Transcription shapes DNA replication initiation and termination in human cells

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Although DNA replication is a fundamental aspect of biology, it is not known what determines where DNA replication starts and stops in the human genome. We directly identified and quantitatively compared sites of replication initiation and termination in untransformed human cells. We found that replication preferentially initiates at the transcription start site of genes occupied by high levels of RNA polymerase II, and terminates at their polyadenylation sites, thereby ensuring global co-directionality of transcription and replication, particularly at gene 5’ ends. During replication stress, replication initiation is stimulated downstream of genes and termination is redistributed to gene bodies; this globally reorients replication relative to transcription around gene 3’ ends. These data suggest that replication initiation and termination are coupled to transcription in human cells, and propose a model for the impact of replication stress on genome integrity.

S
ince the identification of cis-acting sequences responsible for the definition of replication origins in Saccharomyces cerevisiae, considerable effort has been applied to identify analogous determinants of replication initiation in human cells2,3. Although origins have been observed via several independent techniques to be enriched close to transcribed genes and a range of other chromatin features4–7, a coherent model that encompasses both origin specification and activation has not emerged. Furthermore, throughout eukaryotes, many more replication origins are licensed by MCM2-7 loading in G1 than are required to complete S-phase6,7. The pool of excess minichromosome maintenance (MCM) proteins is required for survival when deoxyribonucleoside triphosphates (dNTPs) are depleted by the ribonucleotide reductase inhibitor hydroxyurea (HU)8, and has been proposed to allow the firing of ‘dormant’ replication origins that rescue genome replication after replication fork stalling. The identities of these dormant origins, and how they differ from constitutive origins, have not been defined.

Deoxyribonucleic acid is a one-dimensional template that can be simultaneously acted on by the replication and transcription machineries. The orientation of essential genes in prokaryotes is biased to avoid head-on collisions11; among eukaryotes, budding yeast and Caenorhabditis elegans show statistical orientation bias of the most highly transcribed genes to the co-directional orientation12,13, and substantial co-orientation of transcribed genes with the direction of replication has been noted in human cells12,14. Head-on replication-termination conflicts are deleterious in eukaryotes, leading to increased DNA damage14 and genomic rearrangements15. How origin location is specified to co-orient replication with transcription in diverse human cell types, and the impact of co-directional transcription on replication-fork progression through genes, remain speculative.

Using Okazaki fragment sequencing (Ok-seq) to infer the direction of replication-fork movement, we defined transcription initiation efficiency and gene length as independent determinants of replication origin location and firing efficiency. We found that origin firing occurred close to the transcription start site (TSS) of long, transcribed genes, ensuring co-oriented replication of the most highly transcribed regions of the genome. We also found that, under conditions that stimulate dormant replication origin firing, activation of the most efficient constitutive origins was further stimulated. In addition, we observed widespread localized replication termination at the transcription termination site (TTS) of transcribed genes under unperturbed conditions. Replication termination redistributed to gene bodies during replication stress, resulting in increased replication of gene 3’ ends in head-on orientation.

Results

Using Ok-seq, we and others have reported on replication initiation12,13,14,15, replication elongation12, and lagging-strand processing12,16. To investigate replication initiation and termination in untransformed human cells, we performed Ok-seq on human telomerase reverse-transcriptase-immortalized retinal pigment epithelium (RPE)-1 cells. Previous genome-wide studies of mammalian DNA replication showed limited agreement in origin calls2,3, with the exception that all identified significant enrichment of origins close to transcribed genes. Thus, rather than aiming to identify all of the potential sites at which replication can possibly initiate in the human genome, we focused our analysis on the efficiency of replication initiation and termination in transcribed regions.

Replication initiates in the immediate vicinity of transcription start sites. Rightward-moving replication forks generate Okazaki fragments that map to the Crick strand, whereas leftward-moving forks generate Watson-strand fragments (Fig. 1a). Thus, a replication origin will manifest as an increase in the proportion of Okazaki fragments mapping to the Crick strand. Okazaki fragment distributions from asynchronously dividing RPE-1 cells showed regions of largely unidirectional replication. Not all genes were associated with replication origins, but Okazaki fragment strand transitions often occurred upstream of transcribed genes (two arbitrary, but
Fig. 1 | DNA replication initiates preferentially at transcription start sites. a, The relationship between replication direction and Okazaki fragment strand. b, Distribution of Okazaki fragments and RNA-seq reads from total cellular RNA in RPE-1 cells over 1.5-MB regions around the MCM2 and ROBO1 loci. TSS are indicated, and the percentage of replication forks moving from left to right is shown in orange. Unless otherwise indicated, all data in Figs. 1–7 are from asynchronously dividing human telomerase reverse transcriptase (hTERT)-immortalized RPE-1 cells, and are displayed using 1-kb bins. c, Schematic depiction of expected Okazaki fragment distributions around replication origins. d, Percentage of RPE-1 Okazaki fragments mapping to the Crick strand (indicating rightward-moving replication forks) across a ±50-kb window around annotated TSS in the human genome. All meta-analyses shown in Figs. 1–6 were carried out using one replicate: data from a second biological replicate are shown in Supplementary Figures 1 and 6. c, Percentage of Okazaki fragments mapping to the Crick strand across a ±50-kb window around the TSS of Watson (W) or Crick (C) genes. f, Percentage of replication forks moving from left to right around the TSS of all genes. Here and in subsequent figure panels, data are oriented such that transcription runs from left to right (indicated by T xn L → R). g, Schematic representation of upstream and TSS-proximal replication initiation inferred from data in Fig. 1. h, Replication initiation frequency, calculated as the first derivative of Okazaki fragment strand bias as a function of position, across a ±50-kb window around TSS. Data are smoothed across two 1-kb bins.
representative, 1.5-Mb regions are shown in Fig. 1b). In addition to initiation upstream of TSS, we occasionally noticed Okazaki fragment strand bias transitions consistent with replication initiation immediately downstream of genes (for example, ROBO1 in Fig. 1b). The total RNA sequencing (RNA-seq) data used in this work are from a previously published dataset.

We calculated Okazaki fragment distributions by meta-analysis around specific classes of genomic loci. The efficiency and spatial localization of origin firing will affect the magnitude and gradient of the Okazaki fragment strand bias transition, respectively (schematic in Fig. 1c). As expected, Okazaki fragments showed no strand bias around random genomic loci (Supplementary Fig. 1a). Consistent with global origin activity at TSS, meta-analysis of Okazaki fragment Crick-strand bias over a 50-kb window surrounding all 18,037 annotated TSS showed a symmetrical transition from predominantly leftward- to predominantly rightward-moving forks (Fig. 1d).

However, separate analysis of Watson- and Crick-strand genes revealed profound asymmetry based on gene orientation (Fig. 1e). Thus, we considered Okazaki fragment strand bias relative to gene orientation, such that transcription occurs from left to right (Fig. 1f, schematic in Fig. 1g). All data for TSS analysis were highly reproducible across two biological replicates (Supplementary Fig. 1b–d).

By analyzing the first derivative of replication direction calculated from OF distributions, we were able to directly infer the change in replication polarity—and therefore the extent of replication initiation—at each position relative to the meta-TSS. A positive value for the increase in the proportion of replication forks moving from left to right indicates origin firing. We observed a strong peak of replication initiation within 2–3 kb of the meta-TSS, preceded by a gradual increase over the ~20-kb upstream (Fig. 1b). Replication initiation frequency was consistently negative or zero downstream of TSS, indicating a paucity of replication initiation. Thus, although individually low, but cumulatively high, levels of initiation occurred over a wide region upstream of TSS, initiation was strongly biased to a 2–3-kb region immediately adjacent to the TSS itself and is under-represented or absent in gene bodies. These data are consistent with previous reports that origin firing is predominantly intergenic \[^{\text{5,6}}\], but extend these observations by localizing the preferred site of initiation immediately upstream of the TSS.

Determinants of replication origin firing efficiency at TSS.

We estimated origin efficiencies by calculating the difference in Okazaki fragment strand bias between the region from 50 to 30 kb upstream and the region 1 to 10 kb downstream of the TSS. We present changes in origin efficiency (Δeff), representing the absolute change in the percentage of cells in which a given set of origins will fire. A Δeff of +5% means that origin firing occurs in an additional 5% of cells in the population.

We separated genes into quartiles based on RNA-seq read density from total RNA (fragments per kilobase of transcript per million mapped reads, FPKM) in RPE-1 cells \[^{\text{21}}\]. TSS of genes with higher RNA-seq read density showed significantly greater change in strand bias than TSS of weakly or non-transcribed genes (Fig. 2a,b). We verified the specificity of this transcriptional effect by comparing our Ok-seq data from RPE-1 cells with previously published data from GM06990 cells \[^{\text{22}}\] around TSS of genes that are uniquely active in only one of these cell types (Fig. 2c,d). Genes transcribed above median levels in RPE-1, but below median levels in GM06990 (RPE on, GM off), showed robust origin activity at their TSS only in RPE-1, and vice versa.

We separated transcribed genes (FPKM > median) into quartiles by length and observed that gene length showed a strong positive correlation with origin activity (Fig. 2e,f). Gene length and transcription level were not correlated in RPE-1 cells, and gene length and transcript number therefore independently correlate with origin firing efficiency. Distance to the nearest downstream TSS and transcription termination site (TTS)—both of which are intrinsically dependent on gene length—are substantially stronger determinants of origin efficiency than the distance to the nearest upstream TSS or TTS, which are independent of gene length (Supplementary Fig. 2). Thus, the effect of gene length on origin efficiency cannot solely be explained by decreased passive replication from nearby ‘competitor’ origins. Transcript levels and gene length both correlate with origin activity in HeLa and GM06990 cells using previously published Ok-seq data \[^{\text{22}}\] (Supplementary Fig. 3).

Although (assuming equal RNA decay rates) RNA-seq reports the number of RNA molecules synthesized, the number of RNA polymerase II (RNApoly2) complexes occupying a gene for a given FPKM is linearly related to the length of the gene. We therefore analyzed Okazaki fragment strand bias around TSS separated by transcriptional volume (FPKM × gene length). As expected, activity was strongly correlated with high transcriptional volume (Fig. 2g,h).

We confirmed this by analyzing origin activity as a function of cumulative RNAP2 ChIP-seq signal in the gene body in asynchronous RPE-1 cells \[^{\text{22}}\] (Fig. 2i,j).

We conclude that, in unperturbed human cells, the initiation of DNA replication is strongly biased towards the immediate vicinity of TSS that drive transcription of genes with high RNAP2 occupancy. Although transcription creates conflicts with replication, the coupling of origin firing to RNAP2 occupancy would bias conflicts towards co-directional orientation.

De-localized replication initiation at enhancers.

We reasoned that, in addition to TSS, other accessible chromatin regions, such as enhancers, might similarly act as localized replication origins. Okazaki fragment bias around 27,404 enhancers previously identified in RPE-1 cells \[^{\text{22}}\] was consistent with origin activity (Fig. 3a). We separated enhancers into high versus low transcription based on RNAP2 ChIP-seq \[^{\text{23}}\] (Fig. 3b). Enhancers <50 kb from the nearest TSS showed Okazaki fragment transitions expected for replication origins, but a similar transition was apparent for random sites in the same range (Supplementary Fig. 4), consistent with a ‘bystander effect’ as a result of origin activity of nearby TSS. Thus, we considered only enhancers for which the closest TSS was over 50 kb away. We compared Okazaki fragment distributions around enhancer midpoints to the Okazaki fragment distribution around an identical number of random sites, also >50 kb from TSS (Fig. 3c). A transition in Okazaki fragment strand bias was absent for random sites, but persisted for enhancers, consistent with bona fide origin activity centered on enhancer midpoints and independent of nearby protein-coding TSS (Fig. 3c). As a result of the symmetrical Okazaki fragment signal around enhancers, we calculated relative origin efficiencies using data from ±20 to 40 kb from the enhancer midpoint. Although transcribed enhancers were slightly more active origins than non-transcribed enhancers, both displayed significant origin activity relative to random sites. Origin signal extended over a ~15-kb region on either side of the enhancer. Thus, replication specifically initiates in proximity to enhancers: this effect is slightly stimulated by—but not dependent on—enhancer transcription.

Modulation of constitutive origin efficiency during replication stress.

In response to replication stress, origin firing increases \[^{\text{24}}\]. Origin firing events during growth in hydroxyurea have previously been reported to occur in the vicinity of transcribed genes \[^{\text{24}}\]. We treated cells with 0.2 mM HU for 4 h before Okazaki fragment collection, and additionally depleted either of the Fanconi anemia effector proteins, FANCD2 or FANCI \[^{\text{25,26}}\], by RNA interference (RNAi). This HU treatment decreases average inter-origin distance without activating the DNA damage checkpoint \[^{\text{27}}\]. Knockdown of FANCI increases inter-origin distance in HU, reflecting reduced origin firing, whereas knockdown of FANCD2 weakly stimulates origin firing under the same conditions \[^{\text{27}}\].
We analyzed the effect of hydroxyurea treatment on origin efficiency at TSS separated by transcriptional volume (Fig. 4a,b). We observed a significant increase in origin efficiency for the most highly transcribed genes, which is the most efficient pre-existing origins. We observed no evidence for a widespread increase in intragenic origin firing on hydroxyurea treatment. Indeed, although the
overall pattern of origin use was globally similar in the presence and absence of hydroxyurea, the preferred site of replication initiation moved upstream of the TSS on hydroxyurea treatment, as opposed to downstream (Fig. 4c).

We observed that FANCI knockdown significantly reduced origin firing around high-volume TSS in HU-treated cells (Fig. 4d,e and Supplementary Fig. 1d). We did not observe a significant increase in firing of the most efficient pre-existing origins on FANCIC2 knockdown, although firing efficiency was very modestly increased for less efficient origins (Fig. 4d). The effect of FANCI knockdown was independent of gene length, arguing against a global defect in replication-fork mobility through transcribed regions as a result of reduced FANCI levels (Supplementary Fig. 5). Thus, under conditions of globally increased or decreased origin firing, we found that the firing efficiency of the most active pre-existing origins is the most strongly affected in transcribed regions of the genome. Our data suggest that intragenic replication origins do not contribute significantly to dormant origin firing in RPE-1 cells.

Replication termination is locally enriched at polyadenylation sites of transcribed genes. Sites of replication termination generate a transition in Okazaki fragment strand bias opposite to that observed at replication origins—that is, a decrease in the proportion of replication forks moving from left to right. We queried Okazaki fragment distributions around the annotated polyadenylation site (hereafter referred to as the TTS). The TTS corresponds to the site of pre-messenger RNA cleavage and polyadenylation, and does not necessarily reflect the site of RNA2 dissociation. We quantified replication termination by calculating Okazaki fragment strand bias over the region from −10 to +10 kb relative to the TTS. Differences in termination frequency are presented as Δterm. All data for TTS were reproducible across replicate datasets (Supplementary Fig. 6).

Consistent with an over-representation of replication termination events at the TTS of transcribed genes, we observed a significant, transcription-dependent reduction in replication forks moving in the direction of transcription through the gene body, occurring precisely at the TTS (Fig. 5a). This termination signal was surrounded by an increase in left-to-right fork movement, consistent with diffuse origin firing occurring at TSS a variable distance away (Fig. 5b,d). Termination was significantly higher for more highly transcribed genes. Analogously to replication initiation (Fig. 1h), localized replication termination can be inferred from analysis of the change in replication polarity around a meta-focus. The change in Okazaki fragment strand bias in the vicinity of TTS showed a sharp trough immediately adjacent to the TTS itself, indicating that the peak of replication termination is localized, on average, precisely at the TTS for transcribed genes (Fig. 5c).

In addition to the replication termination signal at the TTS, we observed an origin signal—that is, sustained positive values for the increase in rightward-moving replication—within the region immediately downstream of the gene (Fig. 5b,c). This origin firing signature was apparent only downstream of genes transcribed above the median level (cf. top versus bottom two panels in Fig. 5b,c).

Replication termination at gene 3′ ends is not affected by R-loops. We analyzed replication termination around genes separated based on their probable levels of R-loop formation at TTS in RPE-1 cells. Most differences in R-loop formation between cell types and even species can be attributed to differences in transcription; thus, we separated genes with FPKM > median in RPE into two bins based on the DRIP-seq signal observed ±10 kb from their TTS in HeLa cells to obtain high- and low-DRIP TTS gene sets (Fig. 5e). Genes in our high-DRIP set are significantly shorter than those in our low-DRIP set (Fig. 5f).

Analysis of Okazaki fragment strand bias around the TTS of high- and low-DRIP genes indicates equivalent replication termination frequency and localization regardless of the propensity of the region to form R-loops (Fig. 5g,h). The upward gradient observed upstream of the TTS for high-DRIP genes is a result of origin firing at TSS, which are closer to TTS given the length difference between high- and low-DRIP genes (Fig. 5g). Results from Fig. 5 were reproducible using previously published Ok-seq data on HeLa and GM06990 cells (Supplementary Fig. 7). Thus, although replication termination can occur throughout the genome, termination events are enriched at the 3′ end of transcribed genes; localized termination occurs independently of the gene's propensity to form R-loops.

Global reorientation of replication at gene 3′ ends under replication stress. For highly transcribed genes, on HU treatment we observed an increase in both the overall level of gene-associated replication termination and the fraction of this termination that is intragenic as opposed to localized at the TTS (Fig. 6a,b). Analysis of the change in replication direction (Fig. 6c,d) indicated that replication termination is still elevated around TTS, but also showed a marked increase in replication initiation in the
Fig. 4 | Global modulation of origin activity under conditions that increase or suppress dormant origin firing. a, Percentage of replication forks moving left to right around TSS binned by transcriptional volume, for cells grown in the absence (black) or presence (red) of 0.2 mM hydroxyurea (HU) for 4 h before Okazaki fragment collection. P values and effect sizes were calculated using Kruskal–Wallis relative to the no-HU dataset for each quartile. Δeff and P values between the no-HU and low-HU samples for each transcriptional quartile are indicated in the relevant panels. b, Heat map representation of the change in replication direction around TSS in 0.2 mM HU on FANCI knockdown. Genes are sorted by transcriptional volume (length × FPKM). Values were calculated as %L → R0.2 mM HU – %L → Rno knockdown. c, Heat map representation of the change in replication direction around TSS in 0.2 mM HU on FANCD2 knockdown. Genes are sorted by transcriptional volume (length × FPKM). Values were calculated as %L → R0.2 mM HU – %L → Rno knockdown.

Δeff = 4,541 n = 4,541
Q1 volume = 0.27 (n.s.) P = 1 × 10^-8
Q2 volume = 0.05
Q3 volume = 0.78 (n.s.) P = 3 × 10^-3
Q4 volume = 0.23 (n.s.) P = 1 × 10^-3

Δeff = 4,541 n = 4,541
Q1 volume = 0.27 (p.s.) P = 0.05
Q2 volume = 0.55 (p.s.) P = 0.005
Q3 volume = 0.79 (p.s.) P = 2 × 10^-3
Q4 volume = 0.4% P = 0.05
Fig. 5 | Widespread, R-loop-independent replication-fork termination occurs at the 3’ ends of transcribed genes under unperturbed conditions.

**a.** Percentage of replication forks moving left to right around TTS binned by RNA-seq read depth quartile from ref. 19. P values and effect sizes were calculated using Kruskal–Wallis as for TSS data in previous figures, but using data ±10 kb from the TTS as described in the Results and Methods. ∆term and P values between adjacent quartiles are indicated on the relevant panels and in the Methods. **b.** Schematic representation of Okazaki fragment distributions arising from replication termination at TTS. **c.** Replication initiation frequency, calculated as the first derivative of Okazaki fragment strand bias as in Fig. 1h, around TTS binned by total RNA-seq read density in the gene body as in **a.** Negative initiation frequencies correspond to termination. Data are smoothed over two adjacent bins. **d.** Heat map representation of replication direction around TTS: genes are ordered by length. **e.** DRIP-seq signal from (ref. 14) around TTS of actively transcribed genes (FPKM from (ref. 19) > median) separated into high- (purple) and low- (brown) DRIP bins based on DRIP-seq signal ±10 kb from the TTS. **f.** Length distribution of actively transcribed high-DRIP versus low-DRIP genes. The cumulative fraction of high-DRIP versus low-DRIP genes shorter than the length on the x axis is plotted. **g.** Percentage of replication forks moving left to right around TTS of actively transcribed (FPKM from (ref. 19) > median) high-DRIP versus low-DRIP genes. P = 0.85 by Kruskal–Wallis. **h.** Replication initiation (termination) frequency around high-DRIP versus low-DRIP TTS.

region ~20 kb downstream of the TTS relative to untreated cells. To test whether this effect was entirely a result of increased origin firing at proximal TSS in this downstream region, we separately analyzed the change in Okazaki fragment strand bias around TTS of transcribed genes (FPKM > median) with or without a TSS for a protein-coding gene in the 50 kb immediately downstream (Fig. 6e,f). We observed substantial replication initiation downstream of the TTS even in genes with no nearby TSS. Analogous
to efficient origins near TSS (Fig. 4a,c), downstream initiation was more pronounced in HU-treated cells than in untreated cells (Fig. 6f).

Taken together, our data demonstrate that replication origin efficiency increases near active TSS in the presence of HU (Fig. 4a): this increases co-orientation of replication and transcription at
gene 5′ ends (Figs. 4a and 6g). However, HU treatment also increased the efficiency of origins downstream of genes, reducing the proportion of replication forks co-oriented with transcription at gene 3′ ends. This effect was most prominent for long genes (Fig. 6g).

Discussion

Co-orientation of replication and transcription in multicellular eukaryotes. Our data suggest that DNA replication initiation is coupled to transcription initiation, such that origin firing preferentially initiates immediately adjacent to the TSS of genes with high RNAP2 occupancy (Fig. 2, model in Fig. 7). Thus, the most highly transcribed genes will have the strongest bias towards co-directional replication in any proliferative cell type, regardless of its transcriptional profile. Many unicellular organisms define replication origins via the use of cis-acting sequences: in these organisms, the orientation of conflict-prone genes has been biased by evolution, presumably as a result of damage induced by head-on collisions followed by reorientation. However, the use of such cis-acting sequences as the sole means of origin specification in an organism with many transcriptionally distinct proliferative cell types would enforce cell-type-specific head-on replication-transcription conflicts. Thus, although unicellular organisms can co-orient transcription with replication by genome evolution, the alternative strategy—to co-orient replication with transcription—is more robust for organisms with many distinct transcriptomes.

In addition to replication origin activity immediately upstream of highly transcribed genes at TSS, we observed significant initiation...
immediately downstream of TTS. If downstream initiation were as efficient as upstream initiation then genes would, on average, be replicated co-directionally with transcription at their 5’ ends and head-on at their 3’ ends. We did not observe such a pattern in unperturbed cells (Figs. 2 and 5, and Supplementary Figs. 3 and 7). Firing efficiency of TSS-proximal origins must therefore be, on average, higher than that of TTS-proximal ones.

A simple mechanism to explain the increased replication origin activity of the genes occupied by most RNAP2 is that chromatin accessibility determines both MCM2-7 loading and the recruitment of replisome components to licensed replication origins. This mechanism would also provide a rationale for the delocalized origin efficiency observed at enhancers (Fig. 3). Chromatin accessibility has previously been reported as the best correlate of replication timing14. Our data do not preclude preferential initiation at specific sequences in regions of open chromatin defined by a cell’s transcriptional profile—for example, a recent report showed that replication initiation in mouse cells correlates with poly(dA:dT) tracts14. Indeed, given that poly(dA:dT) sequences disfavor stable nucleosome occupancy35, and the human origin recognition complex (ORC) displays increased affinity for A-T-rich DNA35 these sites should experience increased origin recognition complex recruitment and MCM loading.

Replication initiation outside transcribed regions. Gene distribution is not uniform, and there are clearly fewer actively transcribed genes than the number of origins required to replicate the entire human genome. Inactive regions of the genome lack both spatially localized regions of highly accessible chromatin and a requirement to co-orient replication with transcription: thus, specifically (as opposed to randomly) localized replication origins would serve little biological purpose in these regions. Transient chromatin opening, probably driven by low levels of pervasive transcription factor binding or transcription, could ensure a sufficient density of MCM2-7 loading and activation to support genome duplication in these regions through a distribution of origin firing events that largely disfavors initiation at specific individual sites.

Replication termination at gene 3’ ends. Although replication termination can occur throughout the genome, termination events were highly enriched at gene 3’ ends in both unperturbed transformed and non-transformed human cell lines (Fig. 5a–c and Supplementary Fig. 7a,h,e,f). Because replication origins were located at variable distances both upstream and downstream of TTS (Fig. 5d), it is improbable that such localized replication termination would arise passively. The precise termination of replication at TTS can more plausibly be explained by replication-fork stalling or arrest at these sites. RNAP2 and the replication fork normally moved at approximately the same speed in eukaryotes. Replication forks co-oriented with transcription should generally not collide with RNAP2, except when the latter is paused. Gene 3’ ends represent prominent RNAP2 pause sites15. We propose that co-directional collisions with paused RNAP2 are prevalent at TTS and result in localized replication termination at these sites.

Replication-fork stalling resulting from co-oriented collisions with RNA polymerase at gene 3’ ends has been observed in prokaryotes29, but not, to our knowledge, in eukaryotes. Unlike in prokaryotes28, but similarly to replication-fork arrest at transfer RNA genes in yeast21, R-loops do not appear to impede replisome mobility, despite their effect on the ultimate outcome of collisions14,15,39,40. Screens in HeLa cells35 and S. cerevisiae40 identified RNA splicing and 3’ end formation factors as preventative against DNA damage. It will be interesting to determine the relative contributions of R-loop modulation and replisome mobility to DNA damage when messenger RNA processing is impaired.

The frequency of replication termination at TTS was lower than the cumulative initiation frequency at TSS (Fig. 1b), consistent with only a fraction of replisomes stopping at TTS. Regardless, replication fork initiation at TSS followed by stalling or arrest at TTS was able to impair the replication of intergenic regions flanked by convergent, highly transcribed genes. However, the ability of both RNA polymerases14,42 and the replicative helicase43 to push loaded MCM2-7 double hexamers would lead to a redistribution of licensed origins towards these intergenic regions. We predict that a transcriptionally redistributed pool of MCMs, together with MCM loading at these sites as a result of pervasive transcription at gene 3’ ends, generates the TSS-independent pool of replication origins that we observed downstream of active genes (Figs.1b, 5c, and 6c,f, and Supplementary Fig. 7b,f). In support of this hypothesis, TTS-proximal origin activity was observed downstream of genes that were themselves actively transcribed (Figs. 5c and 6c).

Dormant replication origin firing. Under conditions of global origin activation or repression, a proportionally similar activation/repression of all origins would lead to the greatest absolute change in origin efficiency occurring at origins with intrinsically high firing efficiencies. We observed such behavior at TSS on HU treatment (activation) and on depletion of FANCI (repression) (Fig. 4a,b,d). This should globally maintain co-orientation of replication and transcription, especially for short genes and at the 5’ ends of long genes. In HU-treated cells, we also observed increased origin firing downstream of genes even without a proximal protein-coding TSS (Fig. 6f). TTS-proximal origins were active even in the absence of HU, but became more active on HU treatment (Figs. 5c and 6c). Average replication fork speed for RPE-1 cells in 0.2 mM HU is approximately 0.67 kb min⁻¹ as opposed to 1.37 kb min⁻¹ in untreated cells35. Decreased passive replication by oncoming replisomes (resulting from reduced replisome speed) would increase the firing efficiency of all licensed origins—particularly those far from origins that normally fire early and with high efficiency. We propose that at least a significant fraction of dormant origin activation during conditions of replication stress is a result of the increased firing efficiency of pre-existing origins.

Reorientation of replication-transcription conflicts during replication stress. Following treatment with low-dose HU, we observed a global reorientation of replication relative to gene 3’ ends, most prominently for long genes (Fig. 6g). Reorientation of replication is associated with increased origin firing downstream of TTS (Fig. 6c–f). This increased TTS-proximal origin firing could arise from decreased passive replication of these sites as a result of decreased replication fork speed. Another possibility, not mutually exclusive to decreased passive replication, is that origin firing downstream of genes is actively stimulated by global upregulation of origin firing. The time required for RNAP2 or the replisome to progress from the 5’ to the 3’ end of a gene is similar as a result of the matched speeds of these two complexes. Assuming that RNAP2 initiating on replicated DNA cannot co-directionally bypass a replisome, the suppression of downstream origin firing by passive replication would predict that all transcribing RNAP2 has time to clear the gene before downstream origin firing normally occurs. Thus, if origin firing downstream of genes is normally suppressed mostly by passive replication, decreased replication fork speed, or increased intragenic replisome stalling is improbable to lead to increased head-on replication-transcription conflicts. By contrast, the activation of downstream origin firing, independently of decreased passive replication, would drive replication into genes still occupied by RNAP2, increasing head-on conflicts and thereby DNA damage and Ataxia telangiectasia and Rad3 related (ATR) signaling, both of which are strongly orientation dependent41. HU is genotoxic, and long genes show transcription-dependent instability41. Furthermore, a recent preprint suggests that replication timing is stochastic rather than absolute in human cells43, so higher downstream origin firing...
efficiency would also result in the early firing of more downstream origins. We propose that such early firing of origins downstream of highly transcribed genes under replication stress increases head-on conflicts at their 3' ends, contributing to genome instability.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-018-0171-0.

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Author contributions
Y.-H. C., M.K., and P.T. performed the experiments, S.K. and D.J.S. analyzed the data and conceived and supervised the study. All of the authors interpreted the data. D.J.S. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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Methods

Cell culture/EdU labeling. We grew HTERT-immortalized RPE-1 cells (ATCC) in Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (DMEM/F-12) media (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 3% sodium bicarbonate and 1% pen-strep. The cell line was authenticated by short tandem repeat profiling and was tested for mycoplasma using the Roche MycoTOOL detection kit. In brief, exponentially growing cells of 50–60% confluency were plated in 150 mm dishes (approximately 15–20 plates per sample or replicate). Small interfering RNA transfections were done according to27. EdU labeling was done for 2 min (4 min for low-dose hydroxyurea) at 20°C with the most major modification being the use of gel-purified Okazaki fragments were purified and libraries generated essentially as described 7, with the most major modification being the use of gel-purified DNA lysis buffer (10 mM Tris, pH 8, 25 mM EDTA and 100 mM NaCl) with 0.5% (vol/vol) SDS and 0.1 mg ml–1 proteinase K overnight at 50°C. DNA was extracted with an equal volume of phenol-chloroform and precipitated by ammonium acetate and ethanol. After successive washes, DNA was isolated and resuspended in Trit-EDTA (TE) at 4°C. DNA was denatured for 10 min at 95°C, then chilled on ice for 10 min and size-fractionated on a neutral sucrose gradient with a Beckman Coulter Optima XL-100 with SW40T1 rotor at 28,000 xg at 20°C for 16 h, with maximum acceleration and minimum deceleration. The quality and reproducibility of size separation of genomic DNA can be assessed using an alkaline 1.5% agarose gel. Only fractions containing DNA fragments no longer than 200 bp were collected and concentrated. The EdU-labeled DNA was then biotinylated in standard Click-it reaction. The purified DNA was then subjected to RNA hydrolysis with 250 mM NaOH for 30 min at 37°C. DNA fragments were then phosphorylated with T4 polynucleotide kinase before ligation of adaptors using a two-step procedure (before and after streptavidin pulldown). The biotinylated DNA was captured on MyOne T1 Streptavidin dynabeads (Thermo Fisher Scientific) in 10 mM Tris (pH 7.5), 1 mM EDTA, 2 M NaCl, and 0.1% (vol/vol) Tween 20 buffer. PCR was used to survey the Okazaki fragments library and primer removal before analysis by TapeStation (Agilent). Libraries were sequenced using the HiSeq 2500 platform using one lane per sequencing reaction, and reads were aligned to the hg19 build of the human genome using bowtie2, excluding reads that did not map uniquely. Data were binned into 1-kb bins before analysis.

Data analysis. TSS and TTS locations were obtained from the UCSC genome browser http://genome.ucsc.edu. Genes were separated by quartile based on RNA-seq read density, volume (FPKM x length) or RNAP2 Chip-seq of the appropriate cell type. Genes with missing RNA-seq FPKM values were excluded from the analysis. Genes were further separated by length or DRIP-seq read density in HeLa cells, as described in the main text. Enhancer regions were separated by RNAP2 Chip-seq read density and sorted into bins based on distance from nearest TSS using a custom Python script. Random enhancer sites were generated by a Python script that matches the distance distribution of enhancers from nearest TSS while preserving random gene for each site. All plots and heat maps were produced using the packages Python matplotlib and seaborn.

Statistics and reproducibility. Two biological replicates were obtained for all datasets. Data from one replicate are presented in Figs. 1–6; data from the second replicate showed excellent agreement and are presented in the Supplementary Information.

Efficiencies and statistics were calculated for straw bias curves by taking the average difference between ranges downstream and upstream of the site being analyzed. These ranges were: 1–11 kb downstream and 50–30 kb upstream of annotated transcription start sites, ±1–10 kb around annotated transcription termination sites and ±20–40 kb around midpoints of enhancer regions. Efficiencies were calculated from the averaged data as shown in the figures.

When genes were binned by FPKM, n values were: Q1, Q2, and Q3 n = 4,541, Q4 n = 4,542

When genes with RNAP> median were binned by length, n values were: Q1 and Q4 n = 2,271, Q2 and Q3 n = 2,270

When genes were binned by transcriptional volume or RNAP2 ChIP-seq signal, n values were: Q1 and Q4 n = 4,563, Q2 and Q3 n = 4,562

For genes transcribed above median levels in RPE-1 and below median levels in GM06990, n = 324.

For genes transcribed below median levels in RPE-1 and below median levels in GM06990, n = 302.

For enhancers >50 kb from the nearest TSS, n = 10,959. Of these, 4,628 are transcribed above the median level for all enhancers (high RNAP2) while 6,331 are transcribed below the median level for all enhancers (low RNAP2). These enhancers were compared to 10,959 random sites located >50 kb from a TSS.

When genes transcribed above median levels in RPE-1 were binned by R-loop levels in HeLa, 6,078 showed high DRIP signal (high DRIP TTS) and 2,572 showed low DRIP signal (low-DRIP TTS).

P values between adjacent quartiles, calculated by Kruskal–Wallis from the first replicate in each case, are shown in figure panels and listed below. P values below 1 × 10–4 are shown to one significant figure.

Figure 2.

By FPKM: Q1–Q2 P = 7 × 10–4; Q2–Q3 P = 8 × 10–4; Q3–Q4 P = 5 × 10–4

By gene length: Q1–Q2 P = 1 × 10–4; Q2–Q3 P = 7 × 10–4; Q3–Q4 P = 1 × 10–3

By FPKM x gene length: Q1–Q2 P = 2 × 10–4; Q2–Q3 P = 5 × 10–4; Q3–Q4 P = 4 × 10–5

By RNAP2 ChIP-seq: Q1–Q2 P ≈ 7 × 10–2; Q2–Q3 P ≈ 3 × 10–2; Q3–Q4 le 5 × 10–14

RPE-1 Ok-seq, RPE-specific genes versus GM06990-specific genes: P = 2 × 10–14

GM06990 Ok-seq, RPE-specific genes versus GM06990-specific genes: P = 7 × 10–14

Figure 3.

High RNAP2 versus low RNAP2: P = 5 × 10–7

Low RNAP2 versus random sites; P = 2 × 10–5

Figure 4.

By FPKM x gene length:

Q1 no-hydroxyurea versus Q1 0.2 mM hydroxyurea; P = 0.27

Q2 no-hydroxyurea versus Q2 0.2 mM hydroxyurea; P = 0.55

Q3 no-hydroxyurea versus Q3 0.2 mM hydroxyurea; P = 0.05

Q4 no-hydroxyurea versus Q4 0.2 mM hydroxyurea; P = 1 × 10–6

Q1 FANCI RNAi 0.2 mM hydroxyurea versus Q1 0.2 mM hydroxyurea; P = 0.78

Q2 FANCI RNAi 0.2 mM versus Q2 0.2 mM hydroxyurea P = 0.02;

Q3 FANCI RNAi 0.2 mM versus Q3 0.2 mM hydroxyurea; P = 0.001

Q4 FANCI RNAi 0.2 mM versus Q4 0.2 mM hydroxyurea; P = 2 × 10–4

Q1 FANCD2 RNAi 0.2 mM hydroxyurea versus Q1 0.2 mM hydroxyurea; P = 0.003

Q2 FANCD2 RNAi 0.2 mM versus Q2 0.2 mM hydroxyurea; P = 0.04

Q3 FANCD2 RNAi 0.2 mM versus Q3 0.2 mM hydroxyurea; P = 0.001

Q4 FANCD2 RNAi 0.2 mM versus Q4 0.2 mM hydroxyurea; P = 0.23

Figure 5.

By FPKM: Q1–Q2 P = 0.01; Q2–Q3 P = 1 × 10–10; Q3–Q4 P = 7 × 10–8

High DRIP versus low DRIP; P = 0.85

Figure 6.

By FPKM:

Q1 no-hydroxyurea versus Q1 0.2 mM hydroxyurea; P = 2 × 10–5

Q2 no-hydroxyurea versus Q2 0.2 mM hydroxyurea; P = 1 × 10–11

Q3 no-hydroxyurea versus Q3 0.2 mM hydroxyurea; P = 1 × 10–7

Q4 no-hydroxyurea versus Q4 0.2 mM hydroxyurea; P = 3 × 10–5

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data, including raw sequencing reads and tables used to generate source data for graphs in Figs. 1–6, are publicly available under accession number GSE114017. Custom scripts are available upon request from the corresponding authors.
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**Data analysis**: Data were aligned to hg19 using bowtie2, and analyzed using in-house python and R scripts.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | N/A |
|-------------|-----|
| Data exclusions | No data were excluded |
| Replication | A biological replicate dataset was obtained for each condition |
| Randomization | N/A |
| Blinding | Data were not blinded during analysis |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|

- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

| n/a | Involved in the study |
|-----|-----------------------|

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

hTERT RPE-1 was purchased from ATCC

Authentication

The cell lines utilized in our laboratory will be authenticated using DNA fingerprinting. Short tandem repeat (STR) profiling establishes a DNA fingerprint for every human cell line and may be used as a record of the line (Reid et al., Authentication of Human Cell Lines by STR DNA Profiling Analysis. In: Assay Guidance Manual. Sittampalam GS, Coussens NP, Nelson H, et al., editors. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004). We will use STR profiling by multiplex PCR to simultaneously amplify at least eight of the most informative polymorphic markers in the human genome (plus, if necessary, the Amelogenin gene for gender determination). The pattern of repeats results in a unique STR identity profile for each cell line analyzed. The profile can be used as a baseline for comparison with future tests. For ATCC cells, STR is offered at relatively low cost thorough ATCC where experts amplify 17 STR loci (http://www.atcc.org/Services/Testing_Services/Cell_Authentication_Testing_Service.aspx).

Mycoplasma contamination

All cell lines tested negative for Mycoplasma contamination. We use: Frequent assays to detect mycoplasma. At 400X magnification, fluorescent Hoechst 33342 staining reveals mycoplasma infections through their characteristic patterns of particulate or filamentous fluorescence in the cytoplasm. Additionally, cell lines will also be screened for the presence of different mycoplasma strains each month, using the Roche MycoTOOL Detection Kit, which is a sensitive PCR-based method approved by the FDA.

Commonly misidentified lines

(See ICLAC register)

no commonly misidentified cell lines were used in this study