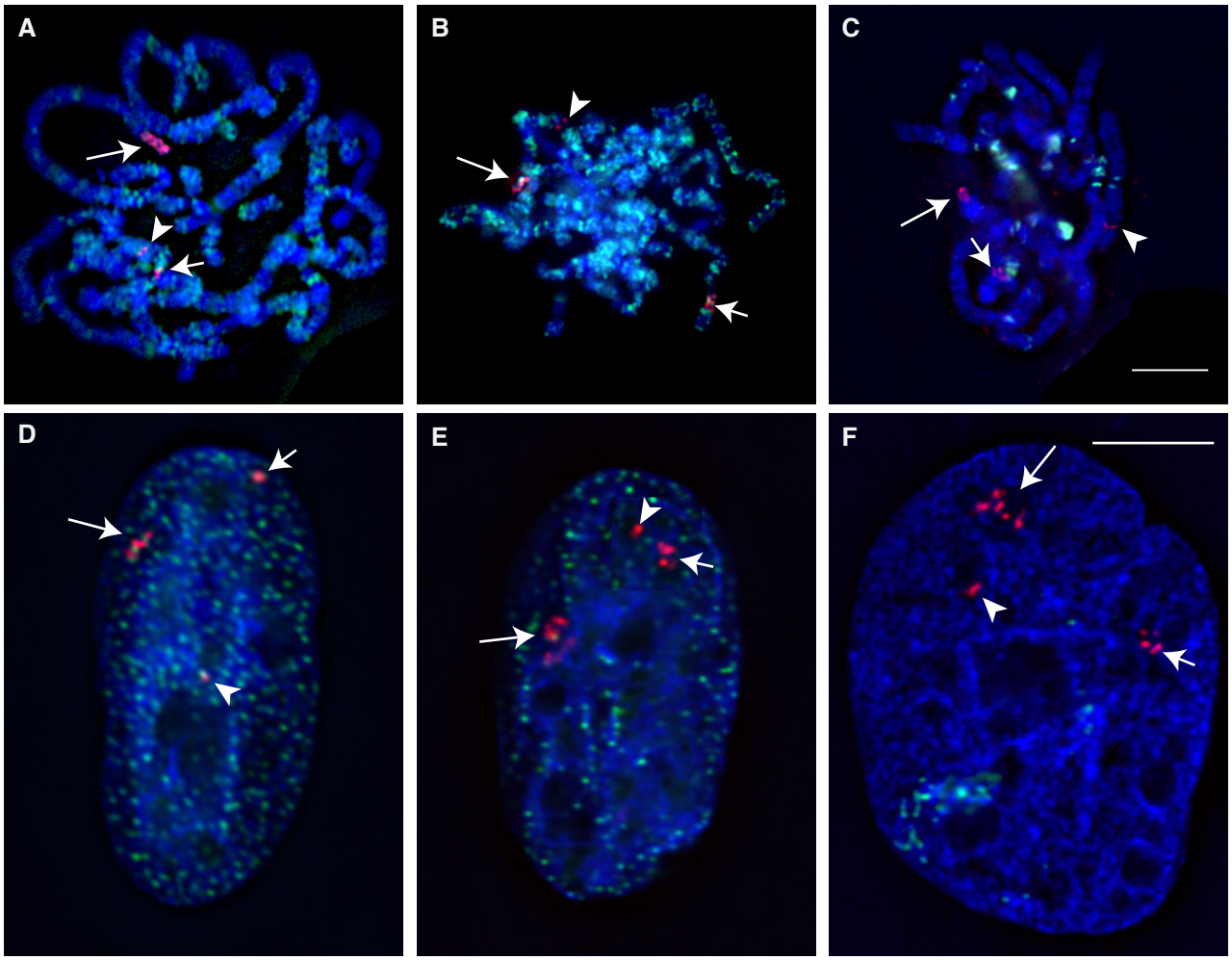
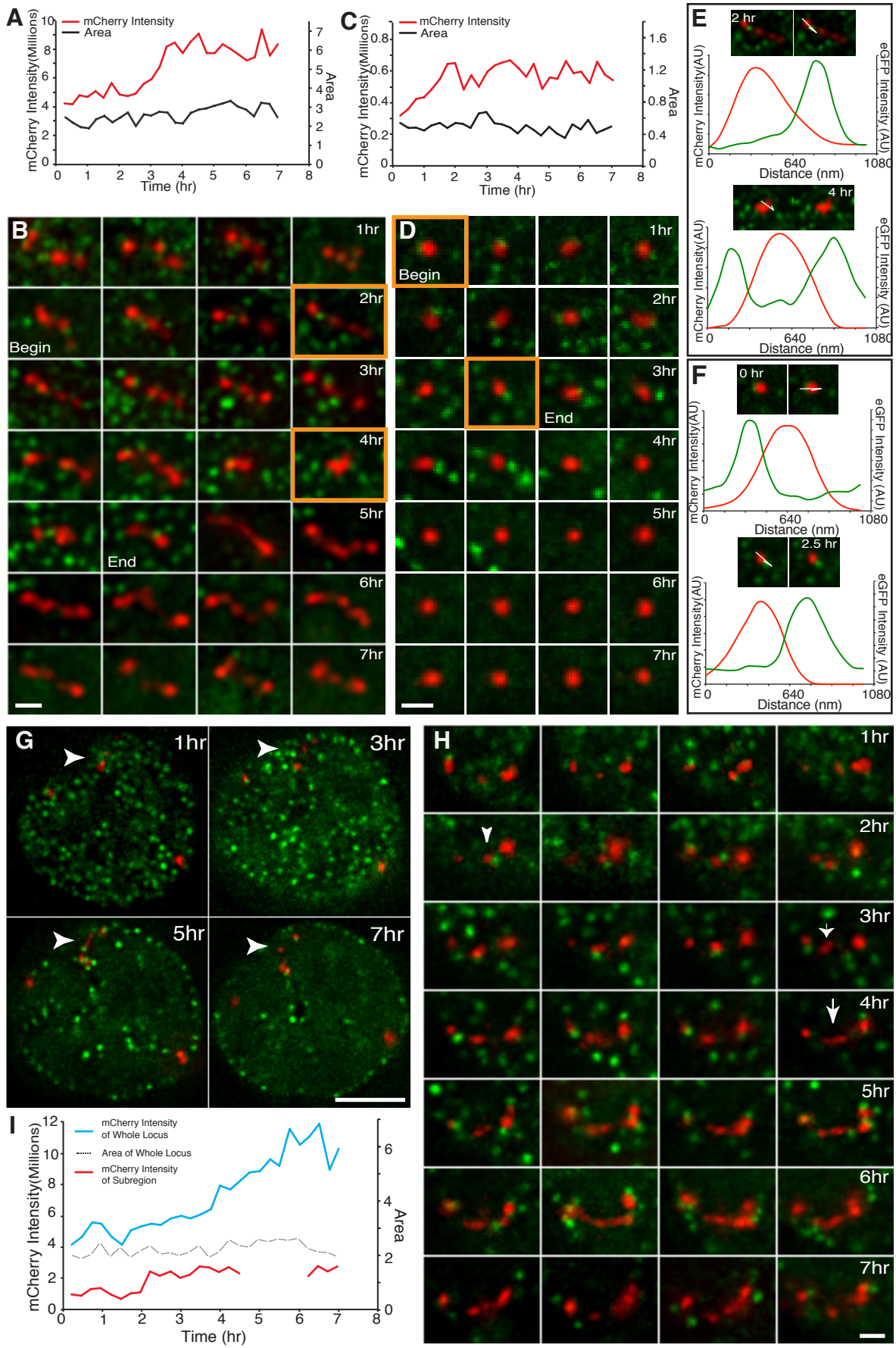
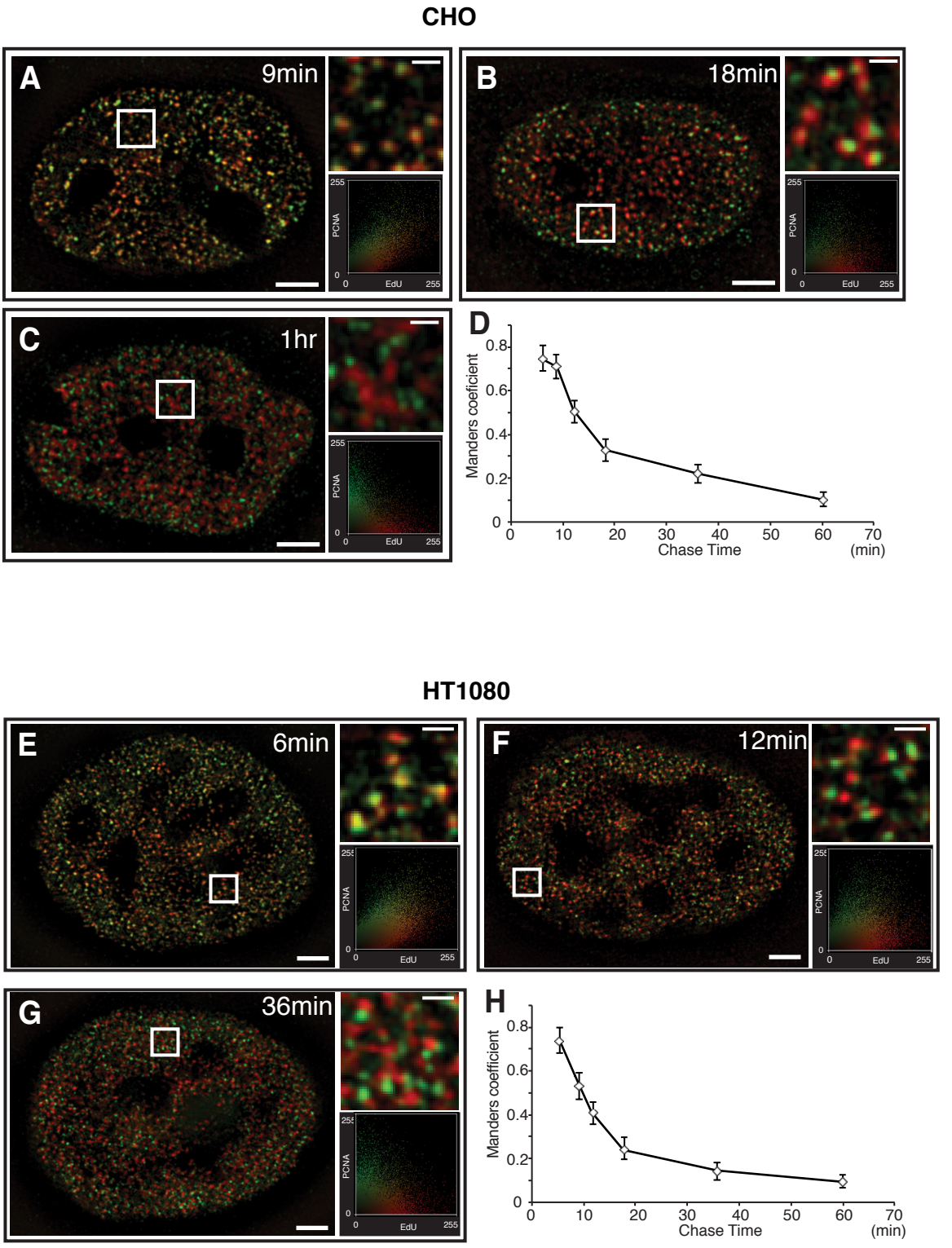
Supplemental Figure 1



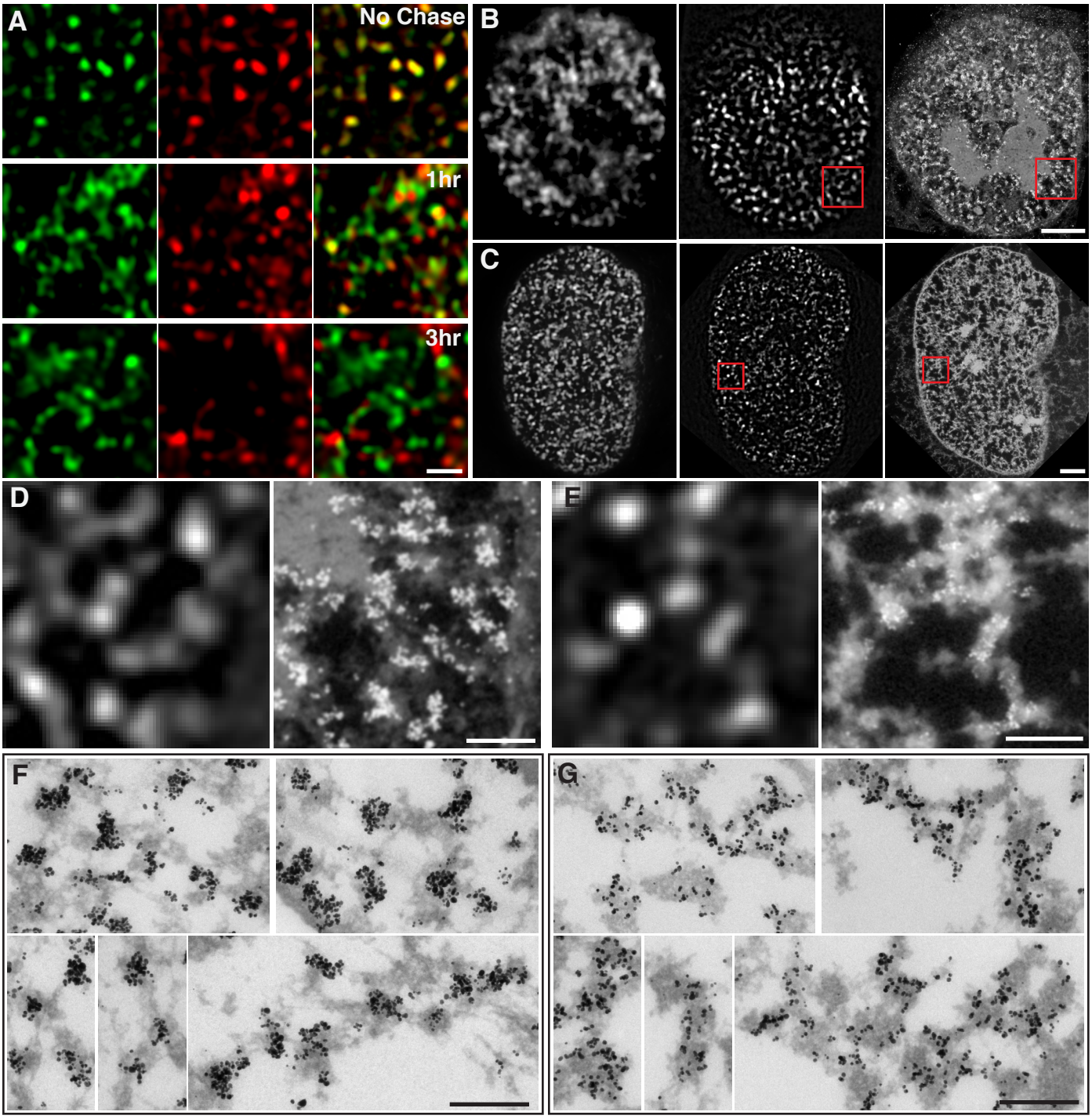
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure Legends:

Figure S1. Related to Figure 1. Three early-replicating, gene-amplified chromosome regions in PDC βA2 CHO cells. (A-C) Pulse-chase labeling of mitotic spreads using 3 min EdU pulse and 2-14 hrs chase. Mitotic spread (DAPI, blue) of PDC βA2 cell clone shows three gene-amplified chromosome regions (mCherry-LacI, red)- loci 1-3 named in order of decreasing size (locus 1, long arrow; locus2, short arrow; locus 3, arrow head) and EdU incorporation (green, Click-It Alexa Fluro 488), tagged chromosome regions. Percentage of each locus that overlaps with EdU incorporation was measured as a function of chase time (Fig. 1G) to determine replication timing of each locus. (D-F) Early replication of amplified chromosome regions (Fig. 1G) based on pulse-chase and mitotic chromosome labeling is also consistent with presence or absence of co-localization of GFP-PCNA foci (green) with mCherry-LacI loci (red) in early (D), middle (E), and late S-phase nuclei (F). GFP-PCNA foci localize adjacent to or overlapping with loci 1-3 in early S phase, but only locus 1 co-localizes with GFP-PCNA in a subset of middle S-phase cells; no overlap is seen in late S-phase. Scale bars: 5 μm.

Figure S2. Related to Figure 1. Additional live-cell imaging examples: loci 1 & 3 (A&C) Doubling of mCherry-LacI signal (red) for locus 1 (A) and locus 3 (C) show DNA replication timing for these loci. During DNA replication the projected areas (black) of these loci remain unchanged. Time-series is from same cell as shown in Fig. 1 for locus 1. (B&D) Images showing locus 1 (B) and locus 3 (D) at 15 min intervals from 15 min to 7 hrs (arranged from left to right, top to bottom) after release from late G1/early S phase block: mCherry-LacI (red), GFP-PCNA (green). "Begin" and "End" correspond to estimated beginning and end times for DNA replication inferred from measurement of mCherry-LacI signals. GFP-PCNA foci associate with loci during these times. (E&F) Images and intensity line-scans (along white arrows) from orange-framed panels (B&D) showing GFP-PCNA (green, image and line-scans) associated with mCherry-LacI (red, image and line-scans) signals from loci 1 (E) or 3 (F). (G) Replication of locus 1 sometimes continues into first part of middle S phase. GFP-PCNA foci patterns change from early to middle replication pattern between 3-5 hrs but concentration of GFP-PCNA foci (green) over locus 1 (red, mCherry-LacI, arrowheads) is still present at 5 hrs but gone at 7 hrs. (H) Projections of optical sections covering the entire locus from consecutive 15 min time points (arranged from left to right, top to bottom) from 15 mins to 7 hrs after release from early-S phase block: GFP-PCNA foci (green), mCherry-LacI (red). GFP-PCNA foci are associated with different regions of locus 1 from ~1.25-6 hrs. One sub-region shows closely associated GFP-PCNA foci between 1.25 hrs (arrowhead) to 3 hrs (small arrow). At 4 hrs this region (larger arrow) shows local extension/decondensation. (I) Doubling of overall mCherry-LacI signal (blue) between 1-6 hrs after release from late G1/early S phase block occurs during time when GFP-PCNA foci are associated with locus 1. Integrated projected area of locus 1 does not change significantly throughout this replication period (grey dash line). Local decondensation at 4 hrs (large arrow,H) of subregion within locus 1 occurs after doubling of its mCherry-LacI signal (red). (Gap in mCherry-LacI represents time period when this subregion merges with other regions of locus 1.) Scale bars: 1 μm (b,d,h), 5 μm (g).

Figure S3. Related to Figure 2. Separation of nascent DNA from PCNA replication foci as function of chase time. (A-C) CHO cells expressing GFP-PCNA (green) after 30s, 10 μM EdU pulse (red) and chase for 9 min (A), 18 min (B), and 1 hr (C). Left panel: grazing optical section from early S-phase nucleus, Scale bar = 5 μm; Right panel (top): enlarged region from left panel (boxed region), scale bar: 1 μm; Right panel (bottom): Scatter plot of green (y-axis) versus red (x-axis) pixel intensities. (D) Pearson R value (y-axis) showing loss of correlation between red and green with increasing chase time (x-axis). (E-F) Analogous to (A-D) for grazing optical section of early S-phase HT1080 cells pulsed with 40 μM EdU for 10s and chased for 6 min (E), 12 min (F), and 36 min (G). Scale bars = 5 μm (left panel), 1 μm (right panel, top).

Figure S4. Related to Figure 3. Spreading of replicated DNA within large-scale chromatin fibers in HT1080 cells with traditional pulse-chase conditions. Correlative light and electron microscopy (CLEM) compares immunogold staining against EdU with EdU-Alexa488 signal in HT1080 cells. (A) Comparison by SIM of EdU incorporation versus mRFP-PCNA signal in HT1080 cells pulsed for 15min with 10 μM EdU (green: EdU-Alexa-488, red: PCNA) without chase (top), with 1 hr chase (middle), and 3hr chase (bottom). (B) 15 min EdU pulse without chase- from left to right: wide-field, deconvolved light

microscopy, SIM, TEM. (C) same EdU pulse with 1hr chase- from left to right: wide-field, deconvolved light microscopy, SIM, TEM. (D-E) Red squares (B, C) mark enlarged regions- SIM (left), TEM (right)- for 15 min pulse (D) or 15 min pulse plus 1 hr chase (E). (F-G) Additional immunogold staining TEM examples: 15 min EdU pulse followed by no chase (F) or 1 hr chase (G). Scale bars = 1 μm (A), 2 μm (B,C), 0.5 μm (TEM) (D-G).

Supplemental Experimental Procedures:

Tissue culture and transfection

CHO DG44 PDC cells containing lac operator tagged, homogeneously staining regions (HSRs) generated by gene amplification [S1] were grown in Ham's F12 media without thymidine and hypoxathine and with 10% dialyzed FBS (Hyclone). PDC cells were stably transfected with pcDNA3_NLS_eGFP_hPCNA and p3'SS-dLacI-mCherry using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions and selected with 200μg/ml G418 and 197μg/ml Hygromycin. pcDNA3_NLS_eGFP_hPCNA was derived from pENeGFPPCNA [S2]; eGFP-PCNA was cut from pENeGFPPCNA with BamHI and XbaI and cloned into pcDNA3 (ThermoFisher) cut with HindIII and XbaI using an adapter (AGCTTATGGCTTCGTGGGGATC) to reconstruct the correct start codon for eGFP-PCNA. Individual subclones were obtained by serial dilution of mixed clonal populations of stable transformants. HT1080 cells were cultured in Dulbecco's modified Eagle medium (Invitrogen) plus 10% Fetal Bovine Serum (Hyclone). CHO K1 cells were grown in Ham's F12 media with 10% FBS (Hyclone). All cells were incubated at 37°C in 5% CO_2.

Immunofluorescence

Cells were fixed in PBS for 15 min at room temperature (RT) with 1.6% formaldehyde (Polyscience) or freshly made 2% paraformaldehyde (Sigma), washed 3x 5 min with PBS, and permeabilized for 15 min in 0.1% Triton X-100 (Pierce) in PBS. Cells were blocked in PBS with 5% BSA in 0.1% Triton X-100 (Pierce) for 1hr at RT. Anti-PCNA staining used mouse monoclonal anti-PCNA PC-10 (Abcam) primary antibody at 1:2000 dilution and Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Life Technologies) at 1:1000 dilution. Click-iT (Invitrogen) labeling of EdU with Alexa Fluor 488 or Alexa Fluor 594 followed the manufacturer's suggested procedure. DNA was counterstained with DAPI for 5min at RT (2μg/ml).

Mitotic Spreads

Mitotic cells were collected by mitotic shake-off at selected chase times after pulse labeling with 10μM EdU for 5 min. After shake-off, cells were incubated in 75mM KCl at 37°C for 15min, fixed for 10min 3x in ice-cold fresh 3:1 methanol/acetic acid, and then dropped onto clean glass coverslips. Coverslips were air-dried and stored for 24-48 hours at RT prior to rehydration in PBS with 0.1% Triton X-100 for 10min prior to EdU labeling. Click-iT labeling of EdU with Alexa Fluor 488 followed antibody staining according to manufacturer's instructions.

Cell synchronization

Synchronization of PDC cells at late G1/early S used a mitotic block with 0.6μg/ml nocodazole for 2-2.5hr followed by mitotic shake-off and 3x wash in complete media. Cells were plated in compete media containing 1mM hydroxyurea (HU) for 14 hr. The HU block was released by washing 3x in complete media prior to live-imaging.

CHO K1 cells were synchronized in G1/G0 using isoleucine deprivation [S3]. CHO K1 cells were grown in F12 media with 10% FBS to 50-60% confluency. Cells were then rinsed with PBS and cultured in isoleucine-free F12 media with 10% dialyzed FBS. After 48 hr, isoleucine-minus F12 media was replaced by fresh F12 media with 10% FBS and 1 mM HU. This G1/S block was removed 14 hrs later by washing cells and adding media without HU.

HT1080 cells were synchronized by a double thymidine block. Cells were grown in DMEM media with 10% FBS to 50-60% confluency. 200 μM of thymidine (SIGMA) was added to the media. Approximately 16 hr later, cells were rinsed with PBS and incubated with new media. 10 hr later, 200 μM thymidine was added to the media and cells were cultured for another ~16hrs. Cells were released into S-phase by changing to new media.

Microscopy and data analysis

Wide-field light microscopy images for measuring nuclear EdU incorporation were collected on the Applied Precision Deltavision IX71-Olympus wide-field microscope (GE Healthcare) using a 60X/1.42 PlanApochromatic oil immersion objective. 3D optical section datasets at 0.2μm z-increment were collected, spanning the entire nuclear volumes from randomly selected cells on the coverslip.

Deconvolution used an iterative constrained algorithm in the Softworx software package (GE, Healthcare). Samples were mounted in VectaShield anti-fade solution prior to microscopy.

Samples for Structured Illumination Microscopy (SIM) were mounted in VectaShield (Vector) anti-fade solution. SIM (Fig. 2) was performed on an Applied Precision OMX V3 (GE Healthcare) using a 100x/1.4 NA oil immersion objective. Light from pairs of diode lasers (405 nm and 488 nm, or 488 nm and 592.5 nm) was transmitted on the focal plane of the sample. Image stacks with a z-distance of 0.125 μm were acquired. Emitted light passes through a set of dichroic mirrors directing light to two EMCCD cameras (Cascade II 512). Exposure time was between 30 ms and 100 ms, yielding typically 3000-5000 max counts in a raw image of 16-bit dynamic range. SIM image reconstruction, registration of channels, and data alignment used used Softworx software (GE Healthcare). Alternatively, samples were mounted in Mowiol 4-88 (Calbiochem) with 1% DABCO to prevent bleaching and examined using a Nikon N-SIM (Nikon) with 100x/1.49 NA oil immersion objective, 488nm and 561nm diode laser excitation. Image stacks (z-steps of 0.12μm) were acquired with EMCCD camera (iXon 897, Andor, effective pixel size 60 nm). Exposure conditions were adjusted to get typical yield about 5000 max counts (16-bit raw image) while keeping bleaching minimal (SFig. 4). Image acquisition, SIM image reconstruction and data alignment were preformed using NIS-Elements software (Nikon).

For live-cell microscopy, cells were plated onto glass-bottom microwell dishes (MatTek) and placed within a microscope incubation chamber (Applied Precision) set to 37°C and 5% CO_2. Data was collected on the OMX V3 system with 100x/1.4 NA oil immersion objective and cooled EMCCD (Cascade II 512) using 0.1% and 1% transmittance for 488 and 592 nm lasers, respectively, with exposure times of 20-30 ms (488 nm) and 20-40 ms (592 nm). 3D optical sections (z-step 0.25 μm) were taken every 15min for 7 hrs after release from HU block. Two channels (Ex 488nm, 592.5nm) were acquired sequentially. Deconvolution using iterative, constrained reconstruction and channel registration and alignment was done using Softworx software.

Image analysis

Fluorescence intensity measurements of total intensity were performed using ImageJ using "sum intensity" projections. The integrated intensity within a region of interest (ROI) was measured; this ROI was shifted over an adjacent background region and the integrated intensity summed in the equivalent size background region. This background integrated-intensity was then subtracted from the original ROI integrated intensity to yield a background corrected value. This procedure was adopted to calculate nuclear EdU fluorescence, using the entire nucleus as the ROI, or the total mCherry-LacI signal over chromosome loci. For the latter measurements, an adjacent, nucleoplasmic region with the same size of the target ROI was used for the background signal. For measurements of EdU incorporation per chase time, ~20 cells were analyzed for each chase time point and normalized by the variable exposure times used. Area measurements were made for the selected ROIs. Plotting of values was done using Microsoft Excel. Calculation of the Manders' coefficient for colocalization used the JACoP Plugin for NIH Image J [S4].

Flow cytometry

CHO K1 or HT1080 cells were synchronized at the G1/S boundary and released into S-phase for 2hrs. Pulse-chase conditions for each cell line were as used in our imaging experiments. CHO K1 cells were pulsed with 10 μM EdU for 30s and chased for 3 min, 6 min, 9 min, 18 min or 36 min. HT1080 cells were pulsed with 40 μM EdU for 10s and chased for 3 min, 6 min, 9 min, 18 min or 36 min. After chase, cells were rinsed with ice-cold PBS and trypsinized. Cells were pelleted and stained with Alexa Fluor 488, following the manufacturer's instruction (Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit, Thermo Fisher, Cat # C-10425). We used cells that were not pulsed with EdU but were stained with the Click-iT Alexa Fluor 488 as a negative control. Flow cytometry used a BD FACSCanto RUO Special Order System (Biotechnology Center, UIUC).

Measurement of the intracellular EdU pool levels by LC-MS/MS

Cells were synchronized and pulse-chased as described above. After pulse labeling, CHO K1 or HT1080 cells were chased for 0 min, 3 min, 6 min, 9 min, 18 min or 36 min. Cells were then rinsed with ice-cold PBS, trypsinized, washed with PBS again, and centrifuged to collect cell pellets. Greater than $1x10^7$ cells were collected for each time point and the same numbers of cells were used to extract EdU and nucleotides at each time point. 70% ice-cold ethanol was used to resuspend the cell pellets and precipitate proteins. The resulting solution was vortex-mixed, incubated at -20 °C overnight, and sonicated for 15 min

at 4 °C. Cell extracts were centrifuged at 1,000xg for 5 min at 4 °C. Supernatants were passed through pre-equilibrated Amicon Ultra centrifugal filters at 4 °C to remove macromolecules >3 KDa. The filtrates were evaporated on a Vacufuge Concentrator 5301 machine (Eppendorf) or dried under a stream of nitrogen. The resultant material was reconstituted in 200 µl of mobile phase A and centrifuged at 1,000xg for 5min at 4 °C to remove any precipitation. BrdU was added to the resulted supernatant with a final concentration of 1 µM. 10 µl of the supernatant was injected into LC-MS/MS system.

LC-MS/MS experiments were performed in the UIUC School of Chemistry Sciences Mass Spectrometry Laboratory on a Waters Synapt G2-Si ESI MS electrospray mass spectrometer (Waters). Previously reported HPLC conditions were adopted and coupled to the LCQ ion trap mass spectrometer for the analysis [S5, S6]. The ESI source was operated in the negative mode. All operations were controlled by MassLynx (Waters) software and quantitative analysis was carried out with MarkerLynx software (Waters).

Immunogold Staining

Cells pulse-chased with EdU were permeablized for 1min in PBS* buffer (Ca-, Mg-free PBS with 5mM $MgCl_2$ and 0.1mM EDTA) containing 0.1% Triton X-100 and then fixed in 3.7% paraformaldehyde for 15min at room temperature. After washing 3x 5min in PBS*, EdU was Click-it labeled with Alexa 488-azide (Invitrogen). Samples were washed with PBS* and blocked in 5% BSA in PBS*+0.1%Triton X-100 for 30 min at room temperature followed by staining with anti-Alexa 488 (Invitrogen) primary antibody (1/1000 dilution) at 4°C overnight. Cells were washed in PBS*+0.1% Triton X-100, and stained with secondary goat-anti-mouse Nanogold-coupled Fab' (Nanoprobes) at 4°C for 12 hrs. Samples were then washed in PBS*+0.1%Triton X-100, fixed with 2.5% glutaraldehyde in PBS* for 1 hr at room temperature, quenched with 1mg/ml $NaBH_4$ and processed for silver enhancement using the Dancher procedure [S7].

CLEM

Cells were plated on etched glass-bottomed Petri dishes (MatTek) and labeled and processed for Click-IT and immunogold staining as described above. For SIM imaging, PBS* was supplemented with 1% N-propylgallate. SIM stacks and DIC images of cells displaying early S-phase patterns of replication were imaged. After extensive washes in PBS*, cells were processed for immunogold staining. Embedded cells were located according to grid marks in the Epon wafer and 150-200 nm thick serial sections were cut, stained with 2% aqueous uranyl acetate, and inspected in a JEM-1400 microscope at 100kV. Alignment of SIM and TEM images was performed manually with ImageJ using bicubic interpolation to adjust image shift, rotation, and magnification.

Supplemental References:

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