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Preventing gene silencing with human replicators

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Transcriptional silencing, one of the major impediments to gene therapy in humans, is often accompanied by replication during late S-phase. We report that transcriptional silencing and late replication were prevented by DNA sequences that can initiate DNA replication (replicators). When replicators were included in silencing-prone transgenes, they did not undergo transcriptional silencing, replicated early and maintained histone acetylation patterns characteristic of euchromatin. A mutant replicator, which could not initiate replication, could not prevent gene silencing and replicated late when included in identical transgenes and inserted at identical locations. These observations suggest that replicators introduce epigenetic chromatin changes that facilitate initiation of DNA replication and affect gene silencing. Inclusion of functional replicators in gene therapy vectors may provide a tool for stabilizing gene expression patterns.

It has long been known that transcription and replication are tightly linked. Several[1], but not all[2], developmentally regulated loci exhibit a correlation between transcription and replication timing: activation of silent genes often correlates with a switch to early replication, as shown for the human β-globin[3,4], immunoglobulin IgH[5] and T-cell receptor loci[6]; inactivation of X chromosomes alters replication timing of the inactive chromosome[7]. We have previously shown that sequences from the locus control region (LCR) in the human β-globin locus can delay replication when transferred to an ectopic chromosomal site[8,9]. These observations suggest that replication timing can be regulated dynamically and respond to tissue-specific modulators.

To investigate the relationship between replication timing and gene silencing in a controlled manner, we created isogenic cell lines that vary only in the DNA sequence of a single, constant genomic location. The use of isogenic lines is critical because the chromosomal and cellular environments affect the timing of DNA replication. For the initial studies, we chose a late-replicating site on murine chromosome 15 (random locus 4, or RL4), in which insertion of an antibiotic marker advanced replication timing[8] (**Fig. 1a**). Replication was delayed by replacing the transgene with cassettes that included hypersensitive sites 2, 3 and 4 of the human β-globin LCR (HS432), the human β-globin promoter and a green fluorescent protein (GFP)-coding sequence (**Fig. 1b**,**c**, construct I). The replication delay was orientation specific, correlated with histone deacetylation and loss of transgene expression[8,10], and required the DNA element containing the hypersensitive sites from the LCR as a silencer[9].

Because late replication correlated with gene silencing, we determined whether transcriptional silencing occurred before the replication delay. We used a cell line containing a transgene cassette that

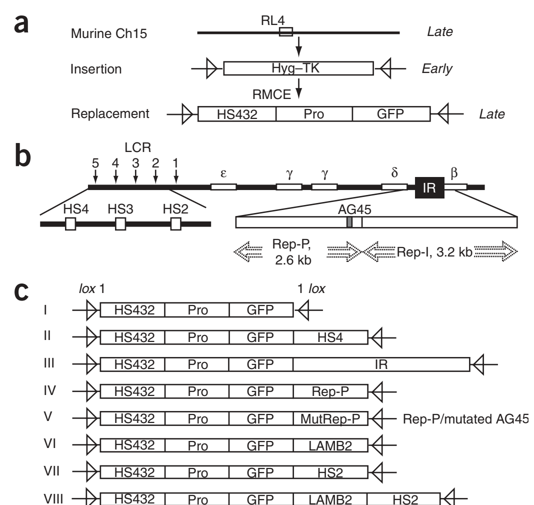

Figure 1 A mammalian experimental system in which replication timing could be altered in a controlled manner. (**a**) The experimental system[8]. The Cre recombinase-mediated cassette exchange (RMCE) technique was used for precise replacement of sequences inserted within a late-replicating site in murine chromosome 15 (RL4). A cassette encoding for antibiotic-resistance markers was first inserted in this locus, then replaced by sequences from the human β-globin locus. Hyg, hygromycin; TK, thymidine kinase; HS432, a miniLCR including DNase hypersensitive sites 4, 3 and 2 (see below); Pro, β-globin promoter; GFP: enhanced green fluorescence protein. (**b**) The human β-globin locus. Sequences used in this study include the locus control region (LCR) core region (DNase hypersensitive sites 4, 3 and 2: HS432); the β-globin promoter (Pro); IR, the replication initiation region; Rep-P, one of the two replicators within IR; mutated Rep-P with 45-bp AG-rich sequence (AG45) replacement (Mut RepP)[13]; and human lamin B2 (*LMNB2*) replicator (positions 3,691 to 4,978, GenBank accession no. M94363). (**c**) Constructs used in this study. These constructs were transfected into MEL cells either at the RL4 site (chromosome 15) or at the RL5 site (chromosome 4)[10].

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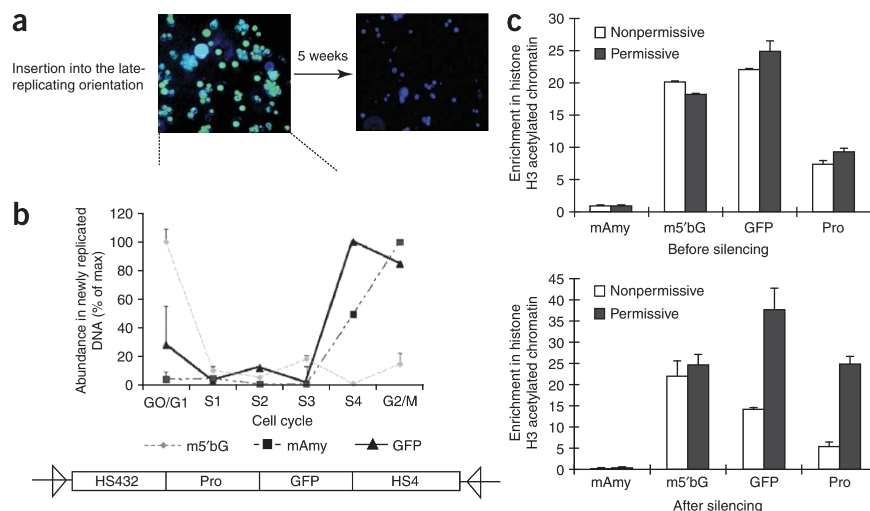

Figure 2 A replication delay precedes transcriptional silencing of gene expression and chromatin condensation in a transgene inserted at the RL4 site. (**a**) GFP expression of MEL cells harboring the miniLCR-pro-GFP-HS4 cassette (Construct II, **Fig. 1c**) at the silent orientation at RL4. The left panel shows cells 3 weeks after transfection (5 weeks before silencing); right panel shows the same clone after silencing, 5 weeks later. (**b**) Replication timing of cells inserted with the miniLCR-pro-GFP-HS4 cassette in the silencing prone orientation 3 weeks after transfection (corresponding to the left panel in **a**). Transgene and host sequences in newly replicated DNA were detected by real-time PCR. (**c**) Chromatin immunoprecipitation (ChIP) analysis of chromatin isolated in cells inserted with the miniLCR-pro-GFP-HS4 cassette in the silencing prone and permissive orientations at different times after transfection (upper panel, before silencing; lower panel, after silencing). Chromatin was isolated with antibodies against acetylated histone H3 and analyzed by real-time PCR. Primers and probes include m5′bG, a murine β-globin sequence, which replicates early in MEL cells; mAmy, murine amylase, which replicates late; GFP and pro are sequences from the transgene (**Supplementary Table 1**).

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included a second copy of HS4, a putative insulator, at the 3′ end (construct II). This transgene exhibited orientation-specific gene silencing after more than 8 weeks (**Fig. 2a**) instead of the 3 weeks required for silencing of construct I[8]. We measured replication timing by determining the abundance of transgene sequences in BrdU-substituted DNA obtained from cells at different stages of the cell cycle. Transgene sequences replicated late in S-phase 3 weeks after transfection (about 5 weeks before silencing, **Fig. 2b**). Five weeks after transfection, transgene sequences were abundant in chromatin containing acetylated histone H3 (**Fig. 2c**) and the abundance of transgene sequences in chromatin containing acetylated histone H3 had progressively declined during the silencing process. These data indicated that the chromatin acetylation status correlated with the transcriptional status of the transgene whereas the replication delay preceded silencing and histone deacetylation. Although the cell number was not sufficient for us to perform a replication timing assay earlier than 3 weeks after transfection, it is likely that the replication delay occurred shortly after insertion of the transgenes.

The location of DNA replication initiation events on chromatin is affected by DNA sequences termed replicators, which contain genetic information that dictates preferential initiation of DNA replication, and on distal sequences, which may reside dozens of kilobases away from replicators[11,12]. In the human β-globin locus, replication starts within a constant region, termed the initiation region (IR), which contains two functional replicators[11,13,14], and is affected by an upstream element, LCR, located 50 kb upstream of IR[11]. Replication starts at IR regardless of gene expression but the locus replicates early during S-phase in cells that express β-globin, and late in cells that do not express β-globin[4,15]. To evaluate the effect of the RL4 site on initiation of DNA replication, we included replicator sequences within transgenes that contained LCR sequences. We first inserted the entire globin IR (construct III) and Rep-P (construct IV), a shorter replicator derived from IR that can initiate replication when transferred to ectopic sites[13,16]. We inserted as a control, a dysfunctional variant of Rep-P (construct V) in which a 45-bp region, essential for replicator activity, was mutated[13]. We also used the Lamin B2 replicator (construct VI), derived from the region between the human lamin B2 *(LMNB2)* and *TIMM13* genes, which initiates replication at ectopic sites[17].

We first determined whether replication was initiated within the replicator-containing transgenes by measuring the abundance of transgene sequences in short, nascent DNA strands from asynchronously replicating cells. Nascent strands were isolated based on their size (600–2,500 bases) and their ability to withstand λ-exonuclease treatment, which does not digest RNA-primed DNA. We found that replication was initiated in transgenes that contained the entire IR, Rep-P and *LMNB2* replicators but not the mutated Rep-P. Replication initiated when the transgenes were inserted in both orientations (**Fig. 3a** and data not shown).

We then compared the expression status and replication timing of replicator-containing and replicator-deficient transgenes that were inserted in the silencing-prone orientation. When transgene I was inserted in the silencing-prone orientation, GFP expression was silenced and replication was delayed (**Fig. 3b**). By contrast, a transgene that contained Rep-P (transgene IV) replicated early (**Fig. 3c**). Although this transgene was inserted in the same silencing-prone orientation as transgene I, it exhibited stable GFP expression for longer than a year. An insertion of the entire IR (transgene III; data not shown) and *LMNB2* (transgene VI; **Fig. 3d**) also prevented replication delay and exhibited stable expression. These data suggested that inclusion of functional replicators in the transgene prevented replication delay and gene silencing. Importantly, replication occurred in late S-phase and gene expression was silenced in cells containing the mutated Rep-P (transgene V; **Fig. 3e**), which did not initiate DNA replication. These observations suggest that a functional replicator is required to prevent replication delay and gene silencing. The prevention of gene silencing by replicators was observed in multiple clones derived from independent transfections (**Supplementary Fig. 1** online).

To determine whether initiation of DNA replication within the transgene affected chromatin condensation, we measured the level of histone acetylation. No histone H3 acetylation was observed in cells harboring transgenes that did not contain a replicator (construct I) at the silencing-prone orientation (**Fig. 4**). When cells were transfected at the silencing-prone orientation with constructs that contained the functional replicators Rep-P (construct IV) and *LMNB2* (construct

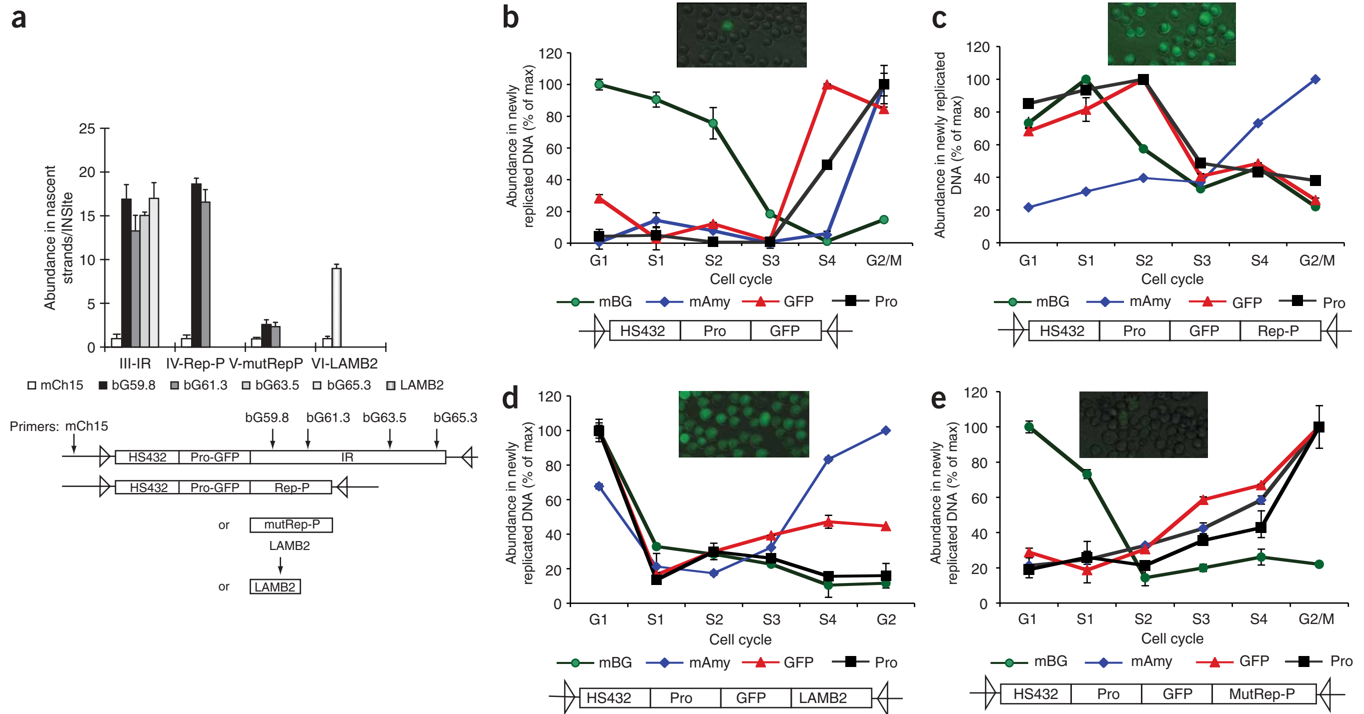

Figure 3 Replication timing of transgenes at the RL4 site in MEL cells. (a) Initiation of DNA replication from transgenes inserted at RL4. The transgenes included the entire IR (Fig. 1c construct III), Rep-P (Fig. 1c construct IV), LAMB2 (Lamin B2 from the *LMNB2* locus) (Fig. 1c construct VI) or mutRep-P, in which a 45-bp sequence essential for initiation was mutated (Fig. 1c construct V). The abundance of the nascent strands in the transgene was tested by real-time PCR. Primers and probes include mCh15, which is genomic DNA of the RL4 region, previously shown to exhibit no DNA replication initiation[8], and bG59.8. bG61.3, bG63.5 and bG65.3, which are from the 8-kb IR of the β-globin locus[13], and LAMB2, which is from the *LMNB2* locus. Replication activity was the relative enrichment of the specific gene to the mCh15. The *x*-axis is labeled with the construct names and numbers as designated in Figure 1c. (b–e) Replication timing and GFP expression of transgenes inserted at the silencing-prone orientation at the RL4 site. As controls, we verified that the murine β-globin locus, an early-replicating locus in MEL cells, replicated early and that the murine amylase locus, a late-replicating locus, replicated late in all the tested cells. These findings confirmed that insertion of a replicator sequence could affect the timing of DNA replication in the vicinity of the insertion site and did not affect global patterns of DNA replication in the host cells (see Fig. 2 and Supplementary Table 1 for primers and probes).

VI), but not the mutated Rep-P (construct V), transgene-specific sequences were enriched in chromatin containing acetylated histone H3 (Fig. 4). Similar data were obtained using antibodies against acetylated histone H4 or methylated histone H3, lys4 (data not shown). All the transgenes exhibited histone acetylation markers of decondensed chromatin when the transgene was inserted at RL4 in the opposite, transcription-permissive orientation (data not shown). These findings suggested that the presence of replicators could prevent histone deacetylation.

All the studies reported above were performed on transgenes inserted into a single, constant genomic site located on murine chromosome 15. To determine whether replicators can prevent gene silencing at other locations, we created a second set of transgene insertions at another site, random locus 5 (RL5), located on murine chromosome 4. Most transgenes inserted into RL5 retain expression in both orientations[10]. However, a transgene containing a second copy of hypersensitive site 2 (HS2; construct VII) exhibited silencing (**Supplementary Fig. 2a** online). When we inserted a functional replicator (*LMNB2*) into the transgene at the RL5 site (construct VIII), the replicator-containing transgene maintained stable expression in both orientations for at least 4 months (**Supplementary Fig. 2a**). Transgenes that contained replicator sequences were enriched in chromatin containing acetylated histone H3 whereas transgenes that did not contain replicators, but contained HS2, were not (**Supplementary Fig. 2b** online). These data demonstrate that replicators can prevent gene silencing and histone deacetylation at the RL5 site, implying that the effects of replicators on gene expression and chromatin structure are not limited to the RL4 site.

Our data suggest that replicators from the human β-globin and *LMNB2* loci interact with sequences from the human β-globin LCR to affect the timing of DNA replication. These two replicators do not share specific consensus sequences but do share some sequence features such as a series of AT stretches near a relatively GC-rich region[18]. An interaction between the LCR and replicators was observed at the native human β-globin locus, where initiation of DNA replication and early replication in erythroid cells require the LCR[11,19]. However, these requirements for the LCR are lost at some ectopic locations: in early-replicating ectopic sites, replicators from the human β-globin locus initiate replication early during S-phase with and without LCR[16], and at other locations LCR may play a role as a silencer and delay replication[9]. The effect of the LCR on replication timing is therefore context-dependent, probably affected by random juxtaposition of regulatory elements at different integration sites. Because the ability to prevent silencing was only observed in functional replicators, our studies suggest that the effects of the LCR on replication are not limited to specific replicators.

Similar to the LCR, other transcriptional control elements may affect replication. Specific examples include the Chinese hamster *DHFR* promoter affecting the location of initiation events within the *DHFR* locus[12,20], promoters affecting initiation activity from

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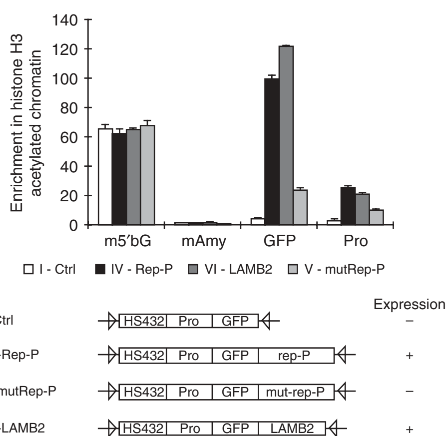

Figure 4 Replicators can prevent chromatin condensation at the RL4 site. A ChIP assay with anti-acetylated histone H3 antibody was carried out to determine whether initiation of DNA replication within the transgene affects LCR-mediated chromatin condensation (see **Figs. 2** and **3** for details of specific transgene and host sequences). Transgene constructs designations correspond to **Figure 1c**. We measured as controls the acetylation of histone H3 at the murine amylase locus, which is not transcribed in MEL cells, and in a sequence from the murine β-globin locus, which is transcribed in these cells[35].

ectopic *c-myc* insertions[21], tethering chromatin modifiers specifying the location of initiation events from the *Drosophila melanogaster* chorion gene replicators[22] and transcription activation at mid-blastula affecting specification of replication origins in *Xenopus laevis* egg extracts[23]. However, although the activation of transcription in the above cases correlated with altered initiation patterns, the causal relationships between the location of initiation events and activation of transcription remain unclear. In the experiments described here, replication delays occurred before gene silencing, suggesting that the replication delays were not caused by changes in transcriptional activity. Observations in the murine immunoglobulin IgH locus, in which an advanced replication time of one of the two IgH alleles in prereplicating B cells occurs before transcription[24], provide another example for changes in replication timing that precede changes in gene expression. The mechanisms by which changes in replication timing affect gene expression are unknown but could involve the differential expression of chromatin modifiers during the cell cycle[1].

We observed that replicators prevented transcriptional silencing of transgenes inserted at two unlinked chromosomal sites. The two sites markedly differ in their effect on gene expression: LCR-containing transgenes inserted at RL4 at the silencing-prone orientation were always silenced, whereas the same constructs often exhibited stable expression at RL5 and silencing was limited to constructs that included HS2 at the 3′ end of the expression cassette. Replicators could prevent silencing at both sites, suggesting that they exert an epigenetic effect that facilitates early replication and stabilizes gene expression at a substantial subset of chromosomal sites. Because RL4 and RL5 were selected at random, we do not know how frequent such sites are in the mammalian genome. However, the high prevalence of transgene transcriptional silencing in mammalian cells[25–28] suggests that chromosomal loci that are subject to silencing are common in mammalian chromosomes and often serve as integration sites for gene therapy vectors.

An important implication of this study is that inclusion of replicators within transgenes can stabilize gene expression and prevent gene silencing. Gene therapy that uses randomly integrating vectors, such as lentiviruses, is one of the most promising forms of gene therapy for many diseases. But insertional mutagenesis, particularly activation of oncogenes by enhancers present in the expression cassette, is still an important concern. Transgene silencing increases the risk of insertional mutagenesis because it decreases the average transgene expression per integrated copy. Inclusion of replicators in gene therapy cassettes might therefore improve vector safety because a reduced frequency of silencing should reduce the average number of integrated vectors required to achieve a therapeutic level of transgene. Replicators, along with insulators and matrix attachment regions that can decrease silencing by blocking the spread of heterochromatin[10,27,29–31], might become important *cis*-acting elements that can reduce the risks associated with gene therapy.

METHODS

Plasmids. Rep-P and mutated Rep-P from the human β-globin locus were described previously[13]. Human lamin B2 (*LMNB2*) replicator[17] (positions 3,691 to 4,978; GenBank accession no. M94363) was obtained from genomic DNA of K562 cells by PCR amplification with specific primers. All sequences were verified by DNA sequencing at the National Cancer Institute core facility. Replicator sequences were inserted into the HS432-β-promoter-enhanced green fluorescent protein (GFP) plasmid[10] using standard methodology. The sequences of all the plasmids are available on request.

Cell culture and transfections. MEL cells harboring plasmids described in **Figure 1** were grown in Dulbecco modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated FCS. Cre recombinase-mediated cassette exchange was performed as previously described with the following modifications[10]. The target MEL cells containing the CMV-HYTK cassette flanked by L1-1L *lox* sites inserted at RL4 were cotransfected by lipofectamine 2000 (Invitrogen) with 4 μg of a Cre expression plasmid and 4 μg of an exchange plasmid containing the cassette of interest. Clones that lost the HYTK cassette were selected with 10 nM gancyclovir. Replacement of the HYTK cassette by the cassette present in the exchange plasmid was then verified by PCR.

Fluorescent-activated cell sorting (FACS) analysis. GFP expressions of the transgenes were monitored by FACS. At least three colones for each inserted direction were tested every 2 to 4 weeks until all colones were either completely silenced or up to 6 months. Dead cells were gated out on the basis of morphological parameters (FSC and SSC) and propidium iodide exclusion. The percentage of cells expressing the GFP transgene was estimated using untransfected MEL cells as a negative control.

Replication initiation analyses. Genomic DNA and nascent-strand DNA were prepared as described previously[32]. Briefly, DNA was collected from asynchronous cultures and denatured by boiling followed by rapid cooling, and short DNA strands were fractionated by size on neutral sucrose gradients. DNA strands ranging from 0.6 to 2.5 kb were collected and treated with λ-exonuclease[33,34]. Nascent strands were amplified by real-time PCR in an ABI 7900 thermocycler (Applied Biosystems International) using a series of probe-primer combinations surrounding the inserted replicator and adjacent sequences. The amount of DNA in each sample was quantified by OliGreen analysis (Molecular Probes). Genomic DNA that was not treated with exonuclease was used as a standard for calculating the number of molecules in the template. Genomic DNA from MEL cells was used as a nontemplate control to verify that primers used in the study were specific for the inserted DNA. To verify that the exonuclease treatment eliminated sequences that are not involved in initiating DNA replication, we included origin-proximal and origin-distal primer-probe combinations from the host genome in each nascent-strand analysis. Data from three PCRs for each primer-probe combination were used to calculate the amount of sequence-specific nascent strands[13].

Replication timing analyses. Replication timing analyses were performed as described previously[8] with the following modifications. Cells were labeled with BrdU for 90 min and with 20 mg/ml Hoechst 33342 dye (Molecular Probes) for 30 min before harvesting. Cell cycle fractions were sorted using a Vantage fluorescence-activated cell sorter or elutriator. Newly replicated, BrdU-substituted DNA was isolated by immunoprecipitation with anti-BrdU

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antibodies as described[8]. Each sample was subjected to two sequential rounds of immunoprecipitation, washes and DNA purification. BrdU-incorporated *D. melanogaster* genomic DNA was added as an internal control to each sample before immunoprecipitation. The quantity of newly replicated (BrdU-substituted) DNA was determined with OliGreen and the abundance of mitochondrial DNA sequences (mMT primers) was used to verify that each fraction contained similar quantities of amplifiable DNA strands. Samples containing 8 ng DNA were amplified with a series of primer-probe combinations and analyzed by real-time PCR on an ABI 7900. The quantity of an amplified sequence was calculated using genomic DNA standards with a standard curve from 0.005 to 50 ng per reaction. Each measurement was performed in triplicate. Experiments were performed using each cell line. The relative abundance for each probe-primer combination was calculated as a percentage of the number of molecules amplified from a specific cell cycle fraction by the number of molecules amplified from the cell cycle fraction where amplification was maximal.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were carried out as described previously[8] with the following modifications: at 25 °C, 4–6 × 10^7 MEL cells were fixed for 5 min by the addition of 1% formaldehyde to the growth medium. After a series of washings, cells were sonicated six times (for 20 s at 1-min intervals) with a 2-mm tip of a Sonics & Materials sonicator at the maximum setting. After centrifugation at 20,800*g* for 20 min, the cleared supernatant was adjusted to contain 1× RIPA buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% of a standard protease inhibitor cocktail; Sigma). To reduce nonspecific binding to protein A, we precleared chromatin with 100 µl UltroLink immobilized protein A (50% slurry in RIPA buffer; Pierce) for 1 h at 4 °C with rotation. A solution of precleared chromatin (0.5 ml) was incubated with or without 5 µg antibody and rotated at 4 °C for 12 to 16 h. Antibodies used included anti-acetyl-histone H 3 (no. 06-599), anti-acetyl-histone H4 (no. 06-866), anti-dimethyl-histone H3 (Lys 4) (no. 07-030), all from Upstate Biotechnology. Protein A beads (50 µl) were added to the ChIP mixture and the mixture was incubated for 2 to 4 h. The protein A beads were then washed once with 1× RIPA buffer, three times with 1× RIPA plus 0.5 M NaCl, twice with a Tris-LiCl buffer (10 mM Tris-Cl pH 8.0, 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA) and twice with TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). A volume of 0.5 ml of elution buffer (10 mM Tris-Cl pH 8.0, 200 mM NaCl, 0.5% SDS, 1 mM EDTA) was then added to the protein A beads and this mixture was incubated at 65 °C for 12 to 14 h, followed by treatment with RNase and proteinase K. The DNA was then extracted with phenol/chloroform, precipitated and resuspended in distilled water. The DNA concentration of the samples was determined by Pico green fluorescence (Molecular Probes). Real-time PCR was used to amplify the ChIP-enriched DNA. The sequences of primers and probes are listed in **Supplementary Table 1** online.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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