Fig S1

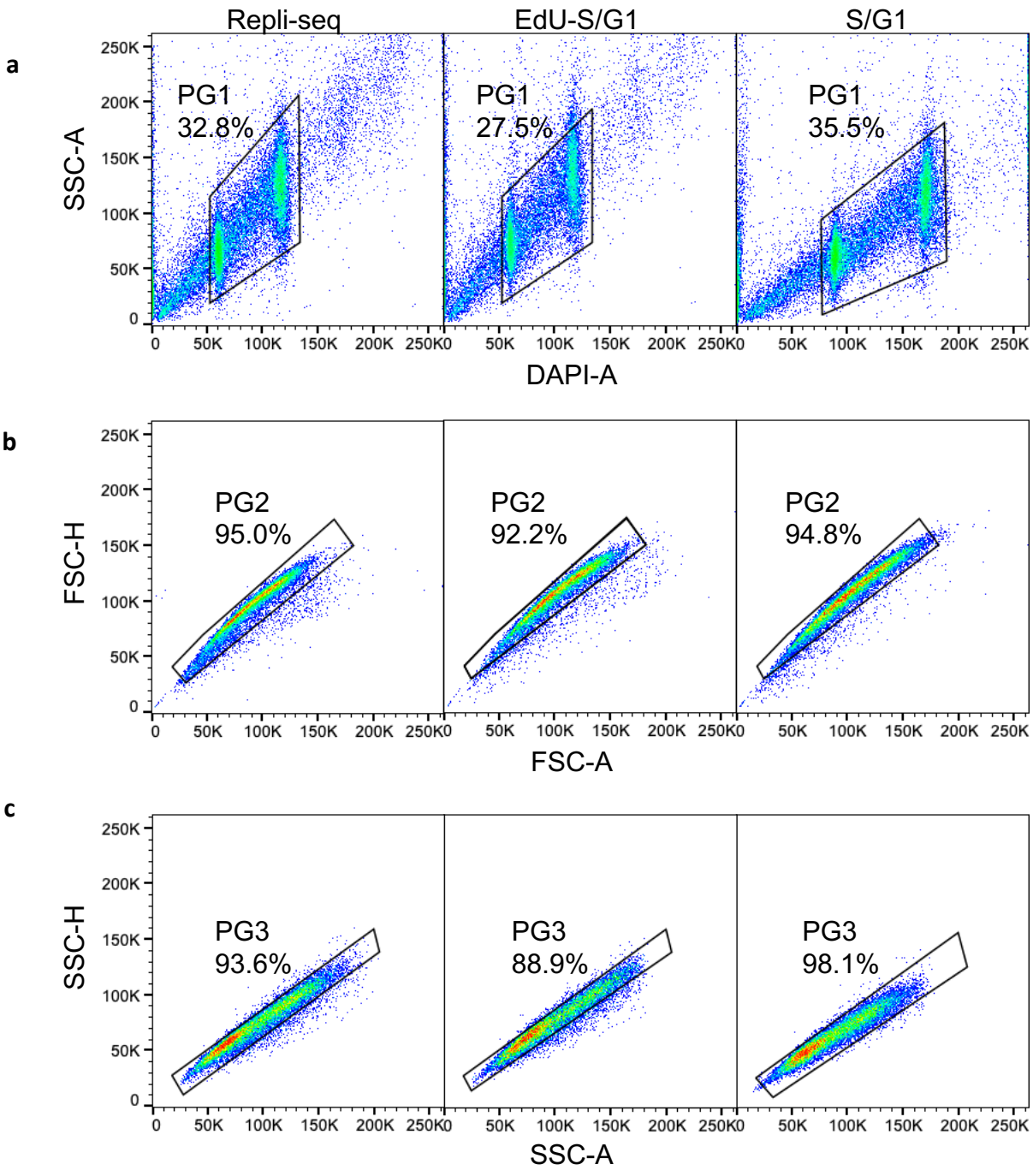

a
Repli-seq
EdU-S/G1
S/G1
PG1
32.8%
PG1
27.5%
PG1
35.5%
SSC-A
DAPI-A
b
PG2
95.0%
PG2
92.2%
PG2
94.8%
FSC-H
FSC-A
c
PG3
93.6%
PG3
88.9%
PG3
98.1%
SSC-H
SSC-A
0
50K
100K
150K
200K
250K

Figure S1. Flow cytometry gating strategy. (**a-c**) The sequential gating strategy used to remove debris, endocycling nuclei, and doublets from Repli-seq (left), EdU-S/G1 (middle) and S/G1 (right) sorted populations. The percentage of nuclei in each gate at each step is shown. (**a**) First, a bivariate plot of DAPI(A) vs SSC(A) was used to select the mitotic nuclei of interest (parent gate 1, PG1) and exclude debris (mostly to the left of the gate) and endocycling nuclei (mostly to the right of the gate). (**b**) Next, a FSC(A) vs FSC(H) plot (of nuclei in PG1) was used to select single nuclei (parent gate 2, PG2) and exclude nuclei doublets. (**c**) Lastly, a SSC(A) vs SSC(H) plot (of nuclei in PG2) was used as an additional selection for single nuclei (parent gate 3, PG3) and exclusion of doublets. The resulting nuclei from PG3 in each RT method are shown in main text Figure 1b-d. This gating strategy ensured that comparable populations of nuclei were used for sorting in the three types of RT experiments.

Fig S2

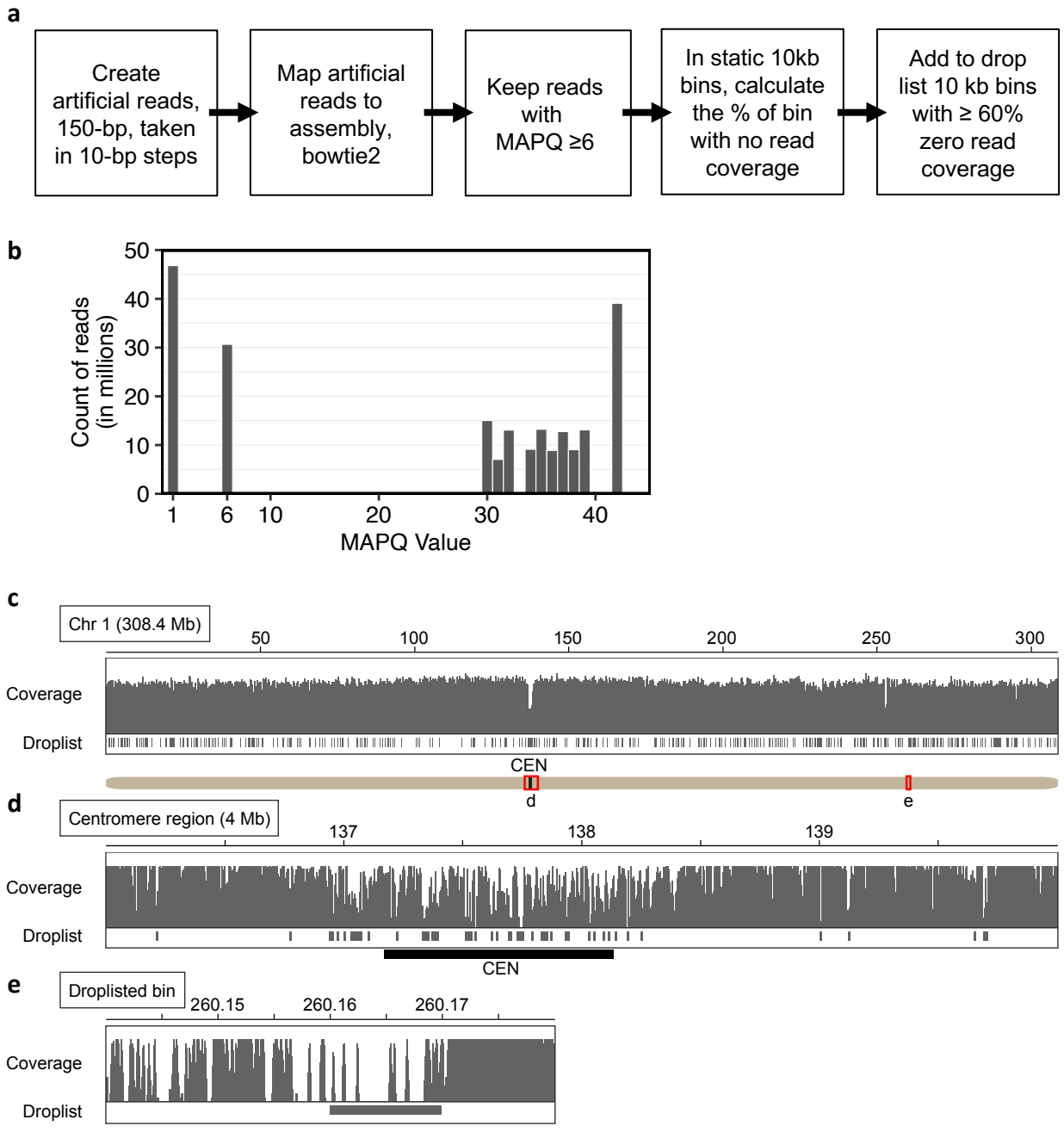

a
Create artificial reads, 150-bp, taken in 10-bp steps
Map artificial reads to assembly, bowtie2
Keep reads with MAPQ ≥6
In static 10kb bins, calculate the % of bin with no read coverage
Add to drop list 10 kb bins with ≥ 60% zero read coverage
b
Count of reads (in millions)
MAPQ Value
c
Chr 1 (308.4 Mb)
Coverage
Droplist
CEN
d
e
d
Centromere region (4 Mb)
Coverage
Droplist
CEN
e
Droplisted bin
Coverage
Droplist

Figure S2. *In silico* analysis of the inherent "mappability" of the reference genome and creating a drop list. The S/G1 methods rely on depletion of reads in a region to indicate late replication timing. Thus, there is a danger of mistakenly defining a low coverage region as late replicating if the low coverage is due to poor mappability. (**a**) An outline of steps used to find regions of the reference genome with inherent low mappability and to create a drop list appropriate for our data analysis. (**b**) The distribution of mapping quality (MAPQ) values from bowtie2 of mapped artificial reads. There are large read populations with a MAPQ of 1, 6, and 30-42. Reads with MAPQ of 1 were filtered out. (**c**) A map of theoretical read coverage on chromosome 1, shown at 1-bp resolution. Droplist regions are shown below in gray. The locations of the enlarged regions shown in panels d and e are indicated on the chromosome coordinate axis, and the centromere is indicated by "C". In a fully mappable genome, the coverage of artificial reads would be 15X throughout the genome. However, due to repetitive sequences, some regions are inherently harder to map uniquely, giving the reads in these regions low MAPQ scores. (**d**) An enlarged 4-Mb region that includes the centromere (black bar) showing the theoretical read coverage and drop listed regions. (**e**) An enlarged 40-kb region in the chromosome 1 longarm showing a close up view of a drop listed 10-kb bin with more than 60% of the bin size with zero coverage by artificial reads.

Fig S3

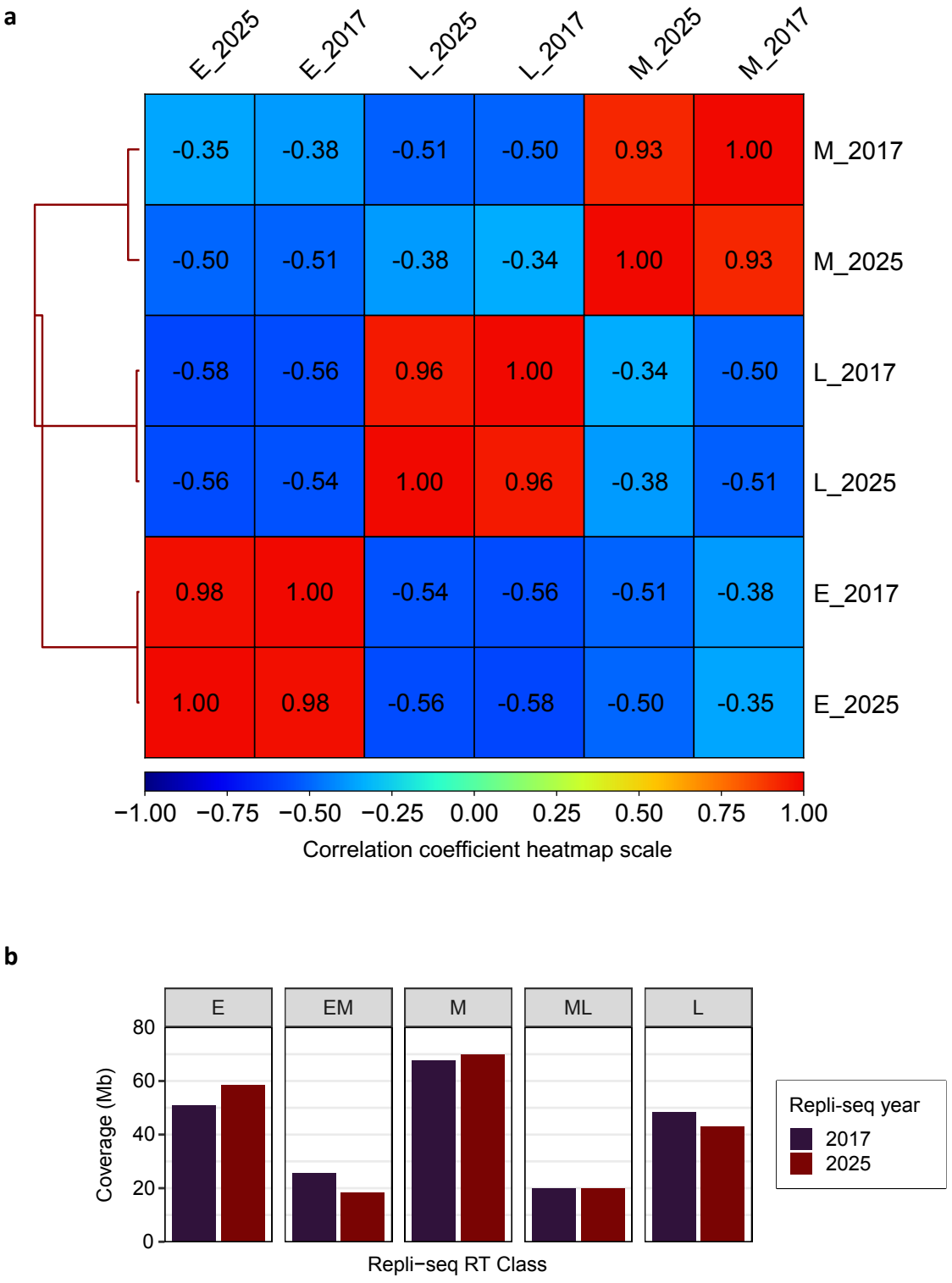
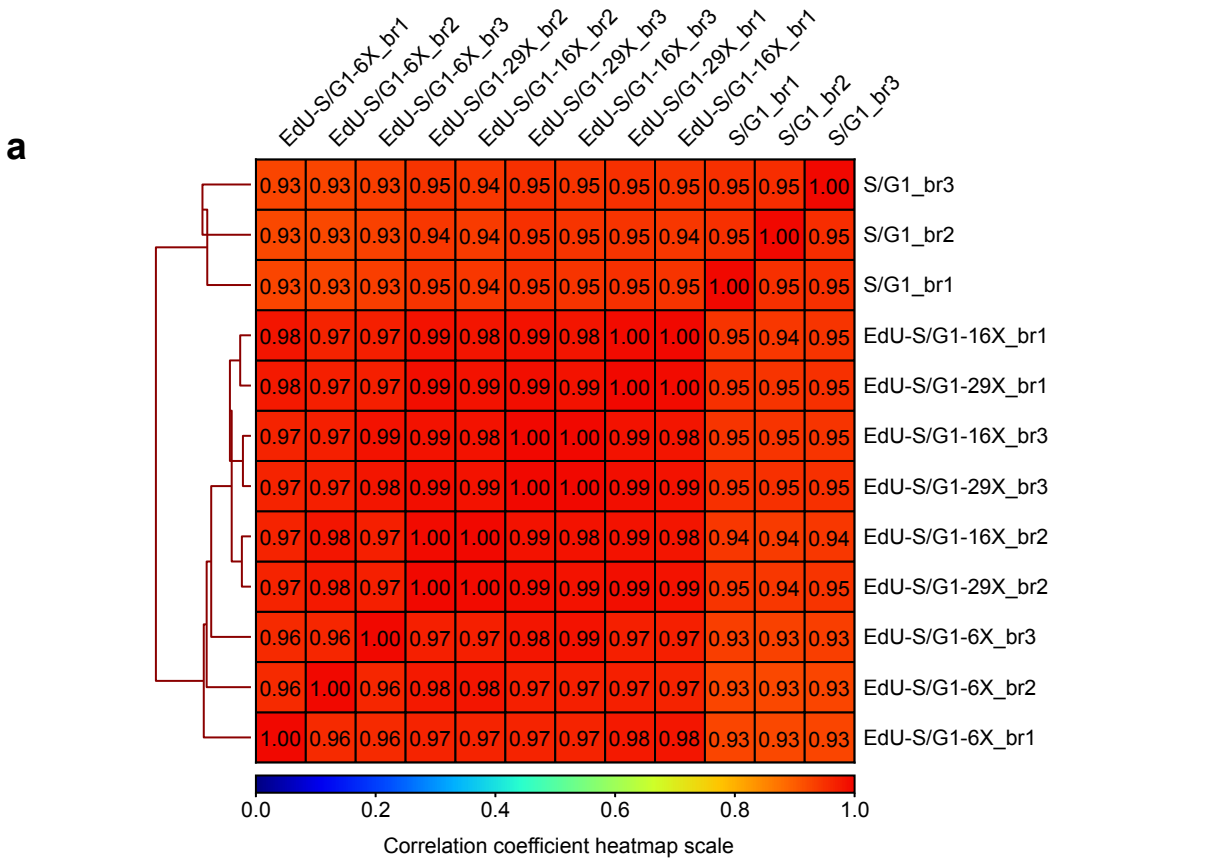
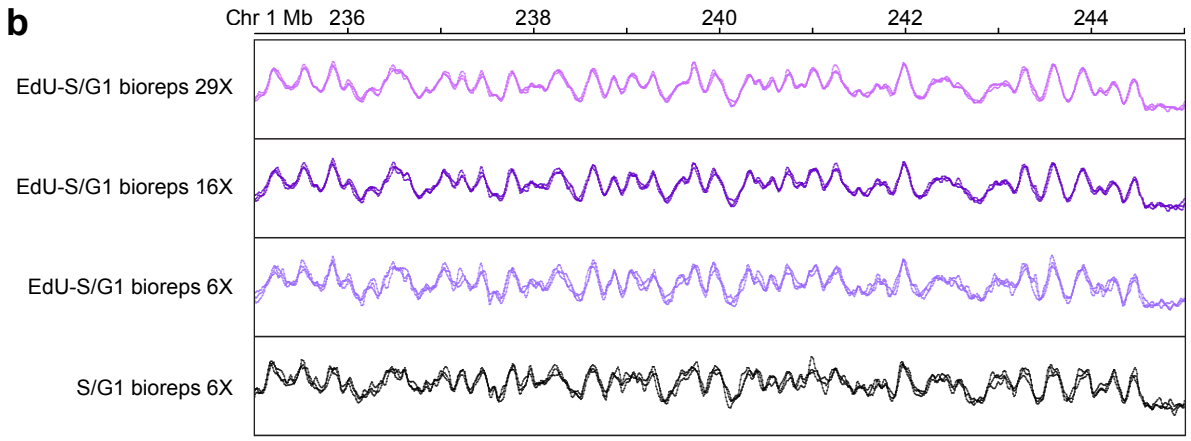

a
E_2025
E_2017
L_2025
L_2017
M_2025
M_2017
-0.35 -0.38 -0.51 -0.50 0.93 1.00 M_2017
-0.50 -0.51 -0.38 -0.34 1.00 0.93 M_2025
-0.58 -0.56 0.96 1.00 -0.34 -0.50 L_2017
-0.56 -0.54 1.00 0.96 -0.38 -0.51 L_2025
0.98 1.00 -0.54 -0.56 -0.51 -0.38 E_2017
1.00 0.98 -0.56 -0.58 -0.50 -0.35 E_2025
−1.00 −0.75 −0.50 −0.25 0.00 0.25 0.50 0.75 1.00
Correlation coefficient heatmap scale
b
E
EM
M
ML
L
Coverage (Mb)
0
20
40
60
80
Repli−seq RT Class
Repli-seq year
2017
2025

Figure S3. Comparison between previously published and current Repli-seq experiments. (**a**) A pearson's correlation coefficient matrix comparing the early (E), mid (M) and late (L) replication intensity signal profiles from our previously published (2017)[9] and current (2025) Repli-seq experiments. (**b**) Barcharts of the coverage of the genome in each RT class defined by Repliscan in the 2017 and 2025 Repli-seq experiments.

Fig S4


a
EdU-S/G1-6X_br1
EdU-S/G1-6X_br2
EdU-S/G1-6X_br3
EdU-S/G1-29X_br2
EdU-S/G1-16X_br2
EdU-S/G1-29X_br3
EdU-S/G1-16X_br3
EdU-S/G1-29X_br1
EdU-S/G1-16X_br1
S/G1_br1
S/G1_br2
S/G1_br3
0.93 0.93 0.93 0.95 0.94 0.95 0.95 0.95 0.95 0.95 0.95 1.00 S/G1_br3
0.93 0.93 0.93 0.94 0.94 0.95 0.95 0.95 0.94 0.95 1.00 0.95 S/G1_br2
0.93 0.93 0.93 0.95 0.94 0.95 0.95 0.95 0.95 1.00 0.95 0.95 S/G1_br1
0.98 0.97 0.97 0.99 0.98 0.99 0.98 1.00 1.00 0.95 0.94 0.95 EdU-S/G1-16X_br1
0.98 0.97 0.97 0.99 0.99 0.99 0.99 1.00 1.00 0.95 0.95 0.95 EdU-S/G1-29X_br1
0.97 0.97 0.99 0.99 0.98 1.00 1.00 0.99 0.98 0.95 0.95 0.95 EdU-S/G1-16X_br3
0.97 0.97 0.98 0.99 0.99 1.00 1.00 0.99 0.99 0.95 0.95 0.95 EdU-S/G1-29X_br3
0.97 0.98 0.97 1.00 1.00 0.99 0.98 0.99 0.98 0.94 0.94 0.94 EdU-S/G1-16X_br2
0.97 0.98 0.97 1.00 1.00 0.99 0.99 0.99 0.99 0.95 0.94 0.95 EdU-S/G1-29X_br2
0.96 0.96 1.00 0.97 0.97 0.98 0.99 0.97 0.97 0.93 0.93 0.93 EdU-S/G1-6X_br3
0.96 1.00 0.96 0.98 0.98 0.97 0.97 0.97 0.97 0.93 0.93 0.93 EdU-S/G1-6X_br2
1.00 0.96 0.96 0.97 0.97 0.97 0.97 0.98 0.98 0.93 0.93 0.93 EdU-S/G1-6X_br1
0.0 0.2 0.4 0.6 0.8 1.0
Correlation coefficient heatmap scale


b
Chr 1 Mb 236 238 240 242 244
EdU-S/G1 bioreps 29X
EdU-S/G1 bioreps 16X
EdU-S/G1 bioreps 6X
S/G1 bioreps 6X

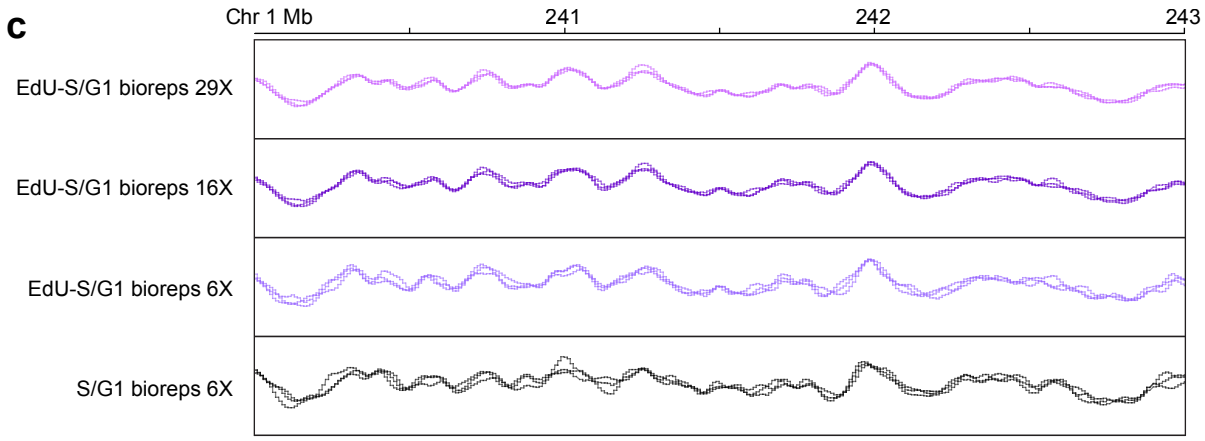

c
Chr 1 Mb 241 242 243
EdU-S/G1 bioreps 29X
EdU-S/G1 bioreps 16X
EdU-S/G1 bioreps 6X
S/G1 bioreps 6X

Figure S4. Pearson correlations between EdU-S/G1 at all coverages, S/G1, and with all Repli-seq signals. (**a**) A Pearson's correlation coefficient matrix comparing all the biological replicates of EdU-S/G1 (including data down sampled to 16X and 6X coverage) and S/G1 data. (**b, c**) Example genome browser regions showing the reproducibility of the biological replicates (profiles overlaid) for both S/G1 and EdU-S/G1 at all coverages.

Fig S5

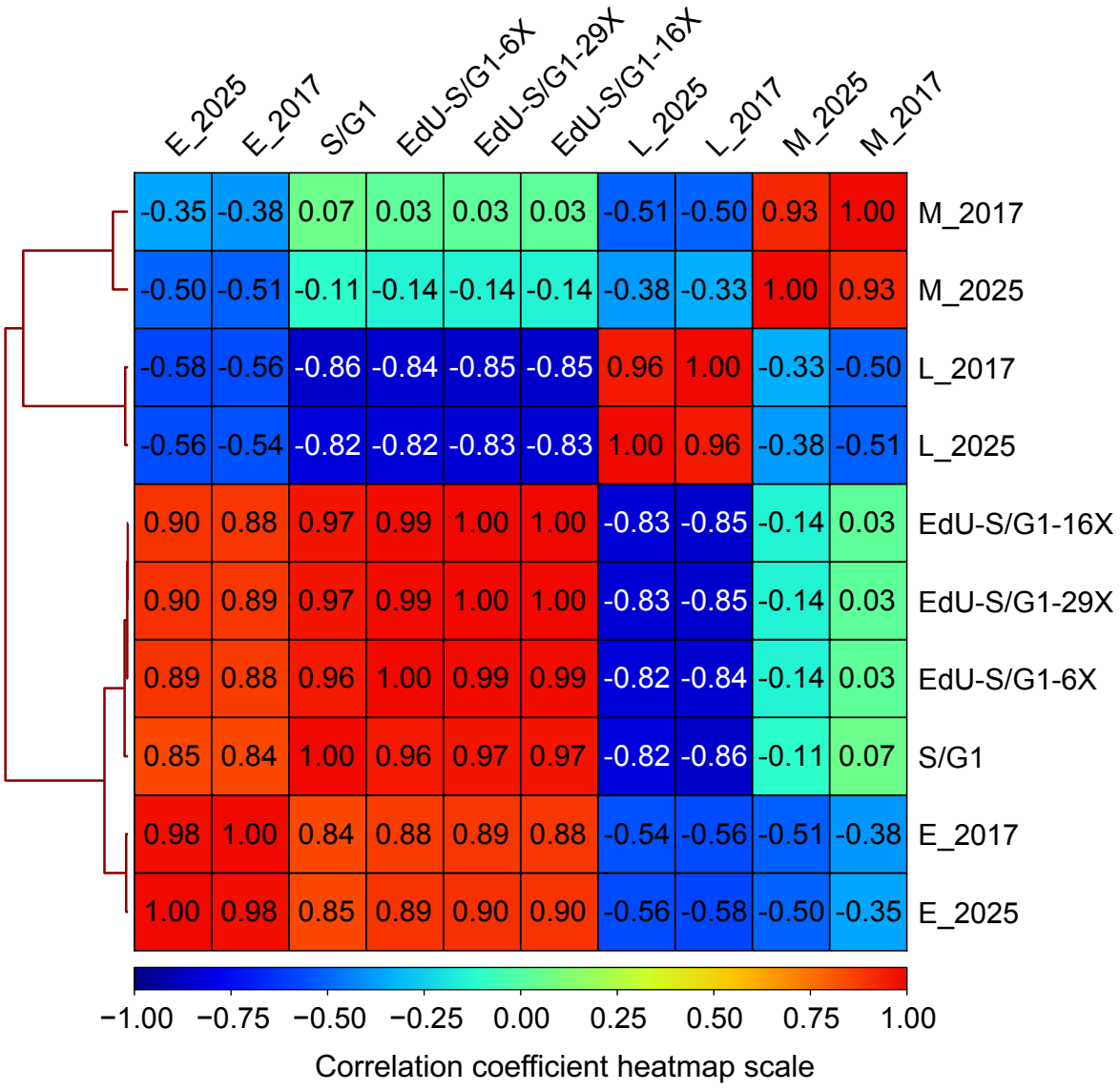

E_2025
E_2017
S/G1
EdU-S/G1-6X
EdU-S/G1-29X
EdU-S/G1-16X
L_2025
L_2017
M_2025
M_2017
-0.35 -0.38 0.07 0.03 0.03 0.03 -0.51 -0.50 0.93 1.00 M_2017
-0.50 -0.51 -0.11 -0.14 -0.14 -0.14 -0.38 -0.33 1.00 0.93 M_2025
-0.58 -0.56 -0.86 -0.84 -0.85 -0.85 0.96 1.00 -0.33 -0.50 L_2017
-0.56 -0.54 -0.82 -0.82 -0.83 -0.83 1.00 0.96 -0.38 -0.51 L_2025
0.90 0.88 0.97 0.99 1.00 1.00 -0.83 -0.85 -0.14 0.03 EdU-S/G1-16X
0.90 0.89 0.97 0.99 1.00 1.00 -0.83 -0.85 -0.14 0.03 EdU-S/G1-29X
0.89 0.88 0.96 1.00 0.99 0.99 -0.82 -0.84 -0.14 0.03 EdU-S/G1-6X
0.85 0.84 1.00 0.96 0.97 0.97 -0.82 -0.86 -0.11 0.07 S/G1
0.98 1.00 0.84 0.88 0.89 0.88 -0.54 -0.56 -0.51 -0.38 E_2017
1.00 0.98 0.85 0.89 0.90 0.90 -0.56 -0.58 -0.50 -0.35 E_2025
−1.00 −0.75 −0.50 −0.25 0.00 0.25 0.50 0.75 1.00
Correlation coefficient heatmap scale

Figure S5. Pearson correlation coefficients at Repli-seq, EdU-S/G1 and S/G1 replication timing values. Notably, there are strong correlations between the Repli-seq early signal and all biological replicates of the two S/G1 methods. A negative correlation between S/G1 data and Repli-seq L data is expected because in S/G1 type methods late replication is identified by a depletion of reads, whereas in Repli-seq, late replicating DNA is identified by elevated read coverage from EdU-labeled DNA regions in late S-phase nuclei.

Fig S6

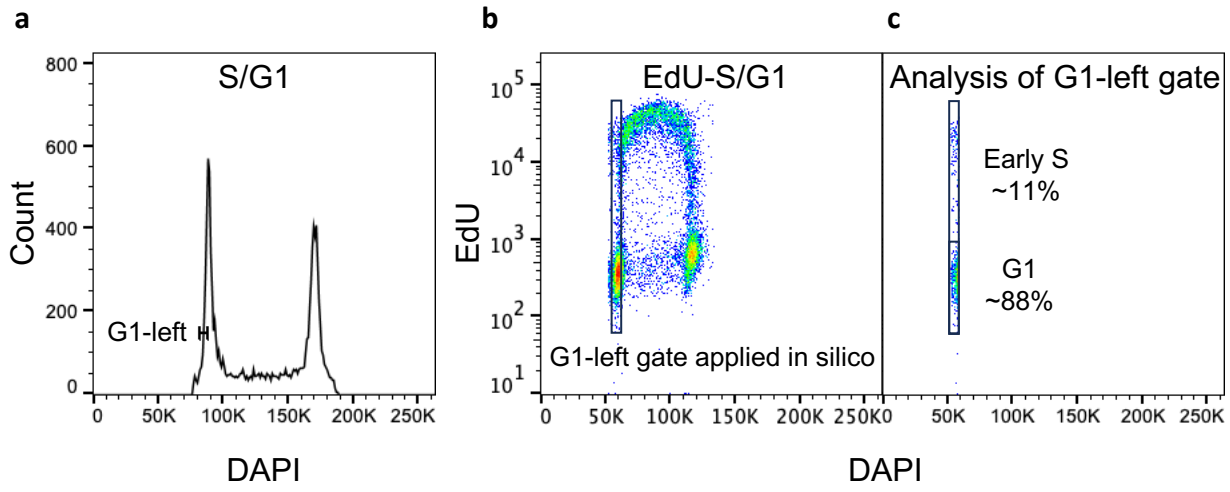
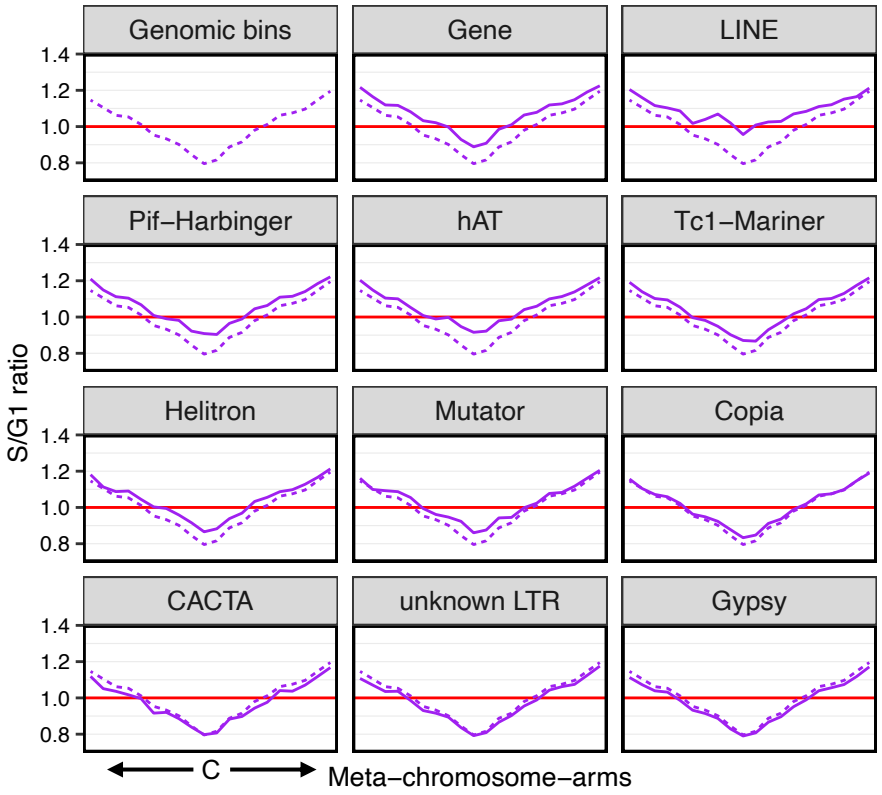


Figure S6. Comparison of the sorted G1 population between S/G1 and EdU-S/G1. Analysis of the G1-left gate from the S/G1 experiment applied *in silico* to the EdU-S/G1 experiment. (a) An example of a flow cytometry histogram plot of the nuclei population used for sorting for the S/G1 experiment (same plot as main text Figure 1d). The gate used to sort the left half of the G1 peak (G1-left) is shown as an interval bar. (b) The G1-left gate used for sorting G1 in the S/G1 experiment is re-created *in silico* (black rectangle) and applied to the EdU-S/G1 nuclei population displayed as a bivariate plot of EdU incorporation (replication, y-axis) vs DAPI (DNA content, x-axis) (same plot as main text Figure 1c). (c) Analysis of the percent of nuclei in the G1-left gate (black rectangle in b) that are replicating vs not replicating based on EdU incorporation. Approximately 88% of the nuclei in the G1-left gate are non-replicating G1 nuclei and ~11% are contaminating nuclei in early S phase.

Fig S7

a


Genomic bins
Gene
LINE
Pif–Harbinger
hAT
Tc1–Mariner
Helitron
Mutator
Copia
CACTA
unknown LTR
Gypsy
S/G1 ratio
1.4
1.2
1.0
0.8
C
Meta–chromosome–arms

b

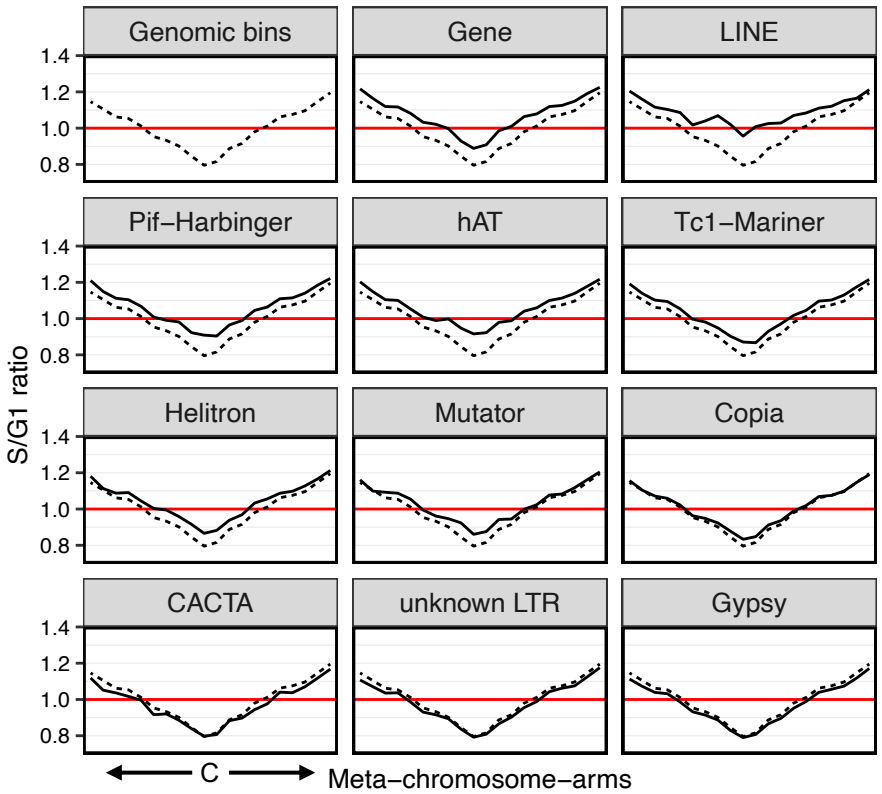

Genomic bins
Gene
LINE
Pif–Harbinger
hAT
Tc1–Mariner
Helitron
Mutator
Copia
CACTA
unknown LTR
Gypsy
S/G1 ratio
1.4
1.2
1.0
0.8
C
Meta–chromosome–arms

Figure S7. Meta-chromosome-arm plots of genomic, genic and TE replication time. Comparison of EdU-S/G1 (**a**) and S/G1 (**b**) signal at genes, hAT and Gypsy superfamilies (solid line) and the genome (dashed line). The centromere (marked C) itself is not given a RT value. All LINE superfamilies were analyzed together. A red line at S/G1 = 1 indicates mid S.

Fig S8

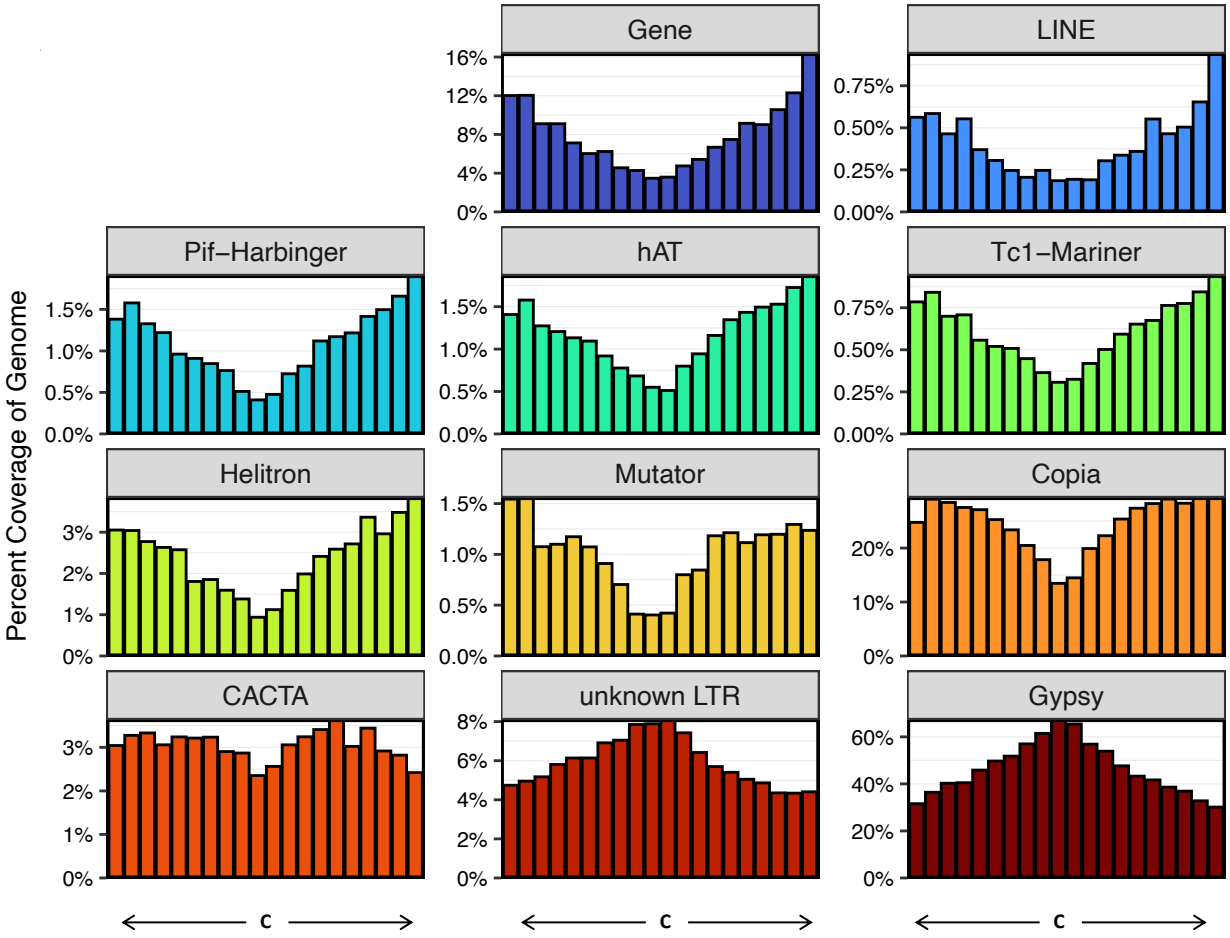

Gene
LINE
Pif–Harbinger
hAT
Tc1–Mariner
Helitron
Mutator
Copia
CACTA
unknown LTR
Gypsy
Percent Coverage of Genome
C
C
C

Figure S8. Genes and Transposable element coverage across relative bins on chromosomes. The percentage of each relative bin from the centromere (see Methods and main text Figure 5) that is covered by genes or TE superfamilies. The LINE superfamilies are analyzed together. The annotated centromere is not represented as a bin, and the bins start at the edge of the centromere. Note that each y-axis scale is different.