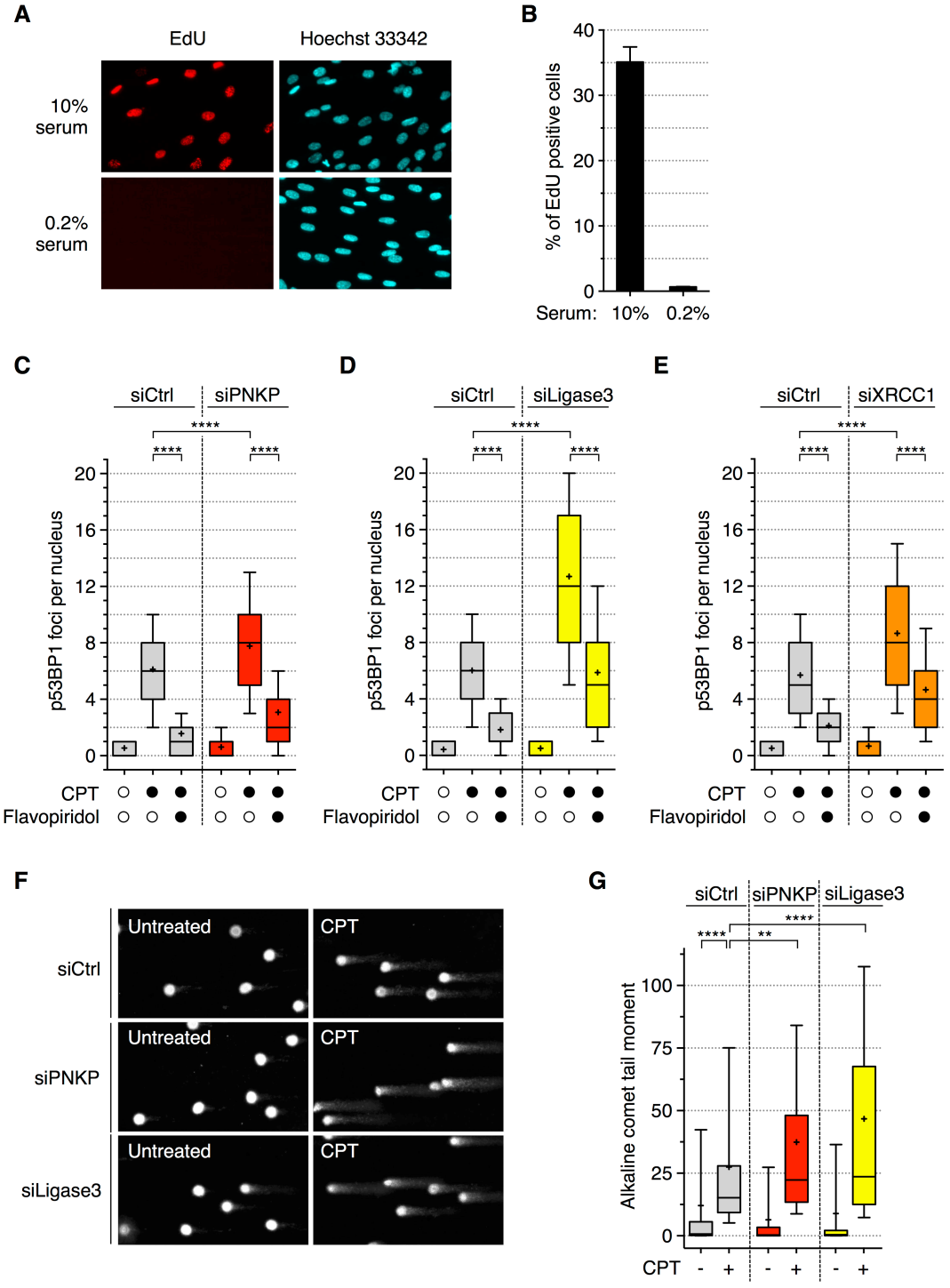


Figure S1. Depletion of PNKP, DNA ligase 3 or XRCC1 increases CPT-induced DNA single-strand breaks and p53BP1 foci in quiescent cells, Related to Figure 1.

(**A,B**) WI38 hTERT cells were cultured in 10% serum or in 0.2% serum for 3 days to induce quiescence. Cells were then incubated with 10 µM EdU for 30 min to label newly synthesized DNA. The incorporated EdU into DNA was detected using the Click-iT EdU imaging kit (red) and DNA was counterstained with Hoechst 33342 (cyan). **(A)** Representative images. **(B)** Percentages of EdU-positive cells (means ± SEM for quadruplicate samples).

(**C-E**) Quiescent cells were treated as in Figures 1F-1K and the number of p53BP1 foci per nucleus is shown. ****$p < 0.0001$ (one-way ANOVA). A representative experiment out of three is shown.

(**F,G**) Detection of SSBs by alkaline comet assays in quiescent cells transfected with the indicated siRNAs before treatment with CPT (25 µM) for 1 h. (**F**) Representative pictures of nuclei. (**G**) Quantification of alkaline comet tail moments. **$p < 0.01$, ****$p < 0.0001$ (one-way ANOVA). A representative experiment out of three is shown.

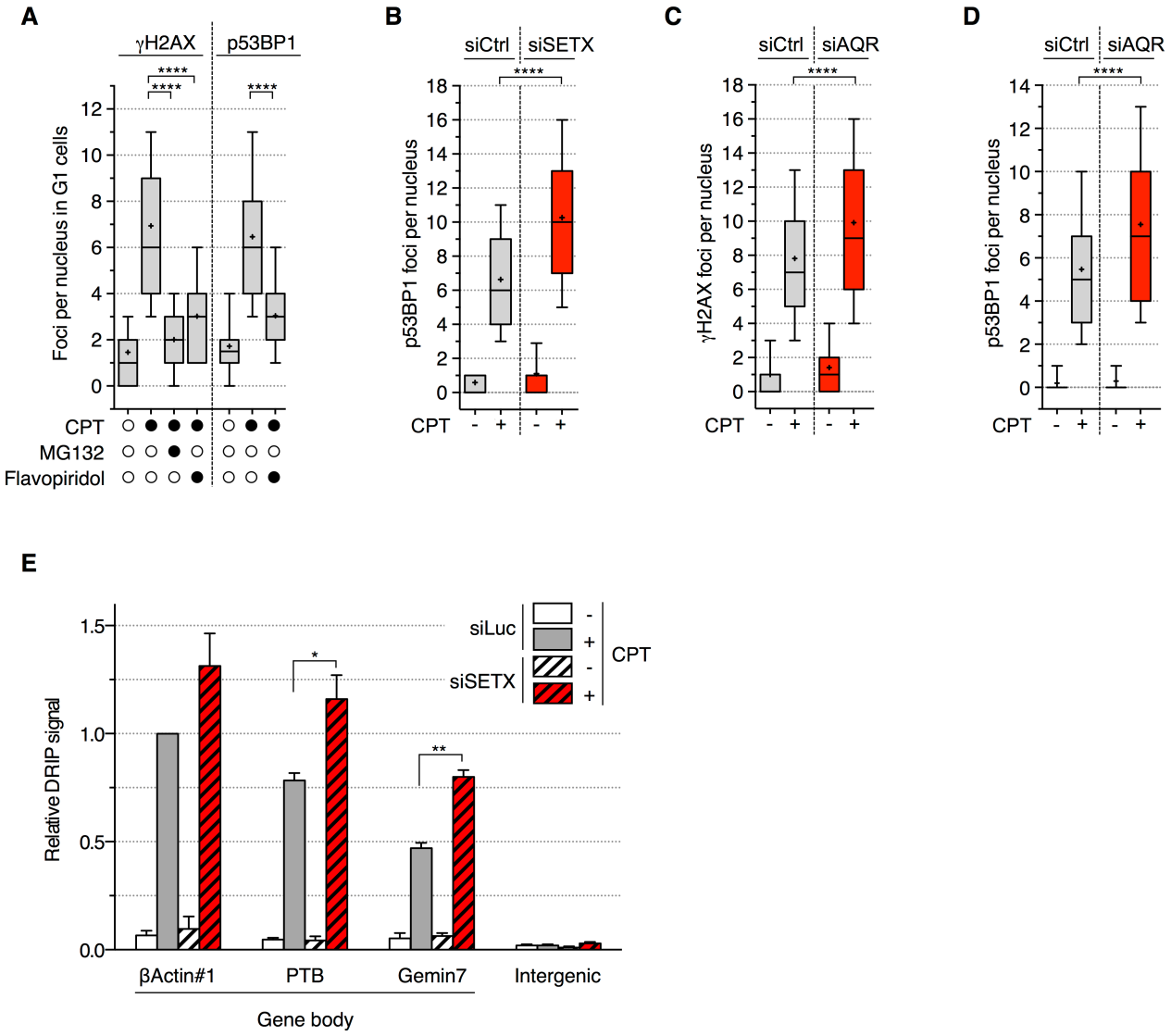


Figure S2. CPT induces γH2AX and/or p53BP1 foci in G1 WI38 hTERT cells, and upon depletion of SETX or AQR in quiescent cells, Related to Figure 2.

(A) WI38 hTERT cells were incubated with 10 μM EdU for 30 min to label newly synthesized DNA before treatment with MG132 (25 μM) or flavopiridol (1 μM) for 1 h and the addition of CPT (25 μM) for 1 h. Cells were then co-stained for γH2AX and p53BP1 and DNA was counterstained with Hoechst 33342. The number of γH2AX and p53BP1 foci per G1 nucleus (EdU-negative and low Hoechst 33342) is shown. ****p < 0.0001 (one-way ANOVA). A representative experiment out of two is shown.

(**B**) Quiescent cells were treated as in Figure 2J. The number of p53BP1 foci per nucleus is shown. ****p < 0.0001 (two-tailed unpaired t test). A representative experiment out of three is shown.

(**C,D**) Quiescent cells were transfected with siCtrl or siAQR, treated with CPT (25 μM) for 1 h and co-stained for γH2AX and p53BP1. The number of γH2AX (**C**) and p53BP1 (**D**) foci per nucleus is shown. ****p < 0.0001 (two-tailed unpaired t test). A representative experiment out of two is shown

(**E**) DRIP analysis in quiescent cells transfected with siLuc or siSETX and treated with CPT (25 μM) for 1 h. Values are normalized to "βActin#1" amplicon from siLuc-transfected cells treated with CPT in each experiment (means ± SEM; n = 3). * p < 0.05, ** p < 0.01 (two-tailed unpaired t test).

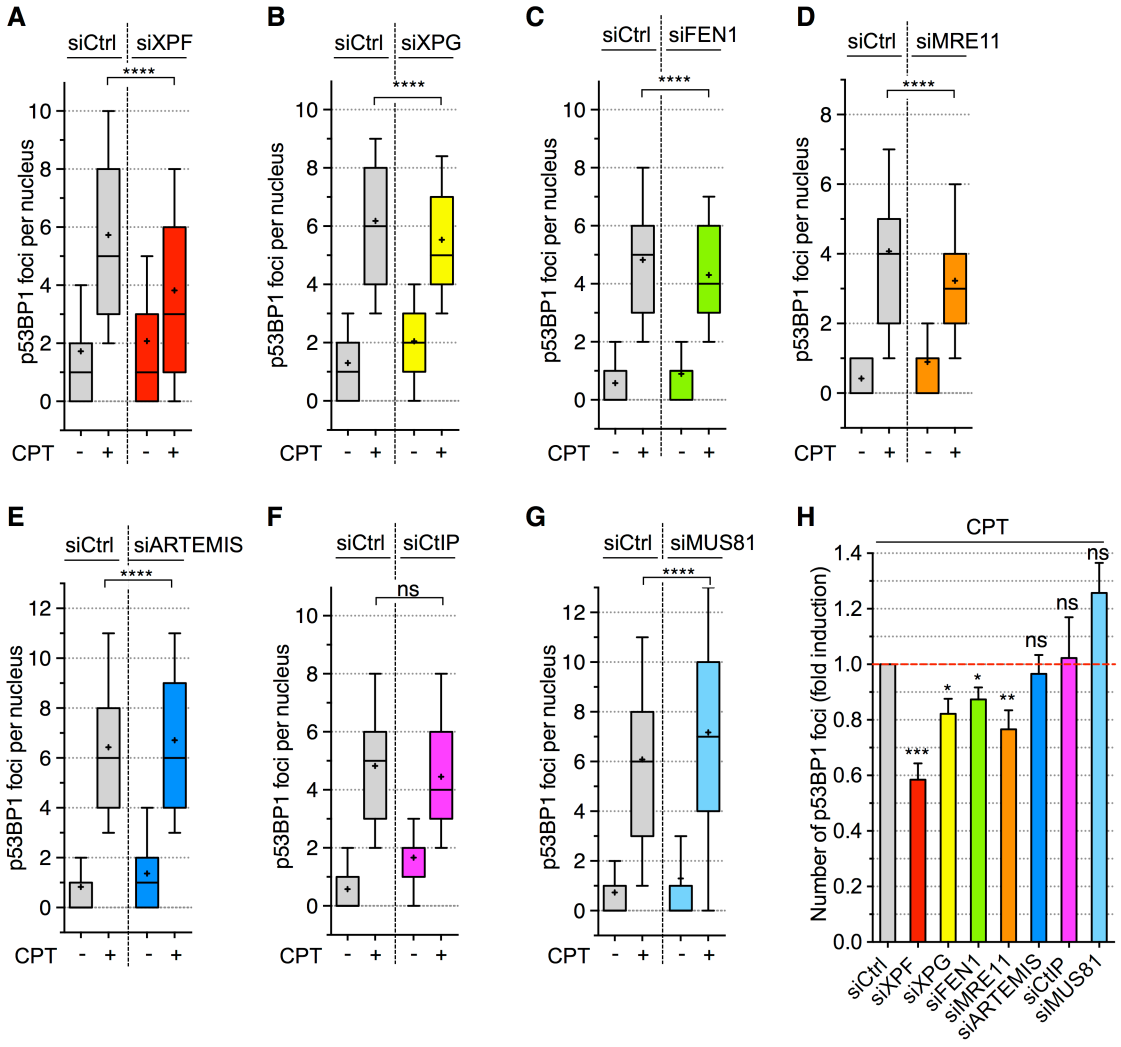
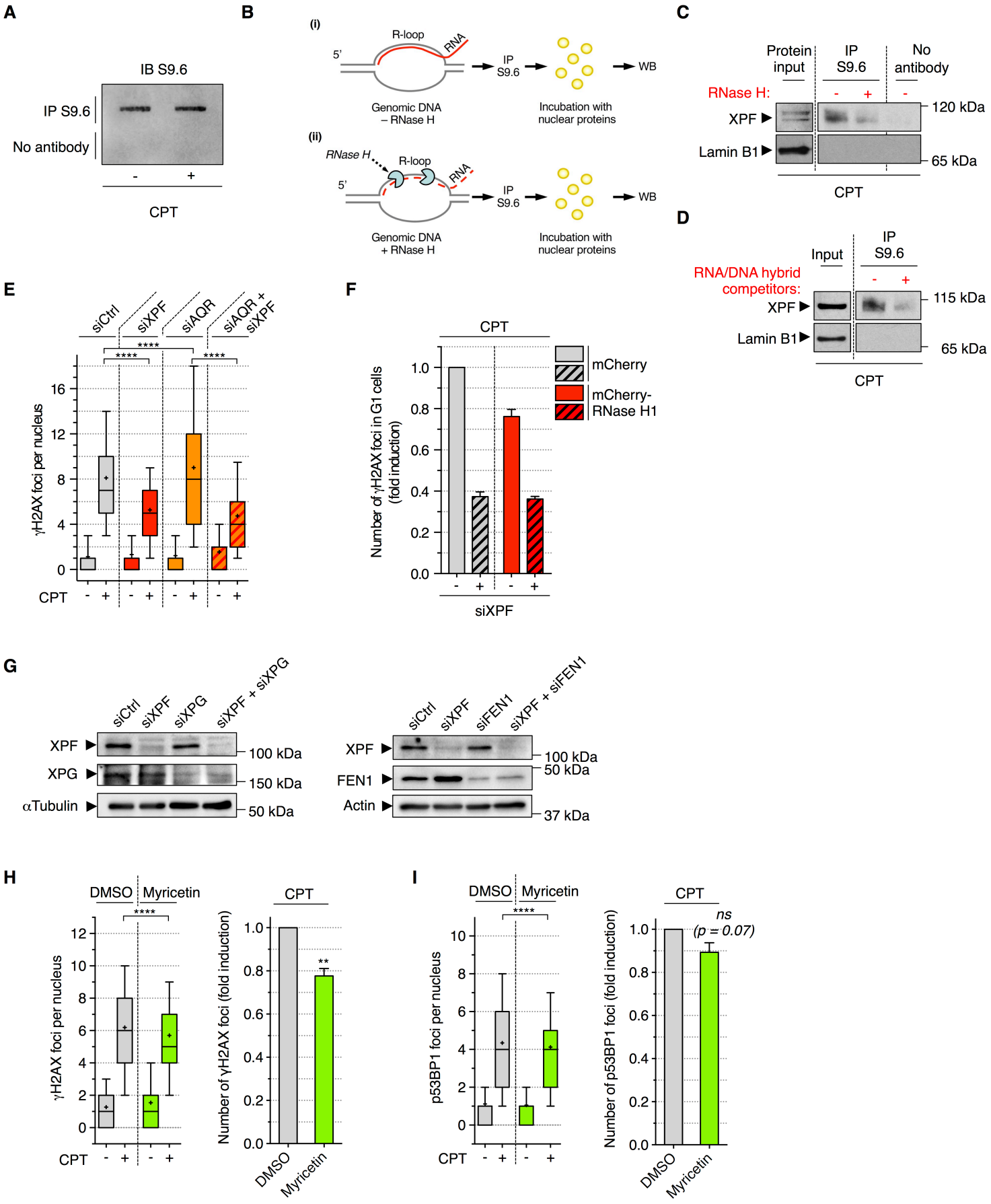


Figure S3. Depletion of the flap endonucleases XPF, XPG, FEN1 and MRE11 reduces the induction of DSBs in CPT-treated quiescent cells, Related to Figure 3.

(**A-G**) Quiescent cells were treated as in Figure 3. The number of p53BP1 foci per nucleus is shown. Ns: not significant, ****p < 0.0001 (two-tailed unpaired t test). A representative experiment out of at least three is shown. The data with siCtrl cells in panels C and F are from the same experiment.

(**H**) The fold induction of p53BP1 was calculated as in Figure 3I (means ± SEM; n ≥ 3). Ns: not significant, *p < 0.05, **p<0.01, ***p < 0.001 (two-tailed unpaired t test).


A
IB S9.6
IP S9.6
No antibody
- +
CPT
B
(i)
R-loop
RNA
5'
IP S9.6
WB
Genomic DNA – RNase H
Incubation with nuclear proteins
(ii)
RNase H
R-loop
RNA
5'
IP S9.6
WB
Genomic DNA + RNase H
Incubation with nuclear proteins
C
Protein input
IP S9.6
No antibody
RNase H: - + -
XPF
Lamin B1
120 kDa
65 kDa
CPT
D
Input
IP S9.6
RNA/DNA hybrid competitors: - +
XPF
Lamin B1
115 kDa
65 kDa
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siAQR
siAQR + siXPF
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γH2AX foci per nucleus
CPT - + - + - + - +
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mCherry-RNase H1
Number of γH2AX foci in G1 cells (fold induction)
siXPF - + - +
G
siCtrl
siXPF
siXPG
siXPF + siXPG
XPF
XPG
αTubulin
100 kDa
150 kDa
50 kDa
siCtrl
siXPF
siFEN1
siXPF + siFEN1
XPF
FEN1
Actin
100 kDa
50 kDa
37 kDa
H
DMSO
Myricetin
****
γH2AX foci per nucleus
CPT - + - +
CPT
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Number of γH2AX foci (fold induction)
DMSO
Myricetin
I
DMSO
Myricetin
****
p53BP1 foci per nucleus
CPT - + - +
CPT
ns
(p = 0.07)
Number of p53BP1 foci (fold induction)
DMSO
Myricetin

Figure S4. R-loop-dependent DSBs are mediated by XPF, XPG and FEN1 in non-replicating cells treated with CPT, Related to Figure 4.

(**A**) Cells were treated and processed as in Figure 4C and IP fractions were immunoblotted with the S9.6 antibody.

(**B-C**) Genomic DNA from quiescent cells treated with CPT (25 μM , 20 min) was either digested (+) or not (-) with RNase H before enrichment for RNA/DNA hybrids with the S9.6 antibody. The immunoprecipitated RNA/DNA hybrids were then incubated with nuclear extracts from quiescent cells treated with CPT (25 μM, 20 min). Bound proteins were analyzed by Western blotting. (**B**) Workflow of RNA/DNA hybrid IP with RNase H digestion based on (Cristini et al., 2018). (**C**) Western blot of RNA/DNA hybrid IP and protein input, resolved on 3-8% Tris-Acetate gel (Invitrogen) and probed with XPF and lamin B1 antibodies.

(**D**) RNA/DNA hybrids in cell lysates (input) from quiescent cells treated with CPT (25 μM) for 20 min were immunoprecipitated (IP) with S9.6 antibody in presence or absence of 1.3 μM synthetic RNA/DNA hybrid competitors. Input and/or IP fractions were resolved on 4-12% Bis-Tris gel (Invitrogen) and probed with XPF and lamin B1 antibodies.

(**E**) Quiescent cells were transfected with siCtrl, siXPF, siAQR, or the combination of siXPF + siAQR together, treated with CPT (25 μM) for 1 h, and stained for γH2AX. The number of γH2AX foci per nucleus is shown. ****p < 0.0001 (one-way ANOVA). A representative experiment out of two is shown.

(**F**) U2OS cells were transfected with siCtrl (-) or siXPF (+) and treated with 2 μg/ml doxycycline for 24 h to express mCherry or mCherry-RNase H1 fusion protein. Cells were then incubated with 10 μM EdU for 30 min to label newly synthesized DNA, treated with CPT (25 μM) for 1 h, and stained for γH2AX. DNA was counterstained with Hoechst 33342. The fold induction of γH2AX foci per G1 nucleus (EdU-negative and low Hoechst 33342) was calculated by subtracting the number of foci of untreated cells from that of CPT-treated cells and normalized to mCherry-expressing cells treated with CPT (means ± SEM; n = 2).

(**G**) Western blot in extracts from quiescent cells transfected with the indicated siRNAs. αTubulin, Actin: loading controls.

(**H,I**) Quiescent cells were treated with myricetin (50 μM) for 1 h before the addition of CPT (25 μM) for 1 h, and co-stained for γH2AX and p53BP1. Left panels: the number of γH2AX (**H**) and p53BP1 (**I**) foci per nucleus is shown. ****p < 0.0001 (two-tailed unpaired t test). A representative experiment out of three is shown. Right panels: the fold induction of γH2AX (**H**) and p53BP1 (**I**) was calculated by subtracting the number of foci of untreated cells from that of CPT-treated cells and normalized to DMSO-treated cells treated with CPT in each experiment (means ± SEM; n = 3). Ns: not significant, **p < 0.01 (two-tailed unpaired t test).

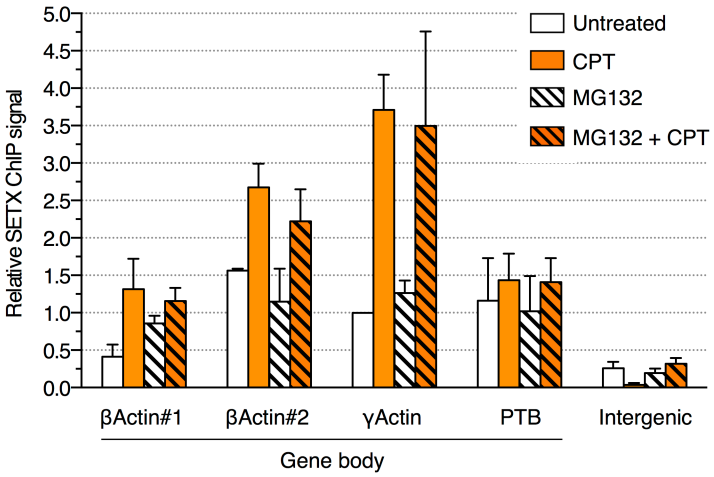

Figure S5. Proteasome inhibition did not prevent SETX recruitment at R-loop positions in CPT-treated quiescent cells, Related to Figure 5.

ChIP analysis of SETX in quiescent cells treated with MG132 (10 μM) for 1 h before the addition of CPT (25 μM) for 1 h. Values are normalized to the ChIP signal of "γActin" amplicon from untreated cells in each experiment (means ± SEM; n = 2-4).

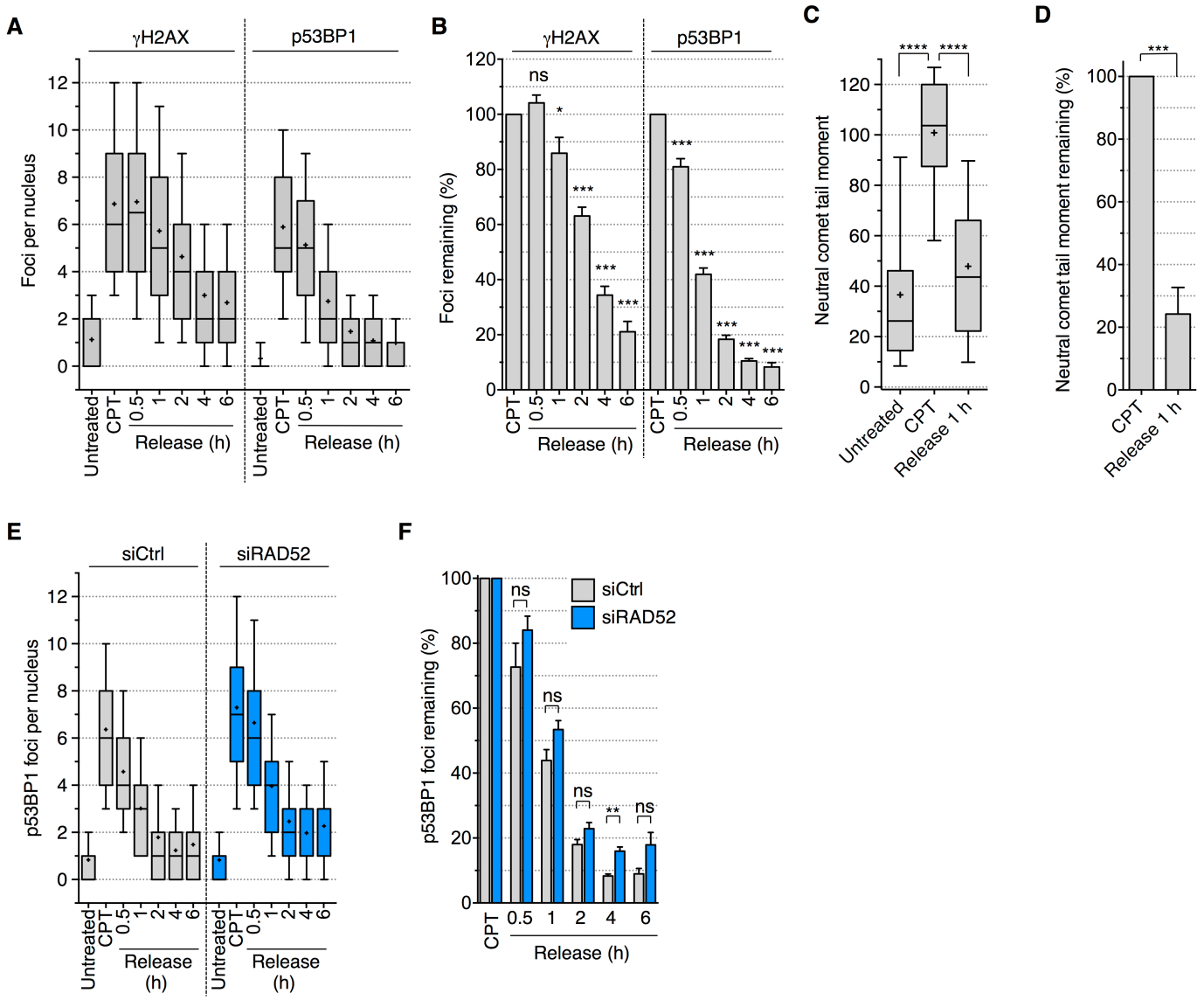

Figure S6. Kinetics of γH2AX/p53BP1 foci reversal and DSB repair in quiescent cells following CPT removal, Related to Figure 6.

Quiescent cells were treated with CPT (25 µM) for 1 h, washed and cultured in CPT-free medium for up to 6 h (release) (see protocole in Figure 6A).

(**A,B**) Cells were co-stained for γH2AX and p53BP1. (**A**) The number of γH2AX and p53BP1 foci per nucleus is shown. A representative experiment out of four is shown. (**B**) The percentages of γH2AX and p53BP1 foci remaining following CPT removal were calculated by subtracting the number of foci of untreated cells from that of treated cells, and normalized to cells treated with CPT in each experiment, which was taken at 100% (means ± SEM; n = 4). Ns: not significant, *p < 0.05, ***p < 0.001 (two-tailed unpaired t test).

(**C,D**) Detection of DSBs by neutral comet assays. **(C)** Quantification of neutral comet tail moments. ****p < 0.0001 (one-way ANOVA). A representative experiment out of three is shown. (**D**) The percentages of neutral comet tail moments remaining following CPT removal were calculated by subtracting the tail moment in untreated cells from that of treated cells and normalized to cells treated with CPT in each experiment, which was taken at 100% (means ± SEM; n = 3). ***p < 0.001 (two-tailed unpaired t test).

(**E,F**) Quiescent cells transfected with siCtrl or siRAD52 were stained for p53BP1. (**E**) Number of p53BP1 foci per nucleus. A representative experiment out of three is shown. (**F**) The percentages of p53BP1 foci remaining following CPT removal were calculated as indicated in panel B (means ± SEM; n = 3). Ns: not significant, **p < 0.01 (two-tailed unpaired t test).

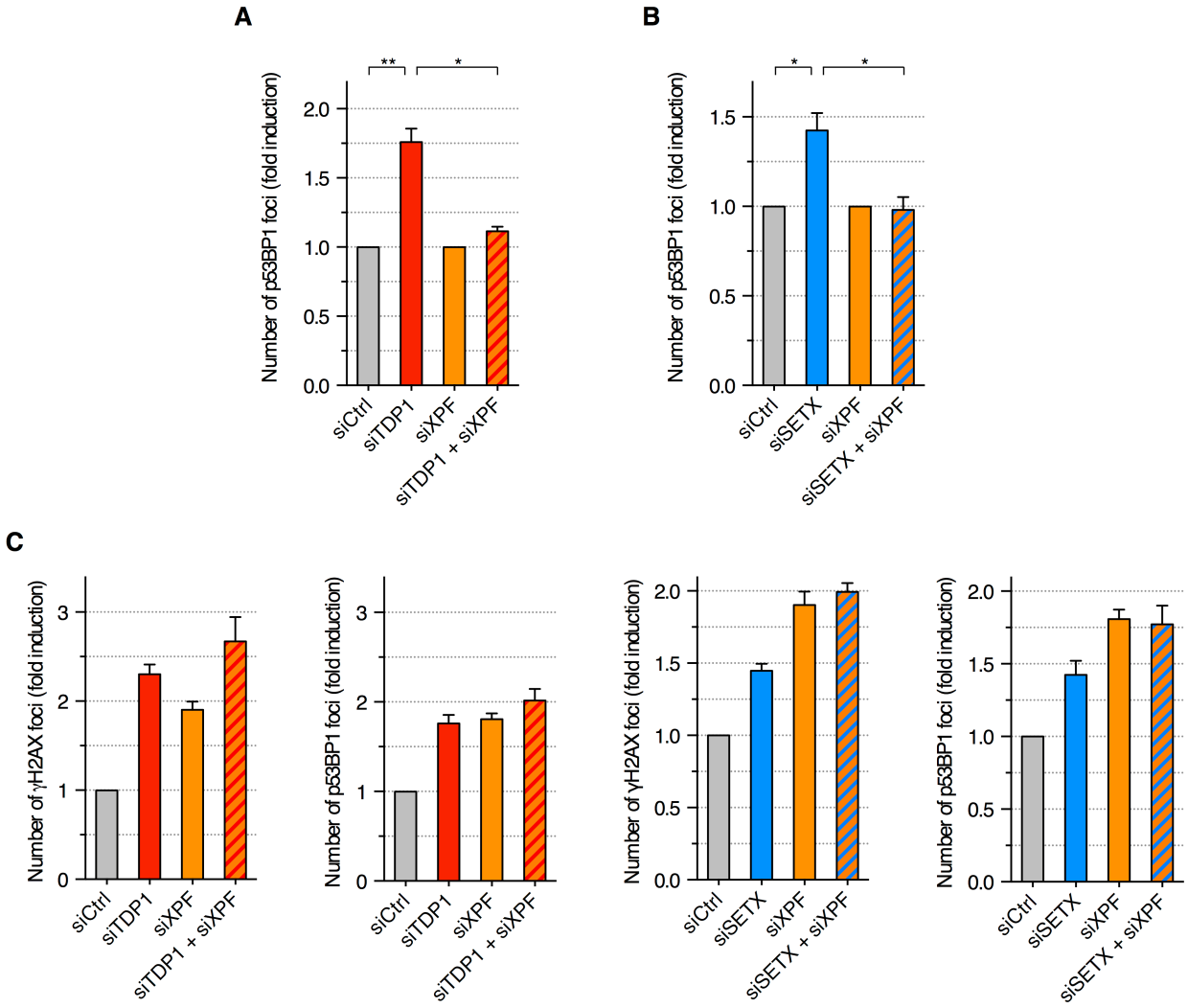

Figure S7. Depletion of XPF prevents the induction of DSBs upon depletion of TDP1 or SETX in quiescent cells, Related to Figure 7.

(A,B) Quiescent cells were treated as in Figures 7A and 7B. In each experiment, the fold induction of p53BP1 was normalized to siCtrl for siTDP1 and siSETX, and to siXPF for siTDP1 + siXPF and siSETX + siXPF (means ± SEM; n = 3). *p < 0.05, **p < 0.01 (two-tailed unpaired t test).

(C) Data points from Figures 7A, 7B, S7A and S7B are normalized to siCtrl.

Name	Forward sequence	Reverse sequence
βActin TSS	CGGGGTCTTTGTCTGAGC	CAGTTAGCGCCCAAAGGAC
βActin Gene body#1	GGAGCTGTCACATCCAGGGTC	TGCTGATCCACATCTGCTGG
βActin Gene body#2	TTACCCAGAGTGCAGGTGTG	CCCCAATAAGCAGGAACAGA
γActin TSS	CCGCAGTGCAGACTTCCGAG	CGGGCGCGTCTGTAACACGG
γActin Gene body	GTGACACAGCATCACTAAGG	ACAGCACCGTGTTGGCGT
PTB Gene body	GCCGTTGGTACAAAGGTAGG	GCCCCTTAGGAATGGAAAAG
Gemin7 Gene body	TCTTCTTCCACCTGGACCAC	GGGACAGAGAGAGTGCCTTG
IL4 Gene body	TTCAGGTGACAAGTGCCACAG	CTGGTTGGCTTCCTTCACAG
Intergenic	ACCCAGCACCCCCTAATACC	AGCCGGACATGCTTCCAGAG

Table S1: Primer sequences, Related to STAR Methods.
Intergenic: genomic region located 6 kb upstream to the TSS of β-Actin.