Supplementary methods

Cell Culture

C127 cells were purchased from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FBS) and 1% Penicillin and Streptomycin.

Mitotic selection

Specifically, exponentially growing populations of C127 cells at 90% confluence in 15 cm plates (Coring) were vigorously tapped 10 times against the wall of a tissue culture hood to remove any dead or otherwise loosely attached cells and the media was replaced with 10 ml of pre-warmed complete medium (DMEM with 10% fetal calf serum) containing 0.05 μg/ml nocodozole (CalBiochem). Plates were returned to a level surface within the incubator for 4 hours. Mitotic cells were selectively detached by tapping the plates six times with moderate strength. Mitotic cells were pooled together while maintaining in nocodozole media and then released for appropriate amount of time, to get synchronous populations at different times during G1.

Mitotic index

Mitotic index was monitored with metaphase spreads, as described (REFS). 1 ml of nocodozole media containing the mitotic cells was diluted with 3 volumes of water and incubated at 37°C for 15 minutes to swell cells. One or two drops of

methanol:acetic acid (3:1) was added. The cells are then pelleted and re-suspended in approximately 0.5 ml of methanol:acetic acid and stored for later for metaphase spread analysis . One to three drops of cells in fixative were dropped onto a slide, allowing them to dry and stained with DAPI stain (Gibco). Mitotic cells were scored as cells containing condensed chromosomes. Using this procedure, 98% of the cells displayed mitotic chromosomes, and were therefore synchronized within a very precise time frame just prior to G1.

3D FISH

C127 cells at 4 hours after synchronization at mitosis were fixed in 4% (wt/vol) formaldehyde in PBS for 10min at room temperature to preserve the 3D structures of nuclei. Fixed cells were dehydrated through a 70% (vol/vol), 90% (vol/vol) and 100% (vol/vol) ethanol series. Cells were then treated in denaturation solution [70% (vol/vol) formamide in 2X SSC] for 10 min at 85 °C, and immersed in 70% (vol/vol) ethanol for 5 min at –20 °C. Cells were dehydrated through a cold 90% (vol/vol) and 100% (vol/vol) ethanol series, and subject to hybridization.

BAC clones used in this study were obtained from BACPAC Resource Center (http://bacpac.chori.org). Purified BAC DNA was labeled with biotin–dUTP or digoxigenin–dUTP using a nick-translation kit (Roche). Labeled probes were then denatured and hybridized to fixed cells at 37°C for 16 h in a moist chamber in

hybridization buffer mixture [50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 2X SSC, 2 µg of human cot1DNA, and 8 µg salmon sperm DNA]. Slides were washed three times with 50% (vol/vol) formamide in 2X SSC for 5 min at 43 °C and three times with 0.1X SSC for 5 min at 58 °C. Slides were then incubated for 1 hour in a blocking buffer [3% (wt/vol) BSA and 0.1% Tween20 in 4X SSC] at 37 °C and incubated with a detection solution[1% (wt/vol) BSA and 0.1% Tween 20 in 4X SSC] containing streptavidin Alexa Fluor 488 (Invitrogen) or anti-digoxigenin–conjugated rhodamine (Roche) for 1 hour at 37 °C. Then, slides were washed three times with 4X SSC and 0.1% Tween 20 for 5min at 43 °C. Cells were counterstained with 100ng/ml DAPI, and images were collected using a Deltavision microscope (Applied Precision) equipped with a cooled charge-coupled device camera (Cool-SNAP HQ; Roper Scientific). The images of 3D-fixed nuclei were captured at different stage positions, processed using Quick projection software (SoftWoRx; Applied Precision), and analyzed using FISH Finder program (Shirley et al. 2011). The size of the nucleus changes during the first 2-3 hours after mitosis therefore the inter-probe distances were normalized to the radius of the nucleus.

4C-seq

4C-seq was performed as previously described with some modifications (Splinter et.al 2012). Specifically, 5 million C127 cells synchronized at a particular cell cycle stage were trypsinized (except for Mitosis and 0.5 hr which were in suspension) and suspended in 2.5 ml DMEM with 10% FBS. Then, 2.5 ml of 2%

formaldehyde in DMEM with 10% FBS was added to bring the final concentration of formaldehyde to 1%. Cells were fixed for 10 minutes at 25°C while rocking. Ice-cold 1 M glycine was added to the cells on ice to a final concentration of 0.125 M to quench the crosslinking reaction. Cells were spun at 200 g for 5 minutes at 4°C and re-suspended in 5 ml ice-cold lysis wash buffer (50 mM Tris–HCl 138 pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1% TX-100 and 1X complete mini-protease inhibitors (Roche). Cells were lysed on ice for 15 minutes, and dounced using a tight piston for 15 strokes to isolate nuclei. Nuclei were spun down at 600g for 5 minutes at 4°C and re-suspended in 0.260 ml ice-cold 1.2x buffer NEB buffer 2, and transferred to non-stick tubes. Cells were brought to 37°C and 7.5 μl 20% SDS was added to each tube, which were then incubated for 50 min at 37°C followed by 10 min at 65°C while vortexing at 900 rpm. 75 μl 20% Triton X-100 was added and tubes were allowed to incubate for another hour at 37°C. 500U of high concentration HindIII (NEB) was added to each tube and the restriction digest reaction was run overnight at 37°C while tubes were vortexed at 900rpm. 500U of high concentration HindIII was again added and incubated for 8 hr. The restriction enzyme was inactivated at 65°C for 25 minutes and digest efficiency was determined as previously descirbed(Splinter et al. 2012). Digested samples were transferred to 15 ml falcon tubes, and 2.85 ml H_2O and 350 µl 10x ligation buffer (NEB) were added to each sample. 8000 CEU high concentration T4 ligase (NEB) were added and samples ligated overnight at 16°C. Ligation efficiency was checked as previously

described(Splinter et al. 2012). 15 μl 20 mg/ml proteinase K were added to the ligated samples and the samples were incubated overnight at 65°C. Subsequently, 15 μl RNase A (Invitrogen, cat# 12091-021) were added to each sample, and samples were incubated for an additional 45 minutes at 37°C. DNA was phenol-chloroform extracted, and precipitated by the addition of 3.5 ml H_2O, 0.75 ml 2M Na-acetate pH 5.6, 3.5 μl glycogen (20 mg/ml, Roche #10901393001) and 17.5 ml 100% ethanol, followed by freezing at -80°C. Precipitated DNA was spun down at 8350g for 20 minutes at 4°C, washed with ice-cold 70% ethanol, and re-spun at 8350g for 15 minutes at 4°C. Upon drying, DNA was re-suspended in 445 μl H_2O, 50 μl 10x DpnII restriction buffer (NEB), and 50U high concentration DpnII (NEB, cat# R0543M) were added to each tube and the DNA was digested overnight at 37°C. DNA was precipitated via addition of 50 μl 3M Na-Acetate, 3.5 μl glycogen (20 mg/ml) and 1.5 ml ethanol, and re-dissolved in 1 ml H_2O. The DpnII-digested DNA was then transferred to a falcon tube to which 5.05 ml H_2O, 0.7 ml 10x ligation buffer, and 8000 CEU high concentration T4 ligase were added for ligation overnight at 16°C. Following ligation samples were precipitated via the addition of 0.350 ml 2M Na-Acetate, 3.5 μl glycogen (20 mg/ml) and 17.5 ml 100% ethanol at -80°C. The precipitated DNA was pelleted and washed as above, and re-suspended in 150 µl 10mM Tris-HCl pH 7.5. Residual salt was removed via OMEGA BIO-TEK PCR purification kit. 4C DNA was quantified by Qubit flurometer.

4C library PCR amplification and Illumina high-throughput sequencing

Inverse PCR primers with Illumina forward and reverse adaptors (Supplementary Table 1) were designed to anneal to a bait locus HindIII/DpnII restriction fragment as described previously(Splinter et al. 2012). A total of 3200 ng of 4C template was used to amplify each bait using Takara HS Ex Taq polymerase system. The PCR program is as follows: 2 min at 94 °C; 30 sec at 94 °C; 1 min at bait specific annealing temperature; 3 min at 72 °C; 29X repeat; 5 min at 72 °C; hold at 4 °C. All 16 PCR tubes were pooled and purified using OMEGA BIO-TEK PCR purification kit.

Size selection of 4C-seq libraries

The purified 4C-seq libraries were size selected between 200bp-600bp using agarose gel stained with SYBR gold stain and gel purified using OMEGA BIO-TEK gel purification kit. 4C-seq library for all six bait regions were quantified using Qubit flurometer after size selection and mixed in equal molar concentration. The final multiplexed 4C-seq libraries were re-purified using OMEGA BIO-TEK PCR purification kit quantified using real time PCR (Kapa biosystems) and the size distribution was analyzed using Bioanalyzer (High Sensitivity DS DNA kit). Samples with ideal size distribution were sequenced using Hiseq 2500 to obtain 50bp reads.

Read mapping and data visualization

50 bp-long single-end reads were first de-multiplexed using their barcodes and assigned to the corresponding time point and 4C bait. Only the reads that

contained one of the valid barcodes was retained for mapping. Each read was mapped to the mouse genome (UCSC mm9) using the short read alignment mode of BWA (v0.5.9) (Langmead et al. 2009) with default parameter settings. Mapping results were post-processed, and only the reads that mapped uniquely with an alignment quality score of at least 30 and an edit distance of at most 1 were qualified for further analysis. Each qualified read was assigned to the nearest HindIII cleavage site which represents a restriction fragment.

For visualization 50 kb upstream and downstream of the bait position was removed from this raw data. Contact counts at HindIII sites for each chromosome were windowed (10 kb windows), normalized for sequencing depth and smoothed using a running mean with a span of 30 windows. The resulting smoothed profiles (contact count) were plotted as a function of distance along the chromosome.

Contact significance calculation

Briefly, for each bait contacts with individual HindIII sites were first binarized and then windowed using multiple consecutive restriction fragments at a window size (w) of w=20 for intra-chromosomal and w=100 for inter-chromosomal interactions. For assigning significance to intra-chromosomal contacts while controlling for the genomic distance effect, the number of contacts within each window of size w=20 is compared to sliding windows of the same size within a

larger window of size W=3000 that is centered on the small window resulting in a z-score for the window of interest. To control for multiple testing, the interacting and non-interacting fragments within the background window of size W=3000 are randomly shuffled 100 times and the z-score threshold at which the false discovery rate (FDR) is 0.1 was identified to define significant contacts at this FDR. For visualization, the z-scores were transformed to p-values with a one-tailed normal test using the p-value package in R. Since the inter-chromosomal contacts do not have a genomic distance effect, the FDRs for their p-values were estimated simply using standard methods (Benjamini and Hochberg 1995).

Calculation of Directionality Index (DI) from 4C data

For each bait region at a given time point DI was calculated using 10 kb windowed and smoothed 4C data as:

$$DI = \left(\frac{(B-A)}{|B-A|}\right)\left(\frac{(A-E)^2}{E} + \frac{(B-E)^2}{E}\right),$$

where A is the sum of contacts (smoothed 4C value) from the bait region to the upstream 2 Mb, B is the sum of contacts from the bait region to the downstream 2 Mb, and E, the expected number of reads under the null hypothesis, is equal to (A + B)/2.

Degree of compartmentalization

50 kb binned Hi-C matrices were used for the genome-wide calculation of degree of compartmentalization. For each 50 kb bin the degree of compartmentalization was calculated as the log ratio of (Sum of contacts to same compartment) and (Sum of contacts to opposite compartment).

Same compartment or opposite compartment were defined as all 50 kb bins in the same chromosome with similar replication timing value (same sign) or opposite replication timing (opposite sign) respectively. Therefore, a score of 1 indicates that the particular bin interacts with both early and late compartments equally.

Segway/ChromHMM analysis

Annotation labels (7 state) for the human cell lines that are jointly inferred by two semi-automated genomic annotation tools were downloaded from (Hoffman et al. 2013): http://ftp.ebi.ac.uk/pub/databases/ensembl/encode/integration_data_jan2011/byDataType/segmentations/jan2011/Combined_7_state. Enrichment/Depletion of each of the seven annotation labels compared to the whole genome coverage of the label was computed for each class of regions at 40 kb resolution that are either constitutively early (CE), constitutively late (CL) or developmentally regulated (either DE or DL if early or late replicating for the specific cell line, respectively). A value of 1 indicates that the abundance/coverage of the corresponding annotation label from Segway/ChromHMM for the specific

replication-based class of regions is exactly proportional to the number of such regions within the whole genome and therefore indicates no enrichment or depletion. A value of 2 indicates the annotation label covers the regions with that replication label twice more than expected by random.

Boxplots of chromatin features

ChIP-seq signal files for various histone modifications and transcription factor binding profiles were downloaded from the link (Bernstein et al. 2012): http://www.broadinstitute.org/~anshul/projects/encode/rawdata/signal/mar2012/pooledReps/bigwig/macs2signal/foldChange/. These files contain the fold-change of observed reads from the ChIP-seq experiments over the expected number of reads based on sequencing depth normalized input experiments. These fold-change values for both histone marks and trans-acting factors were aggregated over each 50 kb non-overlapping genomic window for 2 different human cell lines (GM12878, HeLa). To generate the boxplots in Supplementary Fig 8, these fold-change values per each 50 kb were first divided by the mean value for this ChIP-seq experiment over all 50 kb windows and then log2 transformed. 50 kb regions that are not classified as either CE, CL, DE or DL were excluded from the boxplots but included in the calculation of mean. Plots were generated using R.

Boxplots for Lamin association was calculated in a similar fashion. Association with lamin was measured as the log2 ratio of Lamin DamID signal to its control.

Previously published datasets used in this study

All replication timing data used in this study is publically available at www.replicationdomain.org. Domain-scale segmentation labels are available at http://noble.gs.washington.edu/proj/gbr/). References and GEO accession numbers for all other datasets used are summarized in Supplementary table 3.

REFERENCES

1) Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B Methodol 57: 289–300.
2) Shirley JW, Ty S, Takebayashi S, Liu X, Gilbert DM. 2011. FISH Finder: a high-throughput tool for analyzing FISH images. *Bioinformatics* **27**: 933–8.
3) Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10**: R25.