

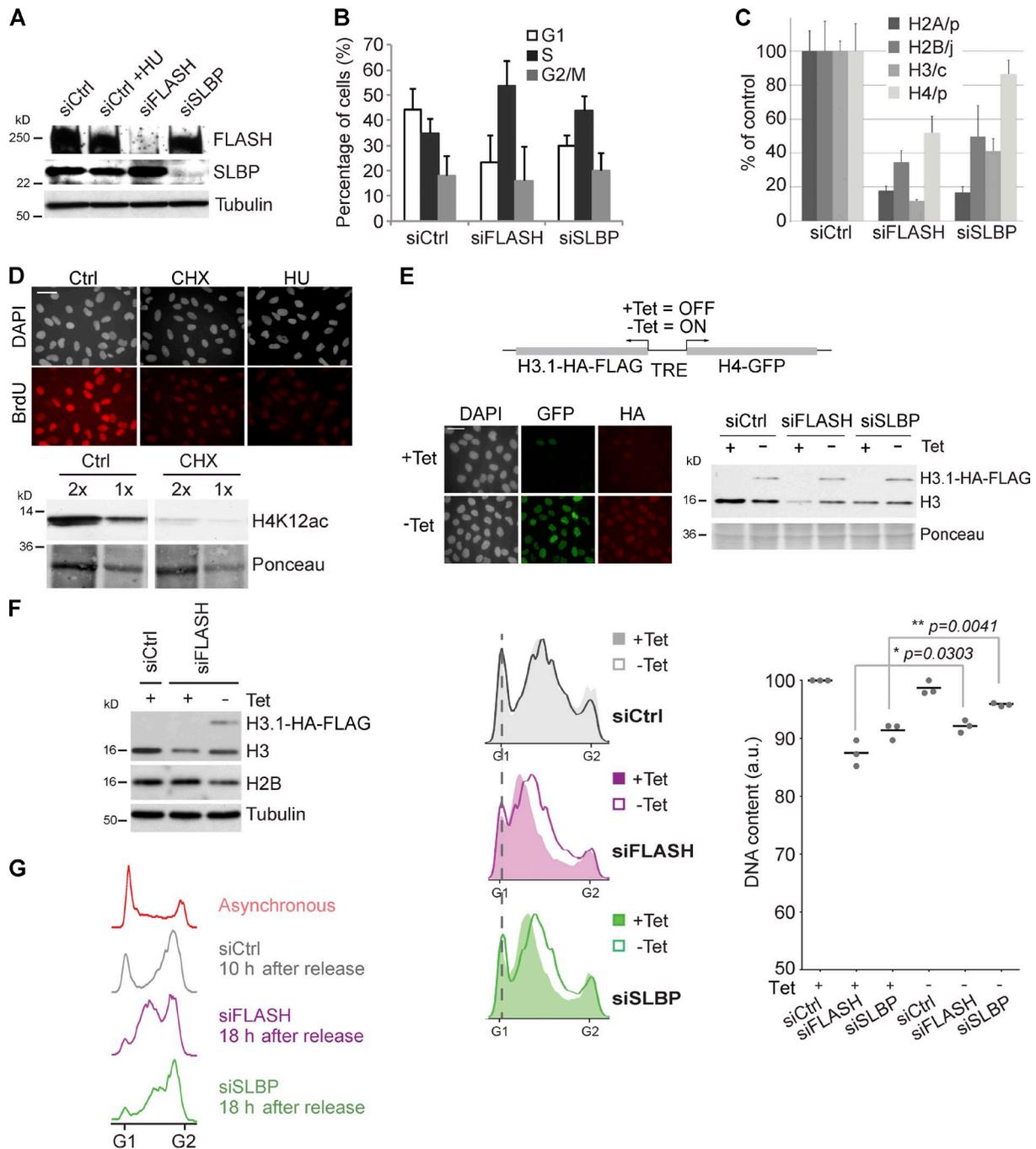
Mejlvang et al., <http://www.jcb.org/cgi/content/full/jcb.201305017/DC1>

Figure S1. Histone deficiency impairs DNA replication. (A and B) U-2-OS cells treated with FLASH and SLBP siRNAs were harvested for Western blotting (A), and cell cycle analysis was by FACS (B). Similar results were obtained with other siRNA/shRNAs targeting FLASH and SLBP (not depicted). The graph illustrates cell cycle distribution. Error bars represent SDs; $n = 4$. (C) Histone mRNA levels. Cells were treated as in Fig. 1 A, and expression of specific histone mRNA was measured by qPCR normalized to GAPDH. Error bars indicate SDs of technical triplicates. One representative experiment out of three biological replicas is shown. (D) DNA replication assayed by BrdU incorporation (top) and Western blot of soluble histones (bottom) in cells treated with CHX or HU for 1 h. (E) Rescue of H3.1-H4 supply by conditional coexpression of histone H3.1 and H4 (Groth et al., 2007). Homogenous expression was verified by immunofluorescence 24 h after induction (-tetracycline [Tet]). For rescue analysis, siRNA-transfected cells were synchronized at the G1/S border by thymidine, and H3.1-H4 expression was induced (-tetracycline [Tet]) for 4–6 h before release. Samples for Western blotting and FACS were harvested 6 h after release into S phase. DNA content measured by propidium iodide staining was quantified in three independent experiments, and significance was evaluated by t test. The means and p -values are indicated. TRE, tetracycline-responsive element. (F) Soluble H2B is reduced upon rescue of H3.1 and H4 expression in FLASH-depleted cells. Soluble protein extracts were analyzed by Western blotting. (G) Cell cycle analysis of siRNA-treated cells released into S phase from a thymidine block. To trap cells in mitosis, 40 ng/ml nocodazole was added 6 h after release. Although control cells accumulated in G2 after 10 h, FLASH- and SLBP-depleted cells were still in S phase 18 h after release. One representative experiment out of two biological replicas is shown. Bars, 50 μ m. a.u., arbitrary unit; siCtrl, siRNA control.

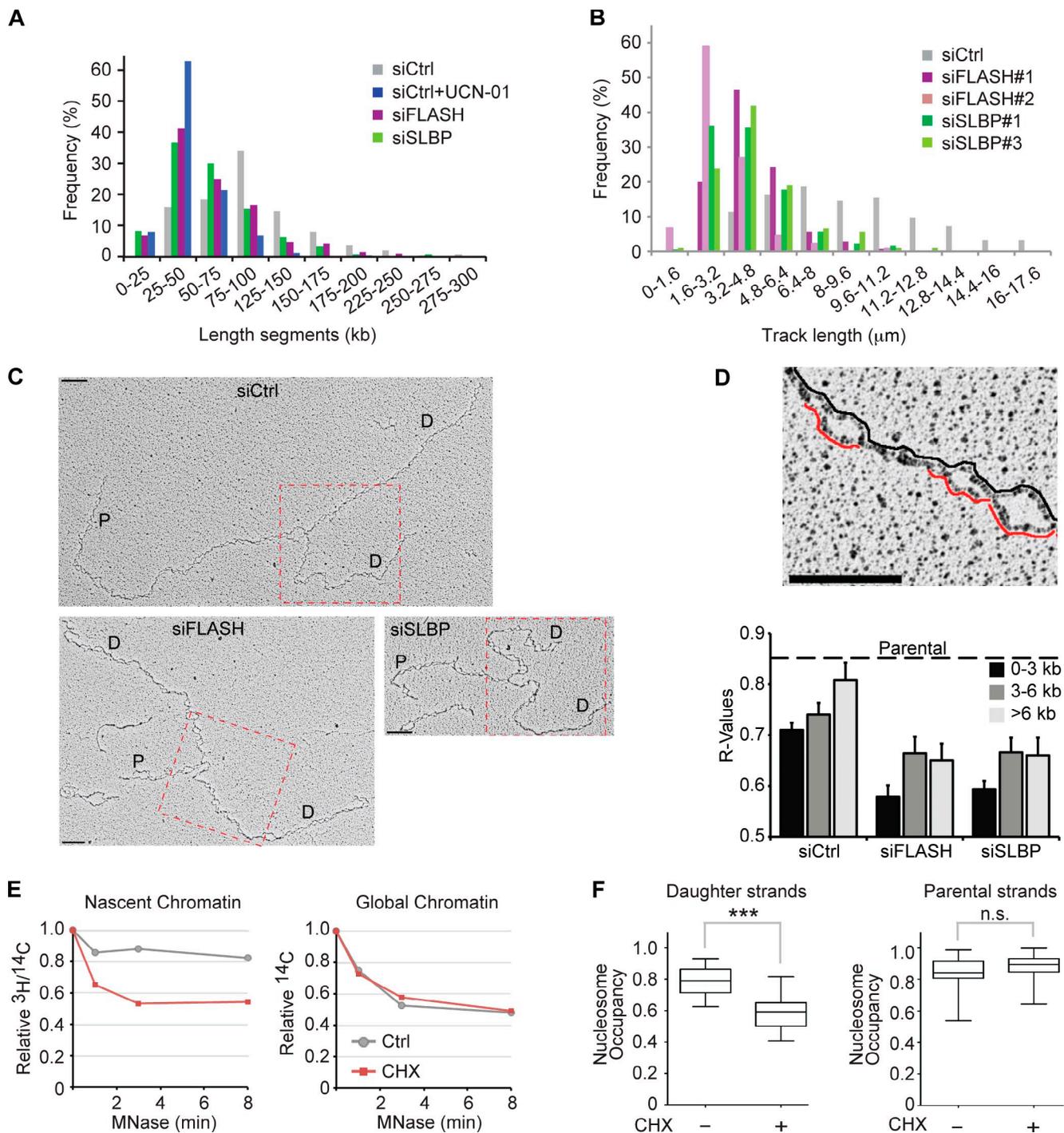


Figure S2. **Single molecule analysis of DNA replication.** (A) DNA combing. Frequency distribution of BrdU track length. $n > 150$. One representative experiment out of two biological replicas is shown. (B) DNA fibers. Frequency distribution of CldU track length after double-pulse labeling. $n > 100$. One representative experiment out of two biological replicas is shown. (C and D) EM analysis of psoralen-cross-linked replication intermediates. (C) Electron micrographs of representative replication intermediates. Regions shown in Fig. 2 B are boxed in red. P and D annotate parental and daughter DNA strands, respectively. Bar, 200 nm (≈ 500 bp). (D, top) Nucleosome occupancy was calculated from the combined contour length of all nucleosome bubbles in a given stretch of DNA (red), divided by the overall contour length of the DNA duplex (black). Bar, 100 nm (≈ 250 bp). (bottom) Nucleosome occupancy on daughter strands in depicted segments. The dashed line indicates nucleosome occupancy on parental strands in control cells. Error bars indicate SEMs. (E) MNase sensitivity of nascent and global chromatin in cells treated with CHX for 1 h. One representative experiment out of two biological replicas is shown. (F) Nucleosome occupancy determined by EM analysis of psoralen-cross-linked replication intermediates isolated from cells treated with CHX for 1 h. Median is displayed. Boxes are 25–75 percentile ranges, and whiskers are 0–100 percentile ranges. Statistics: two-tailed t test; (left) $n > 40$; $***$, $P < 10^{-3}$; (right) $n > 20$; n.s., $P > 0.5$. siCtrl, siRNA control.

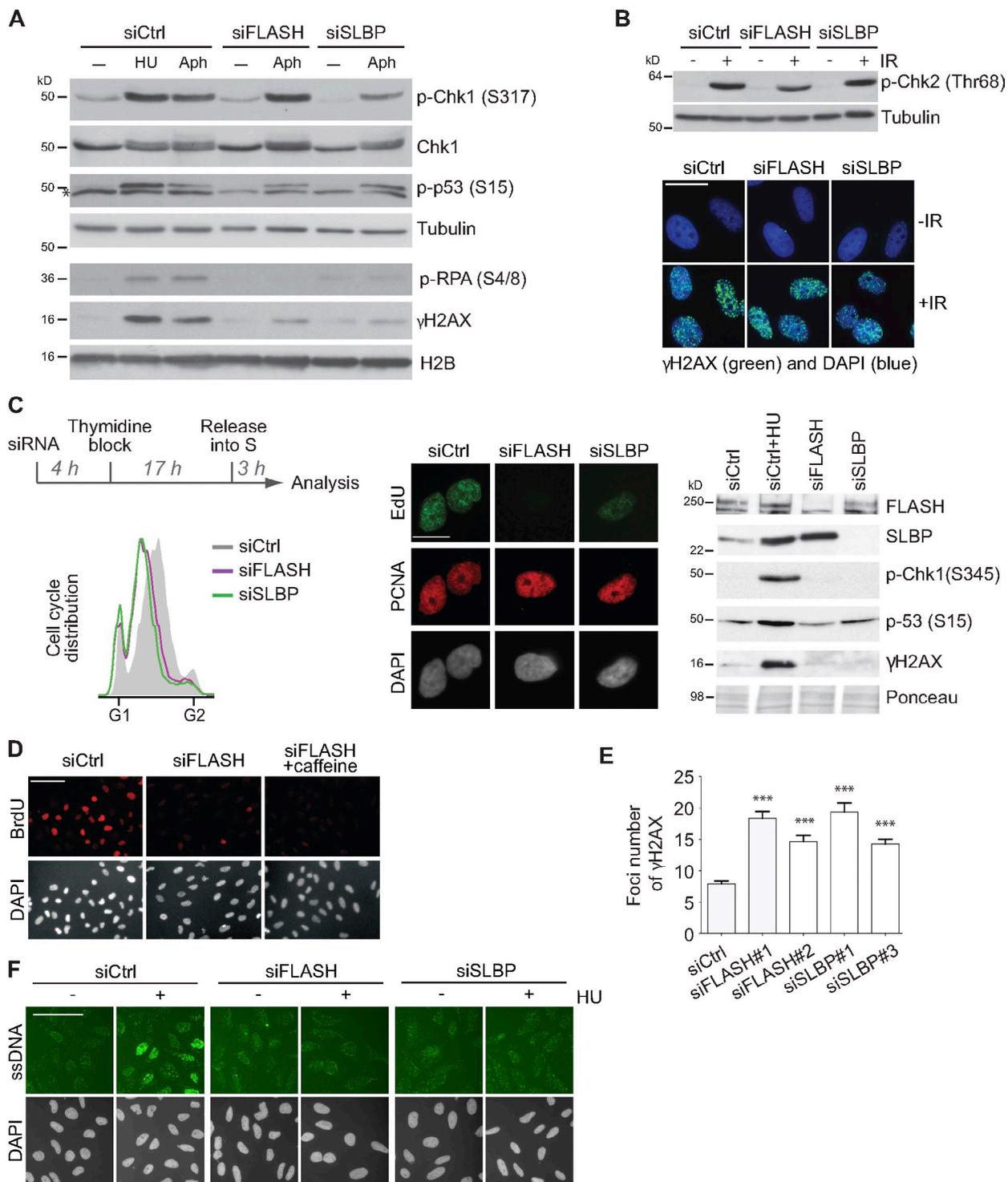


Figure S3. Analysis of DNA damage and checkpoint signaling in FLASH- and SLBP-depleted cells. (A) U-2-OS cells were siRNA transfected and synchronized as in Fig. 1 A. Cells were released into mid-S and harvested for Western blotting. For comparison, cells were treated for 1 h with 4 mg/ml aphidicolin (Aph) or 3 mM HU. The asterisk represents an unspecific band. (B) IR-induced checkpoint signaling in FLASH- and SLBP-depleted cells. 42 h after transfection, cells were irradiated by ionizing radiation (IR; 10 gray) and, 1 h later, harvested for analysis of Chk2 and H2AX phosphorylation by Western blotting (top) and immunofluorescence (bottom), respectively. (C) Analysis of FLASH and SLBP deletion in primary human TIG-3 fibroblasts. (left) Experimental setup and S-phase progression by FACS. (middle) DNA synthesis analyzed by EdU incorporation. (right) Western blot analysis of checkpoint signaling and DNA damage. One representative experiment out of three biological replicates is shown. (D) Replication arrest in response to histone deficiency is independent of ATR/ATM signaling. siRNA-transfected cells were treated 1 h with 10 mM caffeine to inhibit ATM/ATR (Sarkaria et al., 1999) before 10-min BrdU pulse labeling. BrdU was detected after DNA denaturation. (E) DNA damage analysis upon long-term histone deficiency. U-2-OS cells were analyzed for γH2AX by immunofluorescence 48 h after siRNA transfection. Mean number of γH2AX foci per cell is shown. Error bars represent SEMs. Mann-Whitney; $n > 120$; ***, $P < 10^{-3}$. (F) Histone deficiency attenuates HU-induced ssDNA formation. Cells were labeled with BrdU for 24 h before siRNA transfection. Transfected cells were synchronized as in Fig. 1 A, released into S phase for 6 h, and treated with HU as indicated. ssDNA was revealed by BrdU detection under non-denaturing conditions. Bars: (B and C) 20 μm; (D and F) 100 μm. siCtrl, siRNA control.

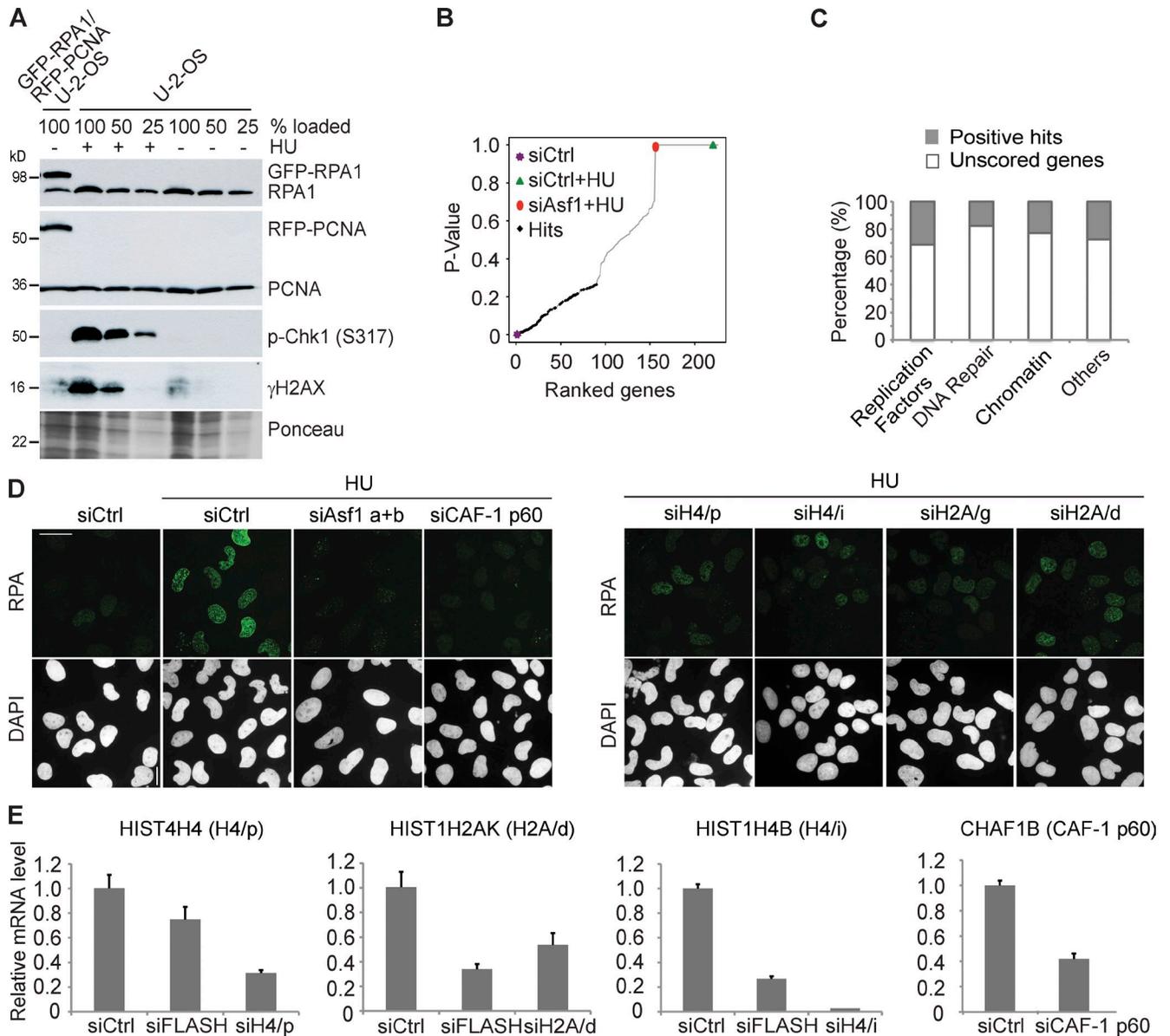


Figure S4. **siRNA screen for factors required for HU-induced DNA unwinding.** (A) Characterization of the GFP-RPA1/RFP-PCNA reporter cell line. GFP-RPA1 and RFP-PCNA are expressed at levels comparable to the endogenous proteins and do not trigger DNA damage. Extracts prepared from U-2-OS cells treated with HU were used as a positive control. (B and C) siRNA screen. GFP-RPA1/RFP-PCNA reporter cells were transfected with a custom-made replication/repair library of 236 genes targeted by three individual siRNAs. Signals were normalized using three controls: scramble siRNA treated with/without HU and siRNA against Asf1a and Asf1b (siAsf1) treated with HU. Individual gene hit calls were made using the RSA method, whereas the significance of the assay signal over all siRNAs targeting one gene was assessed, with the null hypothesis that the signal did not change. As threshold was defined by the score of siAsf1, all genes whose siRNAs scored higher than siAsf1 were not considered as positive and are thus presented with a p-value similar to siAsf1. Hits along with RPA and PCNA scores are shown in Table S2. To strike a reasonable balance between identifying true positives and avoiding false negatives, we combined two independent siRNA screens and used a relaxed significance threshold ($P < 0.1$) combined with the following constraints: the gene had to be considered a hit by at least four out of six siRNAs (three different siRNAs in two independent screens) with $<75\%$ of the activity measurement of the siAsf1 control. (B) RSA analysis for hit selection of one representative screen. (C) Functional classification of positive hits. (D) Validation of siRNA target specificity and attenuation of HU-induced ssDNA exposure for selected hits from the siRNA screen. U-2-OS cells were transfected by siRNAs and, 46 h later, treated with HU for 1.5 h. RPA was detected by immunofluorescence after preextraction. siRNAs against two independent H4 and H2A transcripts were selected based on high ranking in the RSA analysis. Bar, 20 μm . (E) Quantification of mRNA levels by qPCR. After transfection, cells were synchronized to the G1/S border and subsequently released into S phase for 6 h. FLASH depletion was used as a positive control. mRNA levels were normalized to GAPDH. Error bars indicate SDs of technical triplicates. One representative experiment out of two biological replicas is shown. It was not technically feasible to measure H2A/g mRNA levels, as the gene (*HIST1H2AD*) overlaps with *HIST1H3D*, and both transcripts contain the coding sequence of H2A/g. siCtrl, siRNA control.

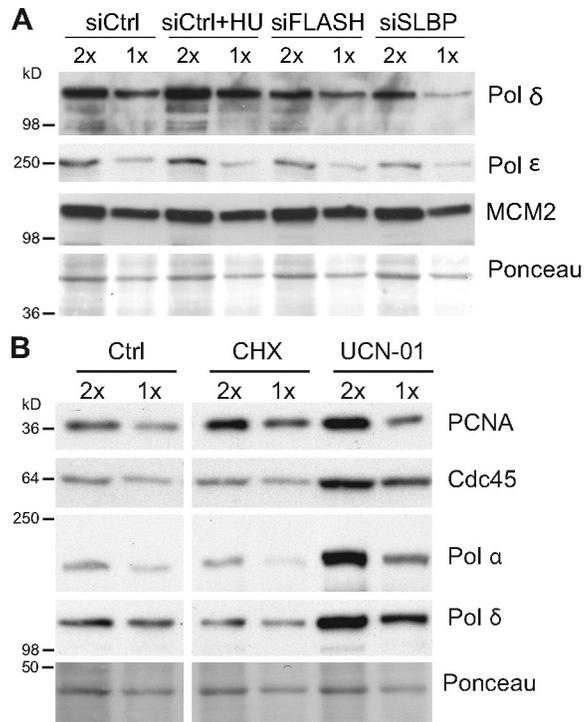


Figure S5. **Analysis of fork components.** (A) Western blot of chromatin fractionated by partial DNase I digestion. Transfected cells were synchronized to the G1/S border and released into S phase for 6 h. Where indicated, cells were treated 1 h with HU. A Ponceau-stained membrane containing the same samples run on a different gel is shown to indicate relative protein levels in the samples. One representative experiment out of three biological replicas is shown. (B) Activation of dormant origins by UCN-01 is associated with additional loading of DNA polymerases, Cdc45, and PCNA onto chromatin. In contrast, inhibition of DNA replication by short-term CHX treatment (Fig. S1 D) leads to PCNA accumulation without affecting Cdc45 levels and polymerase δ . Cells synchronized as in A were treated for 1 h as indicated. Soluble material was removed by Triton X-100 extraction, and chromatin pellets were analyzed by Western blotting. siCtrl, siRNA control.

Table S1. **Antibodies used in this study**

Name	Company/reference	Clone/catalogue number
Asf1 (polyclonal rabbit antibody generated against recombinant GST-Asf1a)	Groth et al., 2005	
α -Tubulin	Sigma-Aldrich	T9026
BrdU	GE Healthcare	RPN202
β -Actin	Sigma-Aldrich	A5441
Chk1	Santa Cruz Biotechnology, Inc.	sc-56291
Chk1 phospho-Ser317	Cell Signaling Technology	2344S
Chk1 phospho-Ser345	Cell Signaling Technology	#2348
Chk2 phospho-(Thr68)	Cell Signaling Technology	2661L
DNA polymerase α	Abcam	ab31777
DNA polymerase δ	BD	610972
DNA polymerase ϵ	BD	611238
FLASH	Provided by V. De Laurenzi ^a	
Flag epitope	Sigma-Aldrich	F3165
γ -H2AX	EMD Millipore	clone JBW301
H2B	Abcam	ab1790
H3	Abcam	ab1791
H4K12ac	Abcam	ab1761
HA	Roche	12CA5
MCM2	Bethyl Laboratories, Inc.	A300-122A
Mouse anti-IIdU	BD	347580
Mouse anti-ssDNA	EMD Millipore	MAB3868
PCNA	Santa Cruz Biotechnology, Inc.	sc-7907
p53 phospho-Ser15	Cell Signaling Technology	9284
RPA phospho-(S4/S8)	Bethyl Laboratories, Inc.	A300-245A
RPA/p34	Thermo Fisher Scientific	MS-691
Rat anti-BrdU	AbD Serotec	OBT0030G
Rat anti-CIdU	AbCys SA	ABC117-7513
SLBP	Abcam	ab56329

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Table S2. **siRNA sequences used in this study**

siRNA ID	Sequence (sense strand)
FLASH#1	5'-CCGCAAGGAUGAAGAAAUA-3'
FLASH#2	5'-AGAUAAAAGACAGUAGGAAA-3'
FLASH#3	5'-UGAUGAAGGACCAGAGAAA-3'
SLBP#1	5'-GGAUGUGAUUUGCAAGAAA-3'
SLBP#2	5'-ACAAAGAAAUGGCAAGAU-3'
SLBP#3	5'-GAGAGAGAAAUCAUCAUC-3'
Asf1a	5'-GAGACAGAATTAAGGAAA-3'
Asf1b	5'-CGGACGACCTGGAGTGGAA-3'
Chk1	5'-GCAACAGUAUUUCGGUAUA-3'
HIST4H4	5'-GGCAUUACAAAGCCGGCGA-3'
HIST1H4B	5'-GACUGUCACUGCCAUGGAU-3'
HIST1H2AD	5'-GGGCAAGUAAAACGAGAAC-3'
HIST1H2AK	5'-GUUCAUUACUCAUACCUCC-3'
CAF1P60	5'-GGAUCUGGAAGGUAGAAA-3'
Control siRNA	5'-CACUUGUCACUACUUUCUC-3'

Table S3. **Quantitative RT-PCR primer sequences**

Genes	Forward primer	Reverse primer
<i>HIST4H4</i> (H4/p)	5'-AGGCGCTGTGATTTAGAAT-3'	5'-GGCTTTGTAATGCCTTGGAT-3'
<i>HIST1H3C</i> (H3/c)	5'-TGCTACTAAGCAGCCCGTA-3'	5'-ACTTCTGGTAGCGACGGATT-3'
<i>HIST1H2BH</i> (H2B/i)	5'-CATGAATTCCTTTGTCAACGAT-3'	5'-GCACGGCTGTCTGGATCT-3'
<i>HIST1H2AG</i> (H2A/p)	5'-CTAAGGCCAAGACTCGCTCT-3'	5'-CTCGGCATAGTTGCCTTTG-3'
<i>HIST1H4B</i> (H4/i)	5'-TTAAGCGAATTTCCGGTTTG-3'	5'-CGTACAGAGTCCGTCCTTGA-3'
<i>HIST1H2AK</i> (H2A/d)	5'-ACAACAAGAAGACCCGCATC-3'	5'-GATATTGGGCAGGACACCAC-3'
<i>CAF1P60</i>	5'-TTTCTTCCACGGACGGTTAC-3'	5'-TGACAGCAGGAGTGATGGAG-3'
<i>GAPDH</i>	5'-TGGTATCGTGGAAGACTCA-3'	5'-CCAGTAGAGGCAGGATGAT-3'

References

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