-through after streptavidin purification; ES: elution from streptavidin sepharose; FTH: flow-through after anti-HA purification; EH: elution form the anti-HA agarose (final eluate). (**e**) RECQ1 interacting proteins identified by mass spectrometry. Following trypsin digestion, the samples were desalted and loaded directly onto a reverse-phase HPLC column coupled to a mass spectrometer. We performed three biological replicate SH-purification experiments, and analyzed each sample once by LC-MS/MS as already described[1]. To eliminate co-purifying contaminant proteins, we generated a database of proteins identified from 3 independent SH–eGFP control purifications analyzed by LC-MS/MS. Proteins identified in bait-specific experiments that were also present in the contaminant database were considered as non-specific binders and removed from the data set. Only the proteins specifically associated with the bait-RECQ1 protein in all three replicates were considered as significant. The total number of peptides identified for each indicated protein in three biological replicates is shown. Additional isoforms of histone H2A were also present (data not shown). (**f**) IPs from 293T-Rex cells using the anti-RECQ1 or the anti-PARP1 antibody. Rabbit IgG IP served as a negative control. (**g**) IPs from U-2 OS cells using the anti-RECQ1 antibody ± PARP inhibitor (50 µM NU1025) and ± DNA damage (2 mM methyl methanesulfonate (MMS) for 30 min, 100 nM CPT for 2 hrs; 2 µM mitomycin-C (MMC) for 2 hrs; or 5 mM H_2O_2 for 30 min). MMS, MMC, and H_2O_2 were all from Sigma.

Supplementary Figure 2



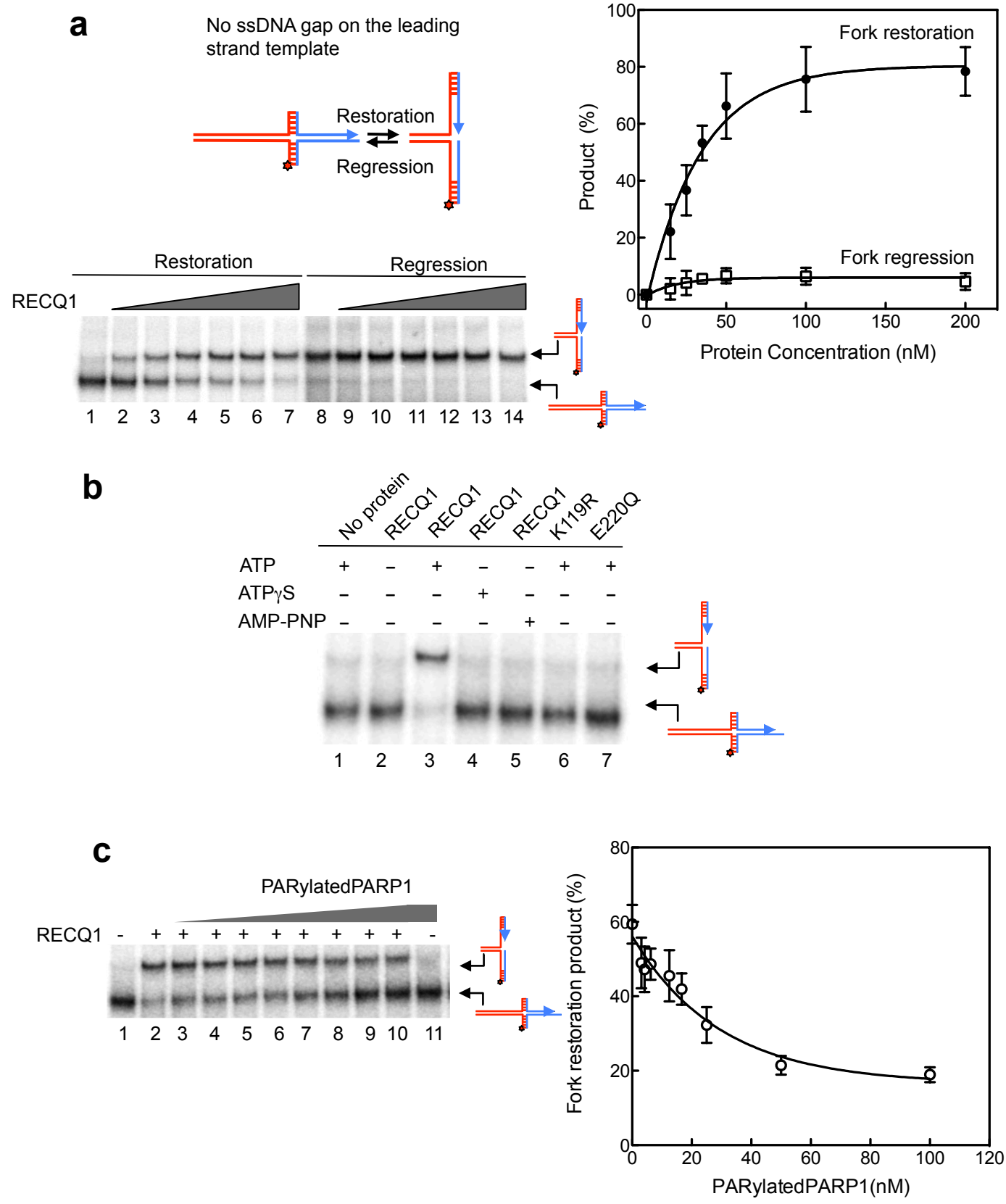
Supplementary Figure 2. RECQ1 interaction with unmodified recombinant PARP1, PARylatedPARP1 and PAR, and *in vitro* analysis of RECQ1 poly(ADPribosyl)ation. (**a**) Far Western analysis of the RECQ1-PARP1 interaction using purified recombinant proteins. Purified human Replication protein A (hRPA) was used as positive control since RECQ1 was previously reported to interact with hRPA [2]. Bovine Serum Albumin (BSA) was used as a negative control. The Hybond-P membrane was incubated with recombinant RECQ1 and western blotting was performed using the anti-RECQ1 antibody. (**b**) GST-pulldown experiments with unmodified recombinant PARP1 (1 μg) or PARylatedPARP1 (1 ug) incubated with GST-RECQ1 (1 μg) or GST alone (1 μg), as a control. 100 μM NU1025 or 200 mM NAD + 100 μM NU1025 were added as indicated. The interaction between RECQ1 and PARP1 is not affected by NU1025. However, it is significantly decreased when the salt concentration is increased from 150 to 500 mM in the washing buffer. Conversely, the interaction between RECQ1 and PARylatedPARP1 is resistant to the washes with 500 mM NaCl. Bound proteins were resolved by gel electrophoresis, and visualized by western blot with anti-PAR + anti-PARP1 antibodies. (**c**) Analysis of RECQ1-PAR binding *in vitro*. Proteins (2 pmol) were dot-blotted onto a nitrocellulose membrane and incubated with ^{32}P-labeled PAR, as described in the Online Methods. BSA and Proliferating Cellular Nuclear Antigen (PCNA) were used as negative controls, while PARP1 and histone H1 were used as positive controls. (**d**) *In vitro* analysis of RECQ1 poly(ADPribosyl)ation. GST-tagged RECQ1 fragments were incubated with PARP (1 μM) and 200 μM of NAD+, and the presence of the PAR polymer was verified by Western analysis. The amount of PARP and GST-tagged RECQ1 fragments used in each experiment was also confirmed by Western analysis using anti-PARP1 and anti-GST antibodies, respectively.

Supplementary Figure 3



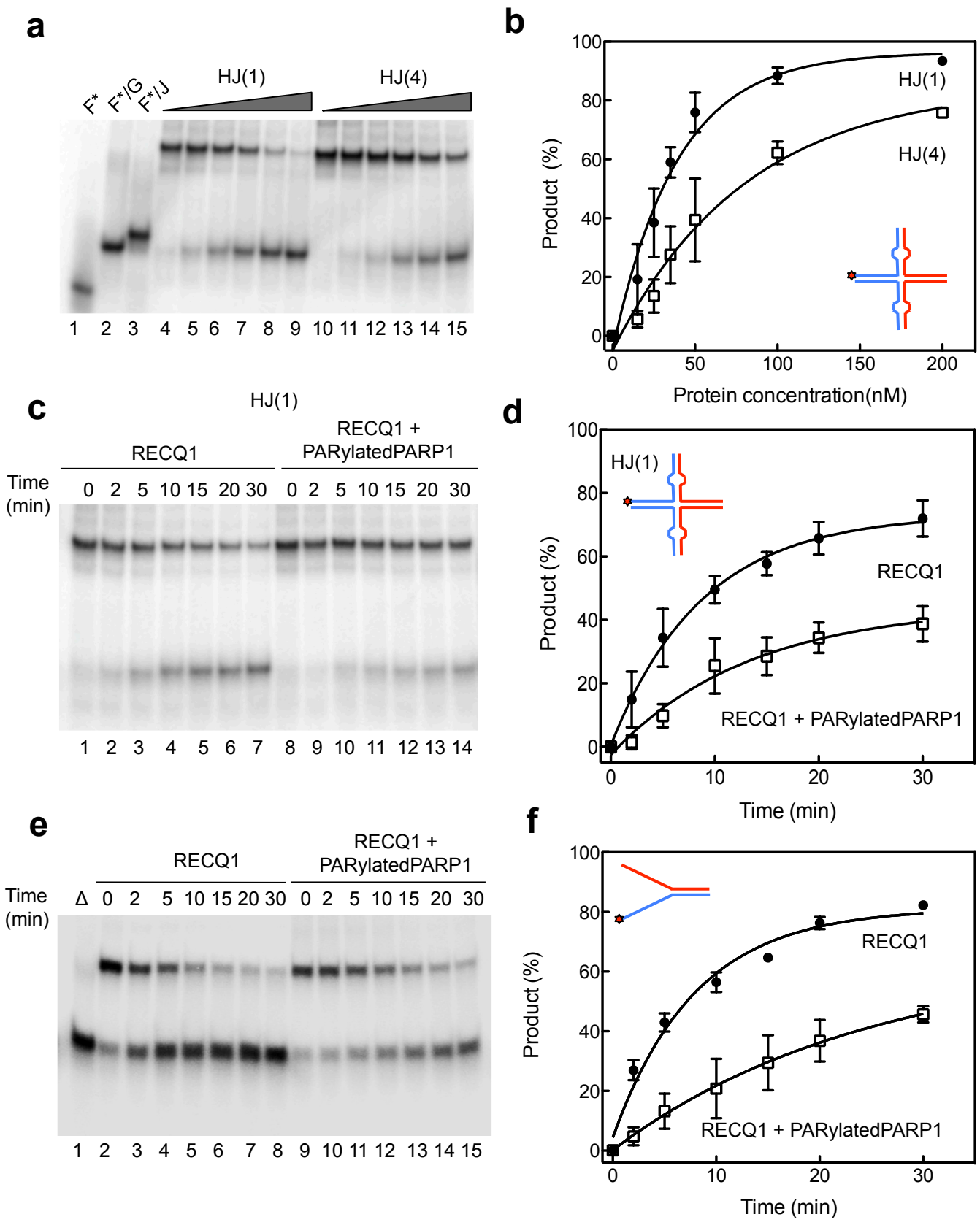
Supplementary Figure 3. DNA damage sensitivity of RECQ1 depleted cells, interaction of RECQ1 with CPT-damaged replication forks, and schematic of all substrates used for the activity assays. (**a**) The Y-axis shows the relative survival of U-2 OS cells expressing a *RECQ1* shRNA or *Luc* shRNA relative to dsRed-expressing U-2 OS cells, following treatment with HU (2 mM, 16hrs), ETOP (1 μM, 16hrs), CPT (10 nM, 24hrs), MMC (750 nM, 1hr), MMS (1 mM, 1hr), and UV (20 J/m^2). (**b**) RECQ1 and PARP1 associate with CPT-damaged replication forks. Forks were isolated by CldU co-immunoprecipitation after 1 hour treatment with 100 nM CPT. The level of histone H3 was used as loading control. (**c**) The substrate used to study fork restoration and regression contained a 6 nt ssDNA gap on the leading strand template of a model replication fork. The two terminal regions of the vertical arms contained different, complementary but mutually exclusive sequences to ensure that the "chicken foot" (or HJ structure) structure is converted to a replication fork structure and prevent complete separation of the two strands. In addition, we inserted a single isocytosine (iso-C) residue in the oligonucleotide that represents a replication fork leading strand (denoted with a circle) and two mismatches on the substrate vertical arms (shown by carets) to prevent spontaneous fork regression and restoration. Oligonucleotide B was end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs), then purified through a Micro Bio-Spin column (Bio-Rad). Fork intermediates were prepared in annealing buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl) by heating the complementary strands at 95^{o}C for 5 min, and then cooling slowly to room temperature. To prepare the branch migration substrates, labeled and a 1.5-fold excess of unlabeled DNA intermediates were incubated in annealing buffer supplemented with 5 mM $MgCl_2$ for 30 min at 37^{o}C, and then for an additional 30 min at room temperature. (**d**) A second substrate used to study for restoration and regression lacks the ssDNA gap on the leading strand template. (**e**) Holliday junction (X-junction) substrate with heterology regions of 1 (oligos F, G, H, I) or 4 bases (oligos F, G, J, K). (**f**) Fork duplex substrate with a duplex region of 20 bp and two ssDNA tails of 30 nt. The sequences of all the oligonucleotides are reported in Supplementary Table 1.

Supplementary Figure 4



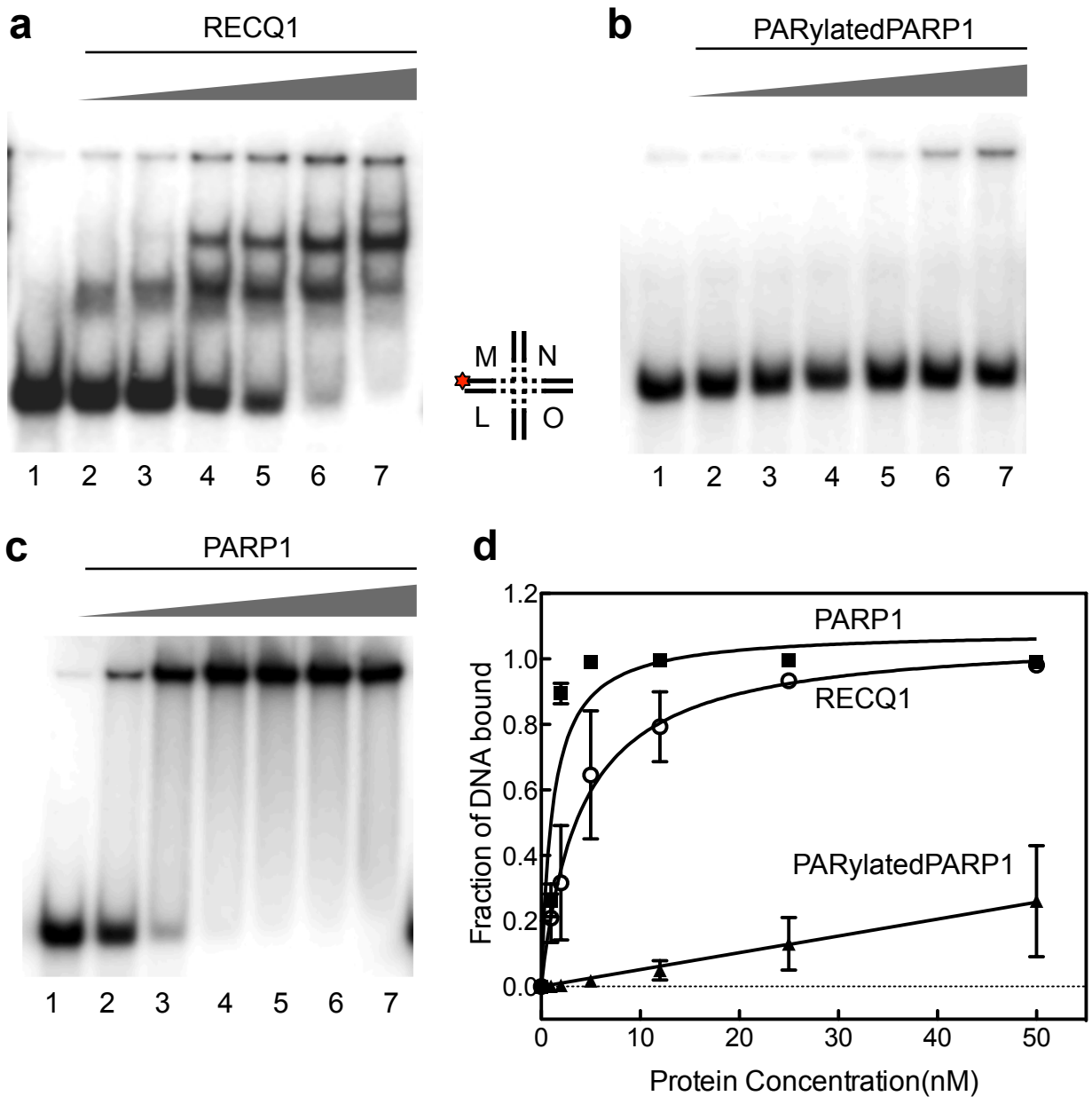
Supplementary Figure 4. Fork restoration and regression assays. (**a**) Fork restoration and regression assays using a DNA substrate that lacks a ssDNA gap on the leading strand template. Lanes 1-7: fork restoration assays performed using increasing RECQ1 concentrations (0, 15, 25, 35, 50, 100, and 200 nM) and a fixed concentration of the chicken foot substrate (2 nM). Lanes 8-14: fork regression assays using increasing RECQ1 concentrations (0, 15, 25, 35, 50, 100, and 200 nM) and a fixed concentration of the replication fork structure (2 nM). All the reactions were stopped after 20 min. Left: Plot of the fork restoration and regression activity as a function of protein concentration. The data points represent the mean of three independent experiments. Error bars indicate s.e.m. (**b**) Fork restoration and regression assays using non-hydrolysable ATP analogs or ATPase deficient RECQ1 mutants. Fork restoration and regression assays were performed in the presence of ATP or different ATP analogs using wild-type RECQ1 (lanes 2-5) or the ATPase deficient RECQ1 mutant, K119R (lane 6) and E220Q (lane 7). The protein concentration was 50 nM for all the experiments. (**c**) Inhibition of the *in vitro* fork restoration activity of RECQ1 by increasing concentrations of PARylatedPARP1. Lanes 1: substrate alone. Lane 2: RECQ1 alone (50 nM). Lanes 3-10: fork restoration assays performed using increasing PARylatedPARP1 concentrations (3.125, 4.16, 6.25, 12.5, 16.6, 25, 50 and 200 nM) and a fixed concentration of RECQ1 (50 nM). Lane 11: PARylatedPARP1 alone (100 nM). All the reactions were stopped after 20 min, and the products were analyzed by electrophoresis in a 8% polyacrylamide gel. The data points represent the mean of three independent experiments. Error bars indicate s.e.m. The substrate used in **b** and **c** was the same utilized for the experiments of Figure 2 and has a ssDNA gap of 6nt on the leading strand template.

Supplementary Figure 5



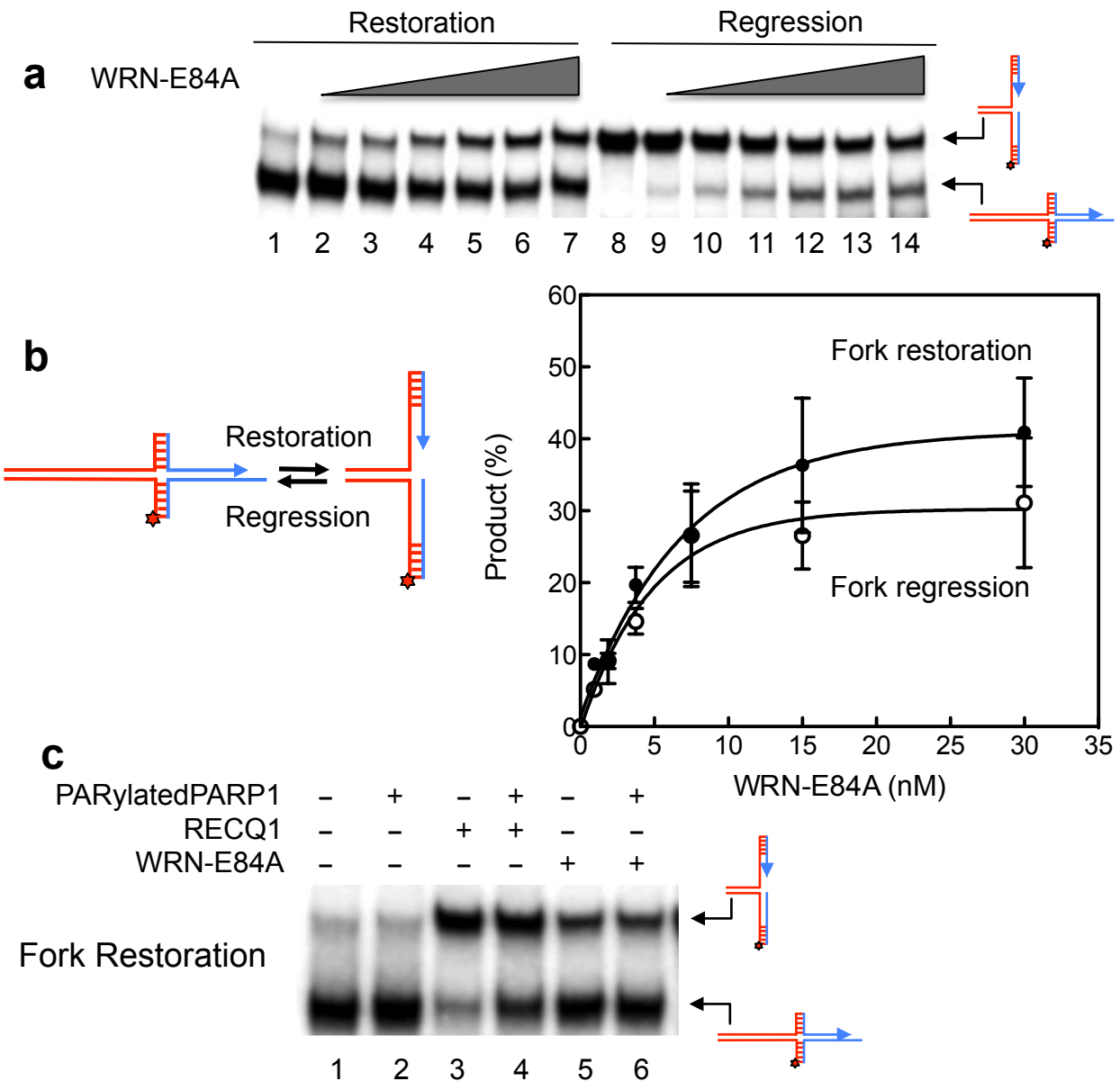
Supplementary Figure 5. Branch migration and unwinding assays using HJ and for duplex substrates. (**a**) Branch migration assays were performed with HJ substrates with heterology regions of 1 (HJ(1)) or 4 bases (HJ(4)). Lanes 1-3: DNA migration markers. Lanes 4-9: branch migration assays performed using increasing RECQ1 concentrations (0, 25, 35, 50, 100, and 200 nM) and a fixed concentration of HJ(1) (2 nM). Lanes 10-15: branch migration assays using increasing RECQ1 concentrations (0, 25, 35, 50, 100, and 200 nM) and a fixed concentration of the HJ(4) (2 nM). All the reactions were stopped after 20 min. (**b**) Plot of the branch migration activity as a function of protein concentration. The data points represent the mean of three independent experiments. Error bars indicate s.e.m. (**c**) Effect of PARylatedPARP1 on RECQ1 branch migration activity using the HJ(1) substrate. Lanes 1-7: kinetic experiments performed using 50 nM RECQ1 and the HJ(1) (2 nM). Lanes 8-14: kinetic experiments performed in the presence of PARylatedPARP1 (50 nM). (**d**) Plots of the branch migration assays performed in the presence and absence of PARylatedPARP1. The data points represent the mean of three independent experiments. Error bars indicate s.e.m. (**e**) DNA unwinding assays using the forked duplex substrate. Lanes 1-7: kinetic experiments performed using 4.5 nM RECQ1 and the forked duplex substrate (2 nM). Lanes 8-14: kinetic experiments performed in the presence of PARylatedPARP1 (4.5 nM). (**f**) Plots of the uwinding assays performed in the presence and absence of PARylatedPARP1. The data points represent the mean of three independent experiments. Error bars indicate s.e.m.

Supplementary Figure 6



Supplementary Figure 6. DNA binding assays at increasing protein concentrations using the HJ probe. EMSA experiments performed using a HJ substrate with a 12-bp homologous core (0.5 nM). Lane 1: substrate alone. Lanes 2-7: experiments at increasing RECQ1 (**a**), PARylatedPARP1 (**b**), and PARP1 (**c**) concentrations (1, 2, 5, 12, 25, 50 nM). PARylatedPARP1 was prepared by incubating PARP1 in the presence of NAD, as described in the Online Methods. (**d**) The plots are the average of three independent experiments. Error bars indicate s.e.m.

Supplementary Figure 7



Supplementary Figure 7. Fork restoration and regression assays using human WRN. (**a**) These experiments were performed using exonuclease-deficient WRN-E84A mutant that allows to follow the branch migration reaction without possible complications arising form the substrate digestion. Lanes 1-7: fork restoration assays performed at increasing WRN-E84A concentrations (0, 0.9375, 1.875, 3.75, 7.5, 15, and 30 nM) and a fixed concentration of the chicken foot substrate (2 nM). Lanes 8-14: fork regression assays at increasing WRN-E84A concentrations (0, 0.9375, 1.875, 3.75, 7.5, 15, and 30 nM) and a fixed concentration of the replication fork structure (2 nM). All the reactions were stopped after 20 min. (**b**) Left: reaction scheme. Right: Plot of the fork restoration and regression activity as a function of protein concentration. The data points represent the mean of three independent experiments. Error bars indicate s.e.m. (**c**) Fork restoration assays performed in the presence (lines 2, 4, 6) and absence (1, 3, 5) of PARylatedPARP1 (50 nM) using wild-type RECQ1 (50 nM, lanes 3,4) or WRN-E84A (20 nM, lane 5,6). All the reactions were incubated for 20 min.

Supplementary Table 1. Sequences of the oligonucleotides used in the study of fork regression and restoration. Bold red letters indicate the nucleotides that form mismatched pairs in the branch migration products.

Name	Length (nt)	Sequence 5' → 3'
A	81	CTT TAG CTG CAT ATT TAC AAC ATG TTG ACC TTC AGT A/isodC/A ATC TGC TCT GAT GCC GCA TAG TGT CAT GCC AGA GCT TTG TAC
B	81	CGG GTG TCG GGG CGC ATG ACA CTA TGC GGC ATC AGA GCA GAT T**G**T ACT GAA GGT CAA CAT GTT GTA AAT ATG CAG CTA AAG
C	43	GTA CAA AGC TCT GGC ATG ATA CTA TGC GGC ATC AGA GCA GAT T
D	50	TCA GTA CAA TCT GCT CTG ATG CCG CAT AGT ATC ATG CGC CCC GAC ACC CG
E	49	GTA CAA AGC TCT GGC ATG ATA CTA TGC GGC ATC AGA GCA GAT TGT ACT G
F	60	CAC TGT GAT GCA CGA TGA TTG ACG ACA GTA GTC AGT GCT GCA GTG GTC AGG TGT CAT CAC
G	60	CCT GCA TAC AGA TGT TGA CCC AGC ACT GAC TAC TGT CGT CAA TCA TCG TGC ATC ACA GTG
H	60	GTG ATG ACA CCT GAC CAC TGC AGC ACT GAC TAC TGT CGT C**G**A TCA TCG TGC ATC ACA GTG
I	60	CAC TGT GAT GCA CGA TGA T**C**G ACG ACA GTA GTC AGT GCT GGG TCA ACA TCT GTA TGC AGG
J	60	GTG ATG ACA CCT GAC CAC TGC AGC ACT GAC TAC TGT C**AC TG**A TCA TCG TGC ATC ACA GTG
K	60	CAC TGT GAT GCA CGA TGA T**CA GT**G ACA GTA GTC AGT GCT GGG TCA ACA TCT GTA TGC AGG
L	50	GAC GCT GCC GAA TTC TGG CTT GCT AGG ACA TCT TTG CCC ACG TTG ACC CG
M	50	CGG GTC AAC GTG GGC AAA GAT GTC CTA GCA ATG TAA TCG TCT ATG ACG TC
N	50	GAC GTC ATA GAC GAT TAC ATT GCT AGG ACA TGC TGT CTA GAG ACT ATC GC
O	50	GCG ATA GTC TCT AGA CAG CAT GTC CTA GCA AGC CAG AAT TCG GCA GCG TC

P	50	GAA CGA ACA CAT CGG GTA CGT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT
Q	50	TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT CGT ACC CGA TGT GTT CGT TC

SUPPLEMENTARY NOTE.

Far Western experiments. The Far Western experiments were performed as previously described [2].

GST pull-down experiments with the recombinant proteins. 1 μg of recombinant PARP1 or poly(ADP-ribosyl)ated PARP1 was incubated with 1 μg of GST-RECQ1, or 1 μg GST alone as control, bound to 10 μl of glutathione–Sepharose beads (Amersham) in binding buffer TNEN (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1.0 mM EDTA pH 8.0, 0.5 % NP-40, 1 mM DTT 1, mM PMSF) supplemented with 0.1 mg/ml ethidium bromide for 2 hrs at 4°C. 100 μM NU1025 or 200 mM NAD + 100 μM NU1025 were added as indicated. The beads were subsequently washed two times in ethidium bromide-supplemented TNEN buffer, and three times with TNEN buffer containing 150 or 500 mM NaCl. Bound proteins were eluted with SDS sample buffer, resolved by gel electrophoresis, and visualized by western blot with the appropriated antibodies.

In vitro poly(ADP-ribosyl)ation assays. To test RECQ1 poly(ADP-ribosyl)ation, GST fusion proteins were incubated with 100 ng of recombinant PARP-1 in activity buffer (50 mM Tris, pH 7.5, 4 mM MgCl2, 50mM NaCl, 200 μM DTT, 0.1 μg/μl BSA, 4 ng/μl DNaseI-activated calf thymus DNA, and 400 μM NAD+). After 10 min at 37°C, reactions were stopped by dilution in GST binding buffer, washed extensively with the same buffer supplemented by 1M NaCl. The (ADP-ribosyl)ated products were resolved by gel electrophoresis and transferred to nitrocellulose membrane for visualization by western blot using an anti-PAR antibody.

Cell competition assays. To study the response of RECQ1-depleted cells to specific genotoxic agents, we utilized a quantitative multicolor cell completion assay where RECQ1 or luciferase depleted cells were mixed in equal amount with U-2 OS cells expressing dsRed following a previously described procedure [3,4]. The mixed cells are treated with the specific DNA replication inhibitor or damaging agent, or left untreated. The relative sensitivity of the RECQ1-downregulated cells was then monitored by flow cytometric analysis of the ratio of uncolored RECQ1- downregulated cells to red fluorescence protein- positive (RFP^+) cells.

Co-immunoprecipitation of RECQ1 with CldU at CPT-damaged Replication Forks. The presence of RECQ1 at CPT-damaged replication forks was assayed as previously described [5]. Briefly, a total of $2x10^7$ HEK293T cells were treated or untreated with 100 nM CPT for 1 hr. CPT was washed away, and cells were labeled with CldU (100 mM) for 40 min. Cells were cross-linked in 1% formaldehyde for 15 min at room temperature and treated with 0.125 M glycine for 15 min at room temperature. Cells were scraped in cold phosphate-buffered saline (PBS). Cytoplasmic proteins were removed by incubation in hypotonic buffer (25 mM HEPES, pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.1% NP-40, 1 mM dithiothreitol, 0.25 mM PMSF, and protease inhibitors) for 10 min on ice. Nuclear-soluble fraction was removed by incubation with nuclear buffer (10 mM HEPES pH 7, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease inhibitors) for 10 min on ice and centrifugation at 13 000 r.p.m. for 2 min. Pellets were resuspended in lysis buffer (10 mM HEPES pH 7, 500 mM NaCl, 1 mM EDTA, 1% NP-40, protease inhibitors (cocktail, Roche)), sonicated, centrifuged for 30 s at 13

000 r.p.m. and the collected supernatant containing crude soluble chromatin was incubated with rat monoclonal anti-BrdU antibody (AbD Serotec) overnight at 4°C and then with protein A/G beads for 4 hr. The IP reaction was washed 2 times with nuclear buffer, 2 times with washing buffer (10 mM HEPES, 0.1 mM EDTA protease inhibitors (cocktail, Roche)), incubated in 2x sample loading buffer for 30 min at 90°C and used for western blot analysis.

EMSA experiments. The 5'-32P labeled synthetic four-way junction (HJ X12) was prepared and purified as previously described [6]. Purified proteins were incubated with 0.5 nM DNA in binding buffer containing 20 mM Hepes-KOH pH 7.6, 75 mM KCl, 2 mM MgCl2, 2 mM ATPgS, 1 mM DTT, 0.25 mM EDTA, 20 µg/ml BSA, 5% glycerol, 0.1% NP-40 for 30 minutes at room temperature. When indicated 200 µM NAD+ and 10 µM Olaparib were added. Protein-DNA complexes were analyzed by electrophoresis in 5% polyacrylamide gels in 0.5x TBE for 3 hours at 4°C.

SUPPLEMENTARY REFERENCES

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