Supporting Information

Deposition bias of chromatin proteins inverts under DNA replication stress conditions

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1. Materials and Methods

1.1. Optimizing co-translational incorporation of azidohomoalanine (AHA)

Histone extraction
Nuclei were isolated as described for the Double-Click-seq experiment. Nuclei were resuspended in 400 μl 0.2 M H₂SO₄ and left at an end-over-end rotator for at least 30 minutes at room temperature. The samples were spun down in a tabletop centrifuge to remove nuclear debris at 16,000 g for 10 minutes. The supernatant (containing histones) were transferred to a fresh tube and while mixing 200 μl trichloroacetic acid (100%) was added drop by drop to the histone solution. The tube was inverted several times to mix the solutions and put on ice for 30 minutes. The histones were spun down in a tabletop centrifuge at 16,000 g for 10 minutes. The supernatant was carefully removed with a pipette and the histone pellet was washed with ice-cold acetone without disturbing it. The samples were centrifuged again at 16,000 g for 5 minutes and washing with acetone was repeated. The supernatant was carefully removed and the histone pellet was air dried for 20 minutes at room temperature. The pellet was then dissolved in an appropriate volume of water (typically 100 μl) and transferred to a fresh tube. The tube was centrifuged at 3400 g for 2 minutes and the supernatant was transferred to a new tube. The non-dissolved pellet contains mostly nonhistone proteins and other debris. The concentration was measured with the Bradford assay.

Propionic anhydride derivatization
Between 1 and 5 μg of the histone sample was aliquoted and diluted with 5 μL of 100 mM ammonium bicarbonate buffer (pH 8). The propionylation mixture was made by vortexing 75 μL of propionic anhydride with 25 μL 2-propanol. Next, 20 μL of this propionylation mixture was added to the histone sample, vortexed and centrifuged for a few seconds. The pH was checked with a pH indicator strip. If the pH was <8, ammonium hydroxide was added dropwise until the pH was approximately 8. Typically, adding 20 μL of ammonium hydroxide was a good starting point for the titration. The reaction was incubated at 51 °C for 20 minutes. The sample was concentrated to about 5 μL in a SpeedVac concentrator at room temperature for about 20 minutes. The sample was diluted with 5 μL of 100 mM ammonium bicarbonate buffer (pH 8) and derivatization was repeated. The reaction was then allowed to cool to room temperature without repeating the concentration step.

In solution trypsin digestion
Trypsin (Promega or NEB) was added to the histone sample at a 1:20 ratio (e.g., 5 mg of trypsin for 100 mg of histones) and the digestion was left at 37 °C for 6 hours. The digestion was stopped by adding 5 μL of glacial acetic acid to reach pH 3, which prevents trypsin from further digesting the histones. The sample was frozen at -80 °C to fully deactivate trypsin.

NanoChip LC–MS/MS QTOF
For the analysis of AHA incorporation of the histone peptides by LC–MS/MS, a quadruple time-of-flight mass spectrometer (QTOF, Agilent 6510) with a liquid chromatography-chip cube (# G4240) electrospray ionization interface was coupled to a nanoLC system (Agilent 1200) composed of a nanopump (# G2226A), a capillary loading pump (# G1376A) and a solvent degasser (# G1379B). Injections were performed with an autosampler (# G1389A) equipped with an injection loop of 40 μL and a thermostated cooler maintaining the samples in the autosampler at 4 °C during the analysis (# G1377A Micro WPS). The instrument was operated under the MassHunter Data Acquisition software (Agilent Technologies, Santa Clara, USA, version B.04.00, B4033.3). A chip (ProtID-Chip-150 II 300A, #
G4240-62006) with a 40 nL trap column and a 75 μm × 150 mm analytical column filled with Zorbax 300SB-C18, 5 μm (Agilent Technologies, Santa Clara, USA) was used for peptide separation. The identification of peptides was based on data collected in auto MS/MS mode (2 GHz) using the following settings; fragmentor: 175 V, skimmer: 65 V, OCT 1 RF Vpp: 750 V, precursor ion selection: medium (4 m/z), mass range: 200–2500 m/z, acquisition rate for MS: 2 spectra/s, for MS/MS 3 spectra/s; MS/MS range: 50–3000 m/z; ramped collision energy: slope 3.8, offset: 0, precursor setting: maximum 3 precursors/cycle; absolute threshold for peak selection was 1000; relative threshold was 0.01% of the most intense peak, active exclusion enabled after 1 selection, release of active exclusion after 0.6 min, precursors were sorted by abundance only. The MS/MS files were stored in centroid and profile mode. MS1 absolute threshold 50 and MS2 absolute threshold 35 were C646 49 2 2 applied to account for detector noise. A static exclusion range of 200–350 m/z for precursor selection was applied. Gas temperature (nitrogen) was 325 °C and gas flow was 5 L/min. The quantification of peptides was based on data collected in MS mode using the same settings except of the mass range 200–3000 m/z; acquisition rate 1 spectra/s. In both cases lock masses 1221.990 m/z (HP-1221; Agilent article number G1982-85001) and 299.294 m/z (Methyl Stearate; Agilent article number G1982-85003) were used to recalibrate spectra during the acquisition.

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by the MassHunter Qualitative Analysis software version B.05.00 (Agilent Technologies) and saved as mgf files. All MS/MS data were analyzed using Phenyx (GeneBio, Geneva, Switzerland); version CYCLONE (2010.12.01.1). The fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 400 ppm were selected for a database search. Oxidation (+15.99) and replacement by AHA (-4.98) of methionine, acetylation (+42.01), methylation (+14.01), dimethylation (+28.03), trimethylation (+42.04), propionylation (+56.03) of lysine, methylation (+14.01), dimethylation (+28.03) of arginine and amidation of aspartic acid (-0.98) were specified in Phenyx as variable modifications. The ratio between AHA labeled and unlabeled peptides of histone H4 (79–92) KTVTAMDVVYALKR was calculated using the areas under the curve in the extracted ion chromatograph to give an estimate of AHA incorporation in new histones. Total histone H4 was calculated by summing the areas of AHA labeled and unlabeled H4 peptides.

1.2. Double-click-seq

Incubation and harvest of cells
The experiments for all treatments were done in duplicate. As a result libraries from two biological replicates were generated for each condition, except p53Null+HU (material lost during library prep stage) and single-click negative control (sample requested during laboratory lockdown). Four T175 flask were each seeded with approximately 3 x 10^6 cells per experiment. After overnight incubation, culture medium was removed and replaced by pre-mixed medium containing 0.95 x volume 100% AHA medium (DMEM, high glucose, no glutamine, no methionine, no cysteine (Gibco) was supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (10,000 units penicillin and 10 mg streptomycin/mL, Gibco), 4 mM GlutaMax™ (Gibco), 0.2 mM L-Cystine 2HCl, 1 mM sodium pyruvate, 0.2 mM AHA and 20 μM EdU) and 0.05 x volume of DMEM (see recipe above). For induction of replication stress either 1 μM olaparib, 100 nM curaxin (CBL0137), 150 or 200 μM hydroxyurea (HU), 0.5 μM ATR inhibitor (VE-821) or a combination was added to the culture medium. Cells were incubated for 24 h, after which the culture medium was removed and cells were washed twice with ice-cold phosphate-buffered saline (PBS). Cells were harvested with Trypsin-EDTA (Gibco) for 5 minutes at 37 °C. Trypsinization was quenched by the addition of medium and cells were collected by
centrifugation at 200 g for 5 minutes. The pellet was resuspended in ice-cold PBS and centrifuged again at 200 g for 5 minutes. The cell pellet was stored at -80 °C until further use.

Nuclei isolation
The cell pellet was resuspended in 1 mL of ice-cold PBS containing 0.1% NP-40 supplemented with protease inhibitor cocktail (PIC) (cComplete™, Mini, EDTA-free, Roche). The cell suspension was vortexed for five seconds at maximum speed. The lysed cell suspension was centrifuged for 10 seconds at 9000 g. The supernatant was discarded and the nuclei were resuspended in 1 mL of ice-cold PBS containing 0.1% NP-40 supplemented with PIC. The washed nuclei were centrifuged for 10 seconds at 9000 g and the supernatant was discarded.

First click reaction
The nuclei were resuspended in 250 µL nuclei buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM HEPES at pH 7.4) supplemented with PIC and 10 µL 100 mM (in methanol) biotin-PEG4-alkyne (Sigma-Aldrich) was added. Next, a pre-mixed solution of 5 µL 50 mM CuSO₄ with 5 µL 250 mM THPTA (Sigma-Aldrich) was added, followed by 10 µL of a freshly prepared solution of 100 mM sodium ascorbate (17). The nuclei were placed in an end-over-end rotator at room temperature for 30 minutes. After that, the click reaction was repeated as described above using fresh reagents. Nuclei were pelleted for 10 seconds at 9000 g (to remove excess biotin, which would interfere with streptavidin bead pull-down later on) and the supernatant was discarded.

MNase digestion
Nuclei were resuspended in 100 µL of MNase buffer (50 mM Tris.HCl, 5 mM CaCl₂, pH 7.9, supplemented with 100 µg/mL BSA) and warmed to 37 °C in a water bath. Then, 60 U micrococcal nuclease (MNase, New England Biolabs, 1U/µL in MNase buffer) was added and cells were incubated at 37 °C for 5 minutes. MNase reactions were stopped by adding EDTA to a final concentration of 5 mM and by cooling on ice. Next, 100 µL PBS supplemented with 0.1% BSA and 0.01% Tween was added and the nuclei were pelleted by centrifugation for 10 seconds at 9000 g.

Streptavidin enrichment of labeled nucleosomes
The supernatant (containing the nucleosomes) was added to 30 µL streptavidin magnetic beads (Dynabeads™ MyOne™ Streptavidin C1, Thermo Scientific, pre-washed three times with PBS according to the instruction manual) and incubated at room temperature for 1 hour in an end-over-end rotator. The supernatant was removed by placing it on a magnet for 3-4 minutes. The beads were washed five times by resuspension in 200 µL PBS supplemented with 0.1% BSA and 0.01% Tween and placing them back on the magnet. After washing, the DNA bound to the captured nucleosomes was released. This was done by resuspending the beads in 100 µL PBS supplemented with 1 mg/mL proteinase K and incubating the suspension for 30 minutes at 50 °C (pipet up and down after 15 minutes to ensure a homogenous mixture). Then, 100 µL 5 M NaCl was added and the mixture was left rotating for 15 minutes at room temperature. Finally, 360 µL (1.8 x volume of sample) Ampure beads (Beckman) was added directly to the bead suspension to purify the released DNA according to the protocol of the manufacturer. The DNA was eluted in 80 µL water.

Second click reaction
1 µL 100 mM (in methanol) biotin-PEG4-azide was added to the eluted DNA, followed by a pre-mixed solution of 5 µL 50 mM CuSO₄ with 5 µL 250 mM THPTA and 10 µL of a freshly prepared solution of 100 mM sodium ascorbate. The sample was placed in an end-over-end rotator at room temperature.
for 45 minutes. To discard DNA fragments larger than approximately 200 bp 70 µL (0.7 x volume of sample) Ampure beads were added and after 5 minutes incubation at room temperature the beads were discarded and the supernatant was transferred to a new tube. Then 180 µL (1.8 x original sample volume) Ampure beads were added and purification was completed according to the manufacturer’s protocol. The DNA was eluted in 55.5 µL water.

End prep and adaptor ligation
End repair, 5’ phosphorylation, dA-tailing and adaptor ligation was performed using the NEBNext® Ultra™ DNA library prep kit for Illumina®. After adaptor ligation the sample was added to 25 µL streptavidin magnetic beads (Dynabeads™ MyOne™ Streptavidin T1, Thermo Scientific) pre-washed three times with B&W buffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl) according to the instruction manual).

Isolation of replicated double stranded DNA fragments
After 15 minutes incubation at room temperature in an end-over-end rotator, beads were washed four times with 200 µl 1x B&WT buffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl, 0.05 % Tween 20) and once with 2 x B&WT buffer.

Isolation of replicated parental strands
The parental strands were then eluted from the beads by incubation with 100 µl alkaline buffer (0.1 M NaOH, 0.05 % Tween 20) for 1 minute (7). Elution of the parental strand was repeated twice for a total of three times and the pH of the combined alkaline supernatants was neutralized by adding acetic acid to a final concentration of 0.1 M and 2 mM EDTA pH 8.0. The sample was diluted to 1 mL with water and concentrated to approximately 20 ul with a Vivaspin® 2 5K centrifugal concentrator according to the manufacturer’s instruction (15 minutes centrifuging at 4000 g in a swing bucket). The DNA was recovered by performing a reverse spin at 3000 g for 2 minutes.

Library amplification and sequencing
Libraries were amplified using the NEBNext® Ultra™ DNA library prep kit for Illumina® using NEBNext® multiplex oligos for Illumina® (index primer set 1 or 2) with 13 PCR cycles. Purification was done as described in the manual (with 0.9 x Ampure beads) and eluted in 30 µl water. Libraries were quantified with Qubit™ dsDNA high sensitivity assay kit (Thermo) and further analyzed with the high sensitivity D1000 ScreenTape system (Agilent). Sequencing was performed on a NextSeq 500 with onboard clustering using a 75-cycle high output v2.5 kit (Illumina) to generate paired-end 41-bp-long reads.

Generating negative control and control of streptavidin enrichment of labeled nucleosomes
The washing fractions of the streptavidin enrichment step of labeled nucleosomes were kept and analyzed with gel electrophoresis (see Figure S1E). Only non-specific protein elution from the beads was observed, indicating that the stringent washing steps were sufficient to primarily enrich nucleosomes over other DNA-protein complexes, as others have observed as well (17). To test for absence of background signal the double-click-seq protocol was followed until streptavidin enrichment of labeled nucleosomes. After DNA elution of the beads, an Ampure bead clean-up, the resulting double-stranded DNA was eluted in 55.5 µL water. This was followed by end prep, adaptor ligation and library amplification according to the NEBNext® Ultra™ DNA library prep kit manual. Sequencing, paired-end, 150 bp, was performed using an Illumina NovaSeq 6000 device. Furthermore, performing double-click-seq with cells incubated with EdU but without AHA or vice versa did not result in DNA amplification.
1.3. Inactivation of TP53 in hTERT-RPE-1 cells

hTERT immortalized human retinal pigmented epithelial cells (hTERT RPE-1, ATCC ®, CRL-4000™) were used. To mutate TP53, a single guide RNA (sgRNA) targeting exon 4 of TP53 (5'-CTGTCATCTTCTGTCCCTTC-3') was cloned into pSpCas9(BB)-2A-GFP (pX458, plasmid #48138, Addgene). pSpCas9(BB)-2A-GFP was a kind gift from dr. Feng Zhang (18). RPE-1 cells were transfected with pX458 using Fugene HD transfection reagent (Promega) according to the manufacturer’s protocol and 48 hours later cells were selected for 3 weeks with 10 mM Nutlin-3 (Selleck Chemicals). TP53 was mutated in exon 4 by a 7 basepair deletion (TCA-TCT-T), leading to a frameshift at codon 97. TP53 mutations in exon 4 were confirmed by Sanger sequencing.

1.4. Quantification and Statistical Analysis

Scripts in this section are available at https://github.com/thamarlobo/histone_deposition_analysis.git

Raw data processing of OK-seq data

Quality trimming of raw OK-seq reads for RPE-1 hTERT cells (GSM3130725 and GSM3130728) was performed using single-end mode implemented in Trimmomatic package (v. 0.36) using recommended settings and a minimum read length after trimming - 20bp. Trimmed sequences were mapped to genome assembly GRCh38 using Bowtie2 mapper (v. 2.2.4). Sorted alignments were marked for PCR duplicates and used for downstream analyses. To prevent problems due to repetitive sequences that are collapsed in the current genome reference (and hence showing extremely high coverage by NGS reads), we first divided the genome into bins of 10 kb and determined the median number of reads mapping to a bin (primary alignments with a minimum mapping quality of 20, PCR duplicates excluded). All reads that mapped to (part of) a bin with either less than a tenth or more than 10 times the median number of reads per bin, were excluded from further analysis. Blacklists for each OK-seq dataset with these bins and the number of reads mapping there can be found in our code repository.

Correlating the OK-seq profiles of cells with and without replication stress

For each mapped OK-seq read (mapq >= 20, duplicates and secondary alignments were ignored) we determined the 5’ start location of the Okazaki fragment that this read must have originated from. We assumed that each fragment had a length of 150 bp. We used GSM3130725 as a reference to divide the autosomes into bins containing 10,000 Okazaki fragment 5’ starts. For each bin we determined the percentage of reads mapped to the forward strand in GSM3130725 and GSM3130728. Bins that contained less than 5000 fragment 5’ starts in GSM3130728 were excluded. The correlation between the percentage of fragments mapped to the forward strand in both samples was calculated using a Spearman rank test.

Identification of replication initiation zones using OK-seq data

Okazaki fragments come from the forward strand in the leftward moving fork of the origin, while they come from the reverse strand in the rightward moving fork. Replication initiation zones were identified by the detection of these strand usage switches using the script detect_strandswitches.pl. For each mapped read (mapq >= 20, duplicates and secondary alignments were ignored) we determined the 5’ start location and 3’ end location of the Okazaki fragment that this read must have originated from. We assumed that each fragment had a length of about 150 bp.

For each genomic region in between two consecutive Okazaki fragments we scored how many fragments mapped to the forward strand in the left flank (FL) and how many fragments mapped to the reverse strand in the right flank (RR), where the flank consisted of a hundred fragments (N =100
fragments per flank). An “origin score” was calculated as \((FL + RR - N)/N\), resulting in a score of zero for a random read distribution and a score of 1 for the perfect origin). Next, the regions were sorted by decreasing origin score. The region with the highest score was marked as a potential initiation zone, and regions for which the origin score was based on at least one of the same fragments as the identified potential initiation zone were subsequently excluded to prevent multiple calls in the same initiation zone. This process was repeated until there were no regions left to assign. When a replication initiation zone was identified in between two overlapping Okazaki fragments, we marked the middle base pair of the overlap. Finally, the significance of every potential initiation zone was determined using a binomial test where the number of successes was defined as FL + RR and the number of trials was the preset number of flanking fragments times two \((N*2)\), using the script get_significant_oris.R.

The resulting p-values were corrected for the number of performed tests using the Benjamini-Hochberg method. Exclusion of potential initiation zones with FDR >= 0.01 and regions with a negative origin score (indicating potential fork merger zones) resulted in a final list of 10,733 identified replication initiation zones. A list of the replication initiation zones can be found in our code repository.

As a control we ran our Perl script while randomly shuffling strand information. Zero replication initiation zones were identified, confirming that our origin detection method is specific and does not identify replication initiation zones in random background data. Only autosomal replication initiation zones (n=9,608) were included into downstream analysis.

1.5. Calculation of replication fork directionality at replication initiation zones

To examine the extent of replication continuation for each separate replication initiation zone, we calculated the OK-seq signal at the detected autosomal replication initiation zones using the script map_seqdata_around_oris_sliding_windows.pl and visualized the results in a heatmap. The signal was computed per 1 kb bins around each replication initiation zone, as the percentage of unambiguously mapped OK-seq reads that correspond to reverse strand fragments. Reads were assigned to bins according to the mapping coordinate of the central point of the fragment they originated from (assuming each Okazaki fragment length of about 150 bp). Due to low read coverage per individual initiation zone, we performed local smoothing, averaging signal from 4 bins upstream to 4 bins downstream (9 bins in total). The heatmap was plotted with the R package pheatmap 1.0.12.

We also calculated the average OK-seq signal across initiation zones using the script map_seqdata_around_oris, and plotted it. The signal was again computed per 1 kb bins around each replication initiation zone, as the percentage of unambiguously mapped OK-seq reads that correspond to reverse strand fragments. Alternatively, for figure S1D we calculated the RFD per 1kb bin as \(\text{RFD} = (F - R)/(F + R)\), where F and R correspond to the number of mapped reads to the forward and reverse strand, respectively. No smoothing was applied here. The signal was averaged across initiation zones.

1.6. Raw data processing of double-click-seq data

The quality of sequencing data and potential contaminations were evaluated by FastQC software (v. 0.11.5). Quality trimming was performed using paired-end mode implemented in Trimmomatic package (v. 0.33). Trimmed sequences were mapped to genome assembly GRCh38 using Bowtie2 mapper (v. 2.2.4). Sorted alignments were marked for PCR duplicates and used for downstream analyses. Data have been deposited to EBI ArrayExpress under accession number E-MTAB-8624.
Calculation of new chromatin protein distribution bias at replication initiation zones

We calculated the average double-click-seq signal across initiation zones using the script map_seqdata_around_oris, and plotted it. The signal was computed per 1 kb bins around each replication initiation zone, as the percentage of unambiguously mapped read pairs that correspond to forward strand fragments. The calculation of partition score (Figure S1C) was performed per 1kb bins as \((\text{F} - \text{R})/(\text{F} + \text{R})\), where \(\text{F}\) and \(\text{R}\) correspond to the number of mapped readpairs to the forward and reverse strand, respectively. Fragments were assigned to bins according to the mapping coordinate of their central points. Read pairs with a mapping quality below 20, discordantly mapped read pairs and read pairs corresponding to fragments shorter than 145 bp apart (that might not originate from a nucleosome-bound fragment) were excluded. The signal was averaged across initiation zones. When combining results from two replicates, averaging was first applied across initiation zones and then across replicates.

We also visualized the double-click-seq signal for selected individual replicates from untreated cells (replicate 1) and in cells treated with ATR inhibitor and hydroxyurea (replicate 2) around each separate replication initiation zone in heatmaps that can be seen in figures 1F and 3D. The script map_seqdata_around_oris_sliding_windows.pl was used for the calculations. The signal was again computed per 1 kb bins around each replication initiation zone, as the percentage of unambiguously mapped read pairs that correspond to forward strand fragments. Due to low read coverage per individual initiation zone, we performed local smoothing, averaging signal from 4 bins upstream to 4 bins downstream (9 bins in total). The result was visualized with the R package pheatmap 1.0.12.

Determining confidence intervals

Since initiation zones differ in terms of their constitutiveness and hence expected strand bias, we used bootstrap analysis for determining confidence intervals. For each replicate 1000 bootstrap replicates were performed to assess the robustness of observed strand bias. We used the range between the 2.5th and the 97.5th percentiles to show 95% confidence intervals on the plots. Scripts for this analysis can be found in our code repository.

Comparing bias at replication initiation zones in GC-rich versus AT-rich regions

The detected autosomal replication initiation zones were categorized into quartiles based on the GC percentage within 10 kb upstream and downstream of replication initiation zones (approximate distance at which we observe apparent strand asymmetry). The four quartiles had a GC percentage of below 35.6% (Q1), 35.6-37.4% (Q2), 37.4-40.3% (Q3) and above 40.3% (Q4). For each group of replication initiation zones the average chromatin protein deposition bias and replication fork directionality were calculated as described in sections above, with the exception that bin size was increased from 1 kb to 4 kb to compensate for lower read depth of quantile data using the script map_seqdata_around_oris_gc.pl.

Comparing bias at replication initiation zones in early replicated versus late replicated regions

The detected autosomal replication initiation zones were categorized into quartiles based on the average 100-cell mid-S RT scores of three hRPE-1 replicates for each initiation zone as downloaded from GEO accession GSE108556. Coordinates were lifted to hg38 using the UCSC Lift-over Genome Annotations tool with default settings. The four quartiles had an average 100-cell mid-S RT score of below -0.52 (Q1), -0.52 to -0.20 (Q2), -0.20 to 0.20 (Q3) and above 0.20 (Q4). A higher score indicates earlier replication. For each group of replication initiation zones the average chromatin protein deposition bias and replication fork directionality were calculated as described in sections above, with
the exception that bin size was increased from 1 kb to 4 kb to compensate for lower read depth of quantile data using the script map_seqdata_around_oris_gc.pl.

Comparing bias at replication initiation zones in transcriptionally active versus inactive regions
The detected autosomal replication initiation zones were grouped into active or inactive regions based on hRPE-1 gene expression as downloaded from GEO accession GSE89413 (mapped to genome assembly GRCh38 using STAR aligner (v. 2.7.3) with --outSAMmapqUnique 50 ), using the script 02a_cluster_expressed.pl. Genes with a minimal expression of 1 FPKM and clusters of neighboring genes with a minimum expression of 1 FPKM each and maximum intergenic distance of 10 kb were regarded as active regions. The remaining sequences were marked as inactive regions. A bed file with the active and inactive regions can be found in our code repository. For each group of replication initiation zones the average chromatin protein deposition bias and replication fork directionality signal were calculated as described in section “Calculation of chromatin protein deposition bias at replication initiation zones” and section “Calculation of replication fork directionality at replication initiation zones” respectively.

Multiple regression analysis to predict the effect of several features on chromatin protein deposition around initiation zones
For each double-click-seq sample we fitted the model $y = \beta + \beta_a a + \beta_b b + \beta_c c$, where $y$= the new chromatin protein deposition bias at an initiation zone, $a$ = the GC-fraction within 10 kb upstream and downstream of the initiation zone, $b$= the average 100-cell mid-S RT scores of three hRPE-1 replicates, $c$= transcriptional status (active or inactive) of the initiation zone. The strand deposition bias of new chromatin protien was calculated as the percentage of reads mapped to the forward strand in the 500:5500 bp region to the right of each origin zone minus the percentage of fragments from the forward strand in the -5500:-500 bp region to the left of each origin zone, divided by two. This means that a positive new chromatin protein deposition bias of maximally 50 indicates a lagging strand bias and a negative new chromatin protein deposition bias of maximally -50 indicates a leading strand bias.

1.7. Reagents and Computational Resources

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Azidohomoalanine    | Santa Cruz Biotechnology | Cat#sc-285947; CAS 942518-29-8 |
| Biotin-PEG4-alkyne  | Sigma-Aldrich  | 764213-10MG; CAS 1262681-31-1 |
| THPTA               | Sigma-Aldrich  | 762342-100MG; CAS 760952-88-3 |
| CuSO₄              | Sigma-Aldrich  | 451657-10G; CAS 7758-98-7 |
| Sodium ascorbate    | Sigma-Aldrich  | A7631-25G; CAS 134-03-2 |
| Dynabeads™ MyOne™ Streptavidin C1 | Thermo Scientific | 65801D |
| Dynabeads™ MyOne™ Streptavidin T1 | Thermo Scientific | 65801D |
| Azide-PEG3-biotin conjugate | Sigma-Aldrich  | 762024-10MG; CAS 875770-34-6 |
1.8. Biological Resources

hTERT immortalized human retinal pigmented epithelial cells (hTERT RPE-1, ATCC ®, CRL-4000™) were grown in T175 flasks at 37 °C with 5% CO₂ with DMEM, high glucose, GlutaMax™, pyruvate (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 units penicillin and 10 mg streptomycin/mL, Gibco). Cells were passaged with Trypsin-EDTA at 70-80% confluency and used for a maximum of 20 passages. Cells were regularly tested for mycoplasma infection.
2. Supplemental figures

Figure S1. Additional information relating to Figure 1. (A) Heatmap of replication fork directionality at replication initiation zones. (B) Average replication fork directionality at replication initiation zones (black line) including 95% confidence interval (dark gray fill). (C) Average bias of new histone deposition at replication initiation zones in the untreated condition calculated as partition score (proportion of forward and reverse read counts). (D) Average replication fork directionality (proportion of forward and reverse read counts) at replication initiation zones. (E) SDS-PAGE result (stained with Coomassie Brilliant Blue) of washing steps of first streptavidin pull-down showing only non-specific protein elution due to the stringent washing conditions.
Figure S2. Average deposition bias of new histones at replication initiation zones in individual replicates (black line) with 95% confidence interval (dark gray fill). (A) Untreated condition, replicate 1. (B) Untreated condition, replicate 2. (C) 1 µM olaparib treatment, replicate 1. (D) 1 µM olaparib treatment, replicate 2. (E) 200 µM hydroxyurea treatment, replicate 1. (F) 200 µM hydroxyurea treatment, replicate 2. (G) 100 mM curaxin treatment, replicate 1. (H) 100 mM curaxin treatment, replicate 2. (I) p53-null cells, replicate 1. (J) p53-null cells, replicate 2. (K) p53-null cells + 150 µM HU treatment. (L) 0,5 µM ATR inhibitor treatment, replicate 1. (M) 0,5 µM ATR inhibitor treatment, replicate 2. (N) 0,5 µM ATR inhibitor + 150 µM HU treatment, replicate 1. (O) 0,5 µM ATR inhibitor + 150 µM HU treatment, replicate 2. (P) Negative control (no second click reaction).
Figure S3. Effect of transcriptional activity, replication timing and local GC-content on the average deposition bias of new histones and replication fork direction in each of the separate replicates; relating to Figure 4. Replication initiation zones were grouped into active and inactive regions depending on hRPE-1 gene expression levels. Alternatively, replication initiation zones were split into quartiles based on their replication timing and GC percentage (Q1-lowest, Q4-highest). Bias of new histone deposition (panels A-D, F-I and K-N) and replication fork directionality (panels E, J and O) at replication initiation zones (solid lines) is plotted together with 95% confidence intervals (light color fill). (A) Effect of transcriptional activity for untreated condition, replicate 1. (B) Effect of transcriptional activity for untreated condition, replicate 2. (C) Effect of transcriptional activity for 0.5 µM ATR inhibitor + 150 µM HU treatment, replicate 1. (D) Effect of transcriptional activity for 0.5 µM ATR inhibitor + 150 µM HU treatment, replicate 2. (E) Fork directionality in regions with active/inactive transcription. (F) Effect of replication timing in untreated condition, replicate 1. (G) Effect of replication timing in untreated condition, replicate 2. (H) Effect of replication timing upon 0.5 µM ATR inhibitor + 150 µM HU treatment, replicate 1. (I) Effect of replication timing upon 0.5 µM ATR inhibitor + 150 µM HU treatment, replicate 2. (J) Fork directionality depending on replication timing. (K) Effect of GC-percentage for untreated condition, replicate 1. (L) Effect of GC-percentage for untreated condition, replicate 2. (M) Effect of GC-percentage for 0.5 µM ATR inhibitor + 150 µM HU treatment, replicate 1. (N) Effect of GC-percentage for 0.5 µM ATR inhibitor + 150 µM HU treatment, replicate 2. (O) Fork directionality depending on regional GC-percentage. Y axis displays percentage of reverse strand reads (panels E, J, O – OK-seq data) or percentage of forward strand reads (remaining panels, double-click-seq data)
Figure S4. Cell cycle analysis by Flow Cytometry. RPE-1 cells were incubated with AHA medium 95% and/or EdU (20 µM) for 24 hours. Cells were harvested and fixed before staining with PI and analyzed by Flow Cytometry. EdU and AHA/EdU treated cells clearly show an accumulation in G2/M while AHA alone had no effect on the cell cycle.
Table S1. Experimental condition, number of input and mapped read pairs for each.

| Experimental condition                  | Number of input read pairs | Informative read pairs* |
|----------------------------------------|-----------------------------|-------------------------|
| Negative control                       | 12,378,575                  | 5,383,622               |
| Untreated replicate 1                  | 40,268,580                  | 19,945,411              |
| Untreated replicate 2                  | 40,733,126                  | 15,373,997              |
| 1 µM PARP inhibitor replicate 1        | 39,553,764                  | 9,215,719               |
| 1 µM PARP inhibitor replicate 2        | 39,027,398                  | 12,835,199              |
| 200 µM HU replicate 1                  | 40,086,193                  | 13,450,567              |
| 200 µM HU replicate 2                  | 36,693,399                  | 16,369,979              |
| 100 nM Curaxin replicate 1             | 44,233,478                  | 19,351,226              |
| 100 nM Curaxin replicate 2             | 40,255,541                  | 22,814,978              |
| 0.5 µM ATR inhibitor replicate 1       | 87,032,554                  | 45,052,785              |
| 0.5 µM ATR inhibitor replicate 2       | 40,936,734                  | 8,764,841               |
| p53-null replicate 1                   | 42,790,748                  | 22,395,773              |
| p53-null replicate 2                   | 42,887,334                  | 22,075,784              |
| 0.5 µM ATR inhibitor + 150 µM HU replicate 1 | 38,277,097                  | 22,369,624              |
| 0.5 µM ATR inhibitor + 150 µM HU replicate 2 | 40,901,843                  | 22,502,139              |
| p53-null + 150 µM HU                   | 22,656,546                  | 4,775,667               |

*concordantly and uniquely (mapq >=20) mapped to autosomes, PCR duplicates excluded, fragment length at least 145 bp.
Table S2. Beta values and p-values resulting from multiple regression analysis. The relationship between the new histone deposition bias around initiation zones and the GC-fraction, replication timing and transcriptional status of the zone were modelled through multiple linear regression. The new histone deposition bias is respectively positive or negative in case of a lagging or leading strand bias. A higher replication timing score indicates earlier replication. Active transcription was taken as the reference transcriptional status. See the supplementary methods for details on the quantification of each feature.

| Library (treatment, replicate) | GC-fraction | Replication timing | Not actively transcribed |
|-------------------------------|-------------|--------------------|--------------------------|
|                               | β           | P-value            | β                        | P-value | β         | P-value |
| Untreated replicate 1         | -20.67      | 3.06*10^{-38}      | -0.40                    | 0.03    | 0.35      | 0.09    |
| Untreated replicate 2         | -4.99       | 5.51*10^{-3}       | -0.31                    | 0.13    | -0.32     | 0.18    |
| 1 µM PARP inhibitor replicate 1| -35.54      | 2.87*10^{-48}      | -0.58                    | 0.03    | 0.03      | 0.92    |
| 1 µM PARP inhibitor replicate 2| -13.91      | 4.98*10^{-12}      | -0.42                    | 0.06    | 0.08      | 0.77    |
| 200 µM HU replicate 1         | 7.01        | 5.02*10^{-4}       | 0.12                     | 0.60    | -0.09     | 0.74    |
| 200 µM HU replicate 2         | 5.98        | 9.58*10^{-4}       | -0.15                    | 0.45    | 0.29      | 0.22    |
| 100 nM Curaxin replicate 1    | 21.45       | 5.88*10^{-39}      | 0.19                     | 0.29    | -0.06     | 0.78    |
| 100 nM Curaxin replicate 2    | 24.76       | 2.93*10^{-55}      | 0.65                     | 2.26*10^{-4} | -0.12 | 0.56    |
| 0.5 µM ATR inhibitor replicate 1| 4.64       | 1.17*10^{-5}       | 0.71                     | 2.32*10^{-9} | 0.10 | 0.46    |
| 0.5 µM ATR inhibitor replicate 2| 12.33      | 2.05*10^{-7}       | 0.79                     | 2.97*10^{-3} | -0.03 | 0.91    |
| p53-null replicate 1          | 2.11        | 0.16               | 0.67                     | 5.85*10^{-5} | 0.34 | 0.08    |
| p53-null replicate 2          | 2.95        | 0.05               | 0.79                     | 2.34*10^{-6} | -0.38 | 0.06    |
| 0.5 µM ATR inhibitor + 150 µM HU replicate 1| 12.53      | 6.69*10^{-16}     | 0.21                     | 0.22    | -0.20     | 0.33    |
| 0.5 µM ATR inhibitor + 150 µM HU replicate 2| 28.74      | 2.71*10^{-70}     | 0.63                     | 4.97*10^{-4} | -0.20 | 0.36    |
| p53-null + 150 µM HU          | 19.07       | 3.44*10^{-6}       | 0.73                     | 0.05    | -0.36     | 0.41    |