

RECQL5 Controls Transcript Elongation and Suppresses Genome Instability Associated with Transcription Stress

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SUMMARY

RECQL5 is the sole member of the RECQ family of helicases associated with RNA polymerase II (RNAPII). We now show that RECQL5 is a general elongation factor that is important for preserving genome stability during transcription. Depletion or overexpression of RECQL5 results in corresponding shifts in the genome-wide RNAPII density profile. Elongation is particularly affected, with RECQL5 depletion causing a striking increase in the average rate, concurrent with increased stalling, pausing, arrest, and/or backtracking (transcription stress). RECQL5 therefore controls the movement of RNAPII across genes. Loss of RECQL5 also results in the loss or gain of genomic regions, with the breakpoints of lost regions located in genes and common fragile sites. The chromosomal breakpoints overlap with areas of elevated transcription stress, suggesting that RECQL5 suppresses such stress and its detrimental effects, and thereby prevents genome instability in the transcribed region of genes.

INTRODUCTION

Over the last decade it has become increasingly evident that transcription is closely integrated with other DNA-related processes, such as chromatin dynamics, DNA replication, and repair. Indeed, the essential process of expressing genes comes at a cost: the movement of RNA polymerases through DNA is associated with genome instability (Aguilera and Gómez-González, 2008; Helmrich et al., 2013), and RNAPII stalling, pausing, arrest, and/or backtracking (hereafter collectively referred to as transcription stress) generates a cellular response akin to the DNA damage response (Wilson et al., 2013).

Transcribing polymerases are potent modulators of other DNA-related processes, such as DNA replication. For example, transcription-associated DNA recombination involves clashes between transcription and replication, and transcription is also associated with mutagenesis and contraction of CAG repeats, as well as breaks at chromosome fragile sites (Aguilera and Gómez-González, 2008; Helmrich et al., 2013). However, the mechanisms underlying transcription-associated genome instability remain largely obscure, and little is known about factors that might have evolved to counteract it.

The RECQ proteins constitute a family of conserved DNA helicases that are important for maintaining genome stability, from bacteria to humans (Chu and Hickson, 2009). The human genome encodes five RECQ family members: RECQL (RECQ1), BLM (RECQ2), WRN (RECQ3), RECQL4, and RECQL5. Mutations in three of these, namely *BLM*, *WRN*, and *RECQL4*, give rise to hereditary disorders associated with cancer predisposition and premature aging (Bloom's, Werner's, and Rothmund-Thomson's syndrome, respectively) (Chu and Hickson, 2009). Moreover, *Recql5* knockout mice exhibit elevated levels of sister chromatid exchange and are predisposed to various types of cancer (Hu et al., 2005; Hu et al., 2007), suggesting that RECQL5 is important for maintaining genome stability as well. Indeed, RECQL5 has been implicated in the prevention of replication fork collapse and the accumulation of DNA double-strand breaks (Hu et al., 2009; Popuri et al., 2012) and is also somehow involved in the suppression or removal of endogenous DNA damage as well as psoralen-induced interstrand crosslinks (Li et al., 2011; Tadokoro et al., 2012; Ramamoorthy et al., 2013). Concomitant mutation in *RECQL5* and another *RECQ* family member typically has a more severe effect than mutation in just one (see, for example, Hu et al., 2005; Popuri et al., 2013), suggesting that RECQL5 is active in pathways distinct from those governed by other RECQ members. However, despite RECQL5 being phenotypically implicated in the preservation of genome integrity at several levels, the mechanistic basis for its function remains poorly understood.

RECQL5 is unique among the RECQ family by interacting with RNAPII, and it harbors two RNAPII interaction domains, which

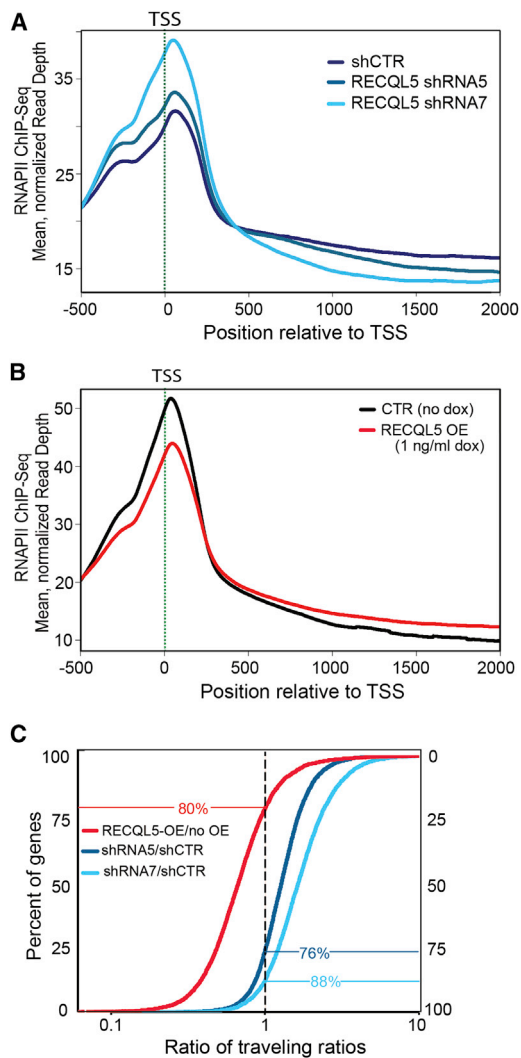


Figure 1. RECQL5 Depletion Changes the Profile of RNAPII across the Genome

(A) Effect of RECQL5 knockdown with two different shRNAs on the RNAPII ChIP-seq profile. TSS, transcription start site. CTR, control.
 (B) As in (A), but after overexpression (OE) of RECQL5 (see Figure S1D).
 (C) RNAPII ratio of traveling ratios for 5,140 genes after knockdown or overexpression of RECQL5, relative to that in the control (set to 1). y axes indicate percent of all genes. See also Figure S1 and Table S1.

are relevant for the phenotypes of different RECQL5-deficient metazoan cell types (Aygün et al., 2008; Izumikawa et al., 2008; Islam et al., 2010; Li et al., 2011). Intriguingly, transcription reactions reconstituted with pure general transcription factors and RNAPII suggest that RECQL5 might act as an inhibitor of transcription (Aygün et al., 2009). However, the physiological relevance, if any, of this surprising observation has not been investigated. Likewise, the mechanistic relationship between the effects of *RECQL5* mutation on genome integrity and RNAPII transcription has also remained unclear. Here, we report that RECQL5 has genome-wide effects on transcript elongation and that it suppresses genome rearrangements associated with

common fragile sites and transcription stress in the body of genes.

RESULTS

RECQL5 Affects RNAPII Progression Genome-wide

Previous experiments indicated that RECQL5 can inhibit transcription, and transcript elongation in particular, in reactions reconstituted with purified transcription proteins (Aygün et al., 2009). To investigate its role in vivo, we performed RNAPII ChIP-seq analysis after RECQL5 depletion. RECQL5 knockdown was achieved using two different lentivirus-driven shRNAs that substantially reduced protein levels, with higher knockdown efficiency reproducibly achieved using shRNA7 than shRNA5 (Figure S1A available online). Upon RECQL5 depletion, a striking genome-wide increase in RNAPII levels over transcription start sites (TSS) was observed, with the increase in peak size correlated with knockdown level (Figure 1A). However, RECQL5 depletion had little effect on total mRNA levels, and there was no correlation between the effect of RECQL5 depletion on promoter-proximal peaks and gene expression (Spearman correlation coefficient -0.05 ; see Figure S1B and Table S1 for expression data), indicating that RECQL5 does not significantly affect transcription initiation frequency.

Intriguingly, an inversion of the relative RNAPII levels across genes was observed approximately 500 bp downstream from the TSS, with less RNAPII being observed in transcribed regions upon RECQL5 knockdown. This effect was again reproducibly more severe with increasing knockdown efficiency, with the highest level of RECQL5 knockdown resulting in a $\sim 25\%$ decrease in RNAPII density in transcribed regions (Figure 1A).

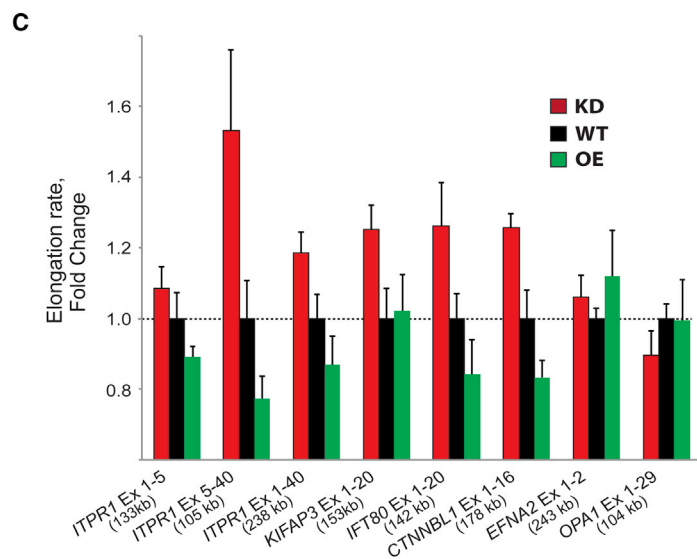
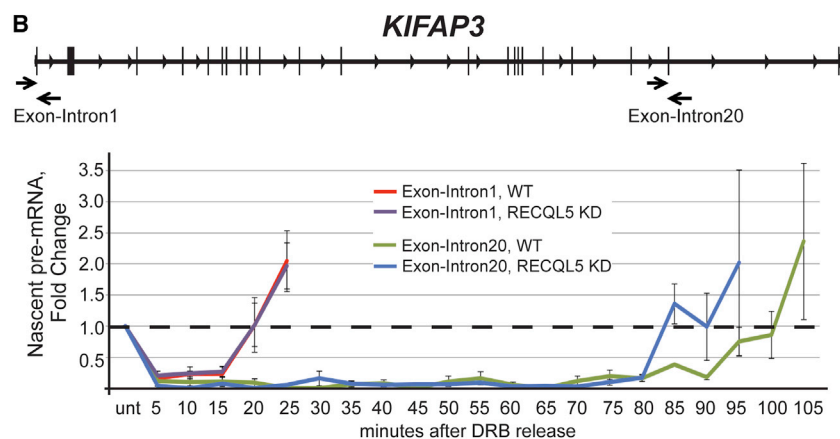
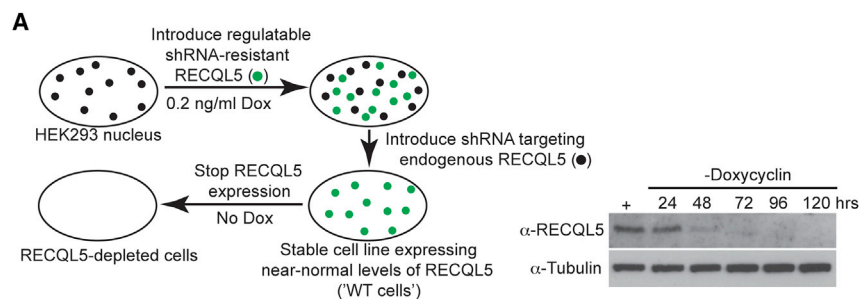
To further analyze quantitatively how RECQL5 affects transcription at the genomic level, the density of RNAPII in the promoter-proximal region relative to the gene body, previously termed the traveling ratio (Reppas et al., 2006), was calculated. In accordance with our observations, the traveling ratio was globally higher in cells lacking RECQL5 compared to control cells, again with a greater effect in cells with less RECQL5 protein (Figure S1C).

We note that RECQL5 chromatin immuno-precipitation (ChIP) and ChIP-seq analysis was also attempted, but that sites of RECQL5 occupancy could not be reproducibly detected, possibly because RECQL5 is lowly expressed and represents a “moving target” without appreciable DNA sequence preference.

Overexpression of RECQL5 Has the Opposite Effect of Its Depletion

We repeated the RNAPII ChIP-seq experiments, but this time after overexpressing RECQL5 (see Figure S1D). Gratifyingly, the effect of RECQL5 overexpression on RNAPII distribution was the opposite of that observed upon knockdown: it reduced RNAPII density over the promoter and TSS and resulted in increased levels across the transcribed regions (Figure 1B). In further support of a genome-wide effect of RECQL5 overexpression, the traveling ratio moved toward lower values under these conditions (Figure S1E).

The traveling ratios given above were for all genes, whether they presented a clear promoter-proximal RNAPII peak or not.



Given that genes with a promoter-proximal peak are more likely to be active, we next focused on 5,140 genes with clear promoter-proximal peaks, and then asked how RECQL5 affects the traveling ratio in this group of genes. Eighty percent (4,092 of the 5,140 genes) had a decreased traveling ratio upon RECQL5 overexpression. Conversely, 76% and 88% had an increased traveling ratio upon RECQL5 knockdown with shRNA5 and shRNA7, respectively (Figure 1C). Importantly, there was a substantial overlap between the genes that responded to either loss or increase of RECQL5 expression:

of Spt5 and Serine 2 of RNAPII's C-terminal domain (CTD), resulting in a failure of newly initiated RNAPII to progress to the elongation phase while permitting mature elongation complexes to complete transcription. DRB thus reversibly blocks new transcript elongation and in effect synchronizes the transcription cycle. Polymerases can then be concomitantly released by inhibitor removal, so that the time-resolved arrival of RNAPII at different intron-exon junctions can be assessed. Figure 2B shows how transcription through the *KIFAP3* gene proceeded after DRB removal. As expected, transcription over the first,

Figure 2. RECQL5 Inhibits Transcript Elongation at a Number of Long Human Genes

(A) A system for regulating RECQL5 expression in cells lacking the endogenous protein.

(B) Nascent mRNA production in different regions of the *KIFAP3* gene after release from DRB-inhibition.

(C) As in (B), but in different areas of different long genes, after either reducing (KD, red), or increasing (OE, green) the cellular level of RECQL5. Average values of a minimum of three independent experiments, with standard errors, are plotted.

among the 4,092 genes whose traveling ratio decreased upon RECQL5 overexpression, 3,641 (89%) were affected in the opposite direction by RECQL5 knockdown with shRNA7 (p value for overlap: 2.08×10^{-9}). Thus, the same set of genes responded to a change in RECQL5 levels.

RECQL5 Decreases the Elongation Rate of RNAPII

We next tested whether RECQL5 might play a role in RNAPII transcript elongation in vivo. In order to first establish an effective and specific system for regulating RECQL5 levels, we created a human cell line in which an shRNA-resistant version of RECQL5 was expressed under the control of a doxycycline-inducible promoter. This allowed stable shRNA knockdown of the endogenous version of RECQL5. In this "RECQL5-shutoff" cell line, RECQL5 is expressed at near-normal levels, but doxycycline removal results in its efficient depletion (Figure 2A). Because the levels of RECQL5 are controlled only by doxycycline, this system effectively controls for off-target effects of shRNA treatment. This cell line was used in all subsequent in vivo experiments.

Singh and Padgett (2009) previously used 5,6-dichlorobenzimidazole 1-beta-D ribofuranoside (DRB) to measure RNAPII transcript elongation rates in vivo. DRB inhibits P-TEFb-dependent phosphorylation

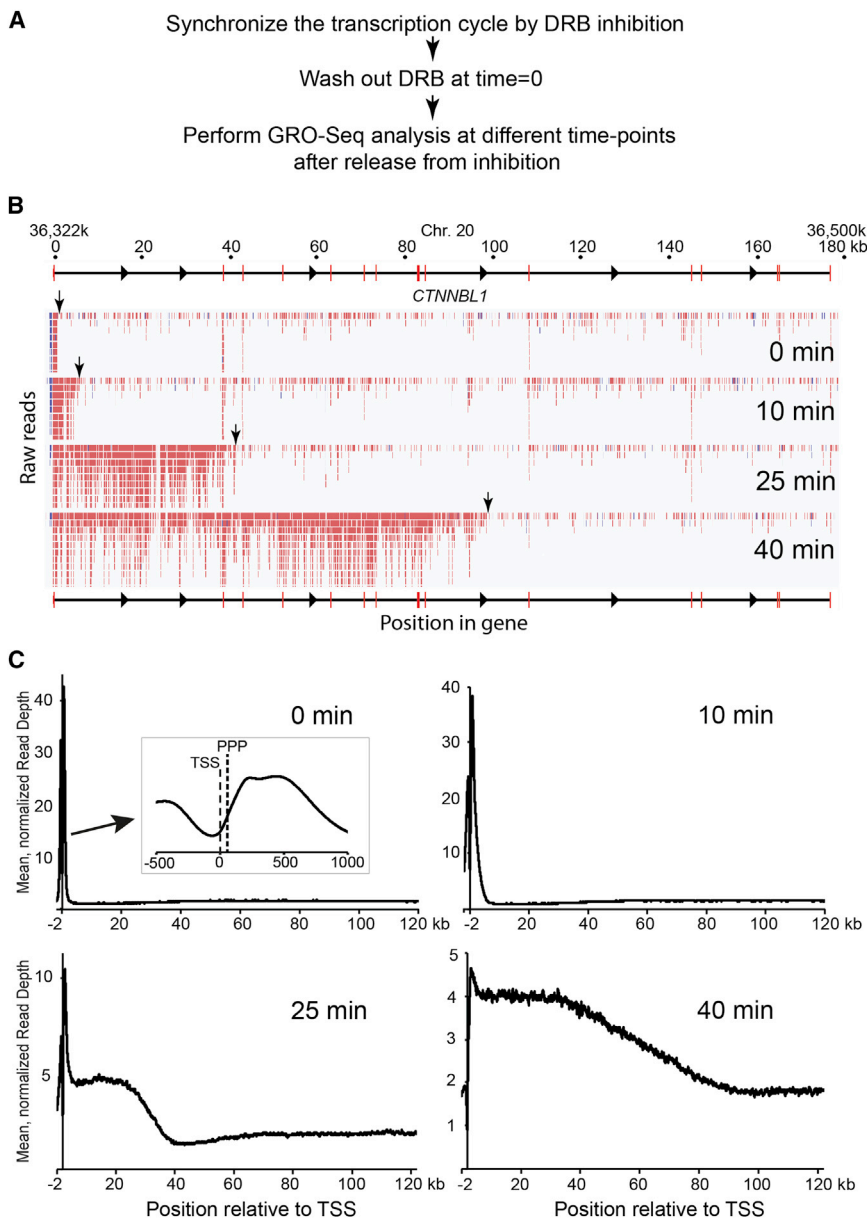


Figure 3. DRB/GRO-Seq to Measure RNAPII Progress across Genes, Genome-wide

(A) Schematic of DRB/GRO-seq.

(B) GRO-seq reads of nascent RNA produced at the *CTNNBL1* gene at different times after release from DRB-inhibition of transcript elongation.

(C) Profile of normalized GRO-seq reads across 8,529 genes in the human genome over time. The insert represents an enlargement of the area close to the TSS. PPP, promoter-proximal RNAPII peak, observed by ChIP-seq.

site effect: it generally reduced elongation rates (compare green and black bars). The decreases were consistently more severe at genes in which greater increases were observed upon RECQL5 depletion (e.g., *ITPRex5-40* and *IFT80*), suggesting that elongation in these regions is particularly sensitive to RECQL5 levels. Conversely, regions in which little or no change in elongation rates was observed upon RECQL5 depletion were also largely unaffected by overexpression (e.g., *EFNA2* and *OPA1*). These results are in line with those obtained by measuring traveling ratios: the same sets of genes are affected by RECQL5 depletion and overexpression. The results are also in agreement with the RNAPII ChIP-seq results above, as faster transcript elongation will result in lower RNAPII density in the transcribed region of genes (Ehrensberger et al., 2013).

DRB/GRO-Seq Measures RNAPII Elongation Rates across Genes, Genome-wide

We next used DRB in combination with GRO-seq analysis (Core et al., 2008), hereafter called DRB/GRO-seq, as a new method to analyze RNAPII elongation

rates genome-wide. In DRB/GRO-seq, the position of RNAPII in the body of genes is analyzed by extending nascent RNA with BrUTP in run-on experiments performed at different times after DRB removal (Figure 3A). BrU-labeled RNA is then purified and subjected to deep sequencing. Transcription at the *CTNNBL1* gene shows the potential of the technique (Figure 3B). Prior to the release from DRB inhibition (time = 0), the vast majority of reads were observed over a narrow area near the promoter. However, as time progressed, deep sequencing reads were observed further and further into the gene (the transcription "wave front" is indicated by vertical arrows). Likewise, when assessing the data as averages across appropriately long genes (8,529 genes, >30 kb), RNAPII activity was observed at the beginning of genes at time = 0, 200–500 nucleotides

promoter-proximal exon-intron junction was indistinguishable between the two cell lines (red and purple graphs). In contrast, pre-mRNA levels at an exon-intron junction 153 kilobases (kb) downstream from the TSS rose markedly earlier in the cell line depleted for RECQL5 (10–15 min; compare blue and green graphs). This corresponds to an overall mean elongation rate across the *KIFAP3* gene of 2.34 kb/min in the knockdown compared to 1.87 kb/min in the WT (i.e., a 25% rate increase in the absence of RECQL5).

We similarly compared elongation rates over seven regions in long human genes, and observed an increased transcript elongation rate at most, but not all of them, with an increase of ~20% on average (Figure 2C, compare red and black bars). Importantly, overexpression of RECQL5 gave rise to the oppo-

site effect: it generally reduced elongation rates (compare green and black bars). The decreases were consistently more severe at genes in which greater increases were observed upon RECQL5 depletion (e.g., *ITPRex5-40* and *IFT80*), suggesting that elongation in these regions is particularly sensitive to RECQL5 levels. Conversely, regions in which little or no change in elongation rates was observed upon RECQL5 depletion were also largely unaffected by overexpression (e.g., *EFNA2* and *OPA1*). These results are in line with those obtained by measuring traveling ratios: the same sets of genes are affected by RECQL5 depletion and overexpression. The results are also in agreement with the RNAPII ChIP-seq results above, as faster transcript elongation will result in lower RNAPII density in the transcribed region of genes (Ehrensberger et al., 2013).

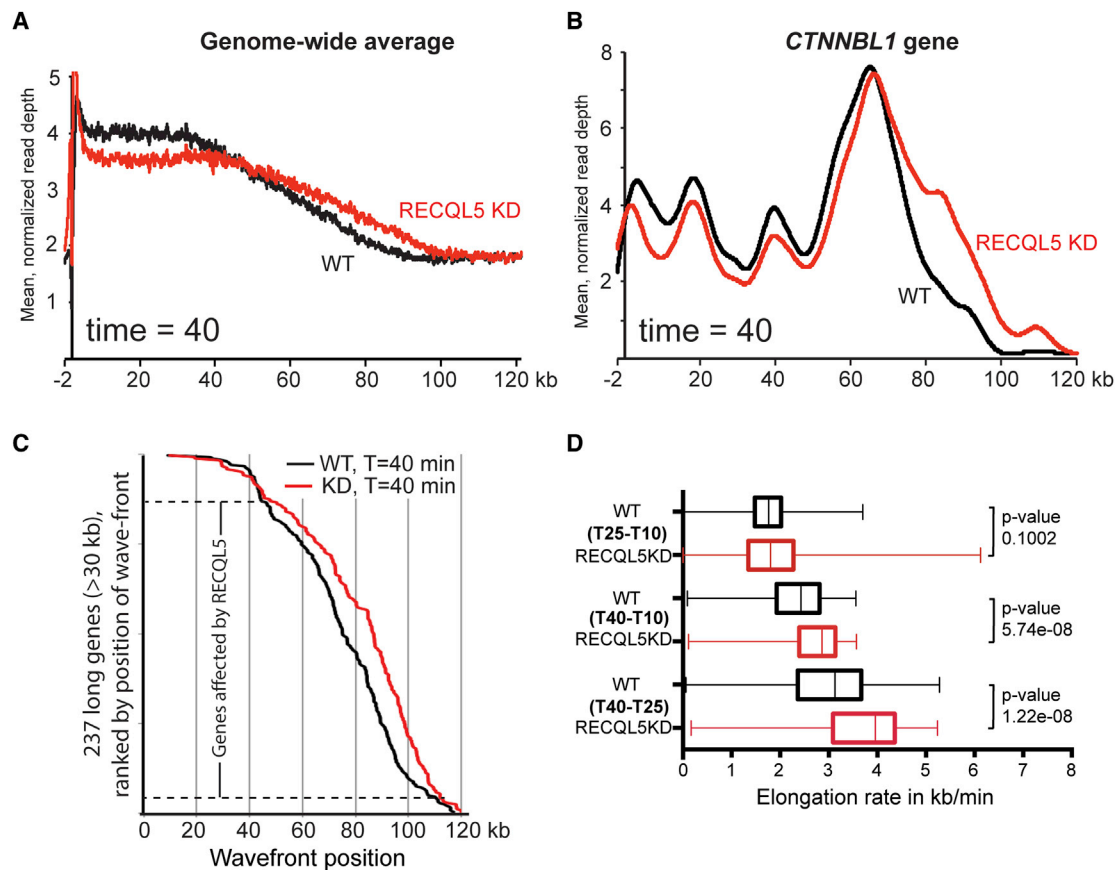


Figure 4. RECQL5 Loss Increases Transcript Elongation Rates across the Genome

(A) Profile of normalized GRO-seq reads across the human genome 40 min after release from DRB-inhibition.

(B) Normalized and smoothed spline profile across the *CTNNB1* gene. The read-depth differs across the gene, giving rise to apparently uneven activity distribution.

(C) Positions of RNAPII wave fronts calculated over appropriately long genes 40 min after DRB removal. p value for differences between data sets $<10^{-4}$.

(D) Transcription elongation rates in the indicated time intervals calculated from the position of RNAPII activity wave fronts. All genes included had to be long enough for transcript elongation to not have reached the TTS at the time of measurement, so fewer genes could be tested at later time points (see [Extended Experimental Procedures](#)). Box-plot representation shows median values \pm 25% quartiles in the box and minimum/maximum distribution of the values in the whiskers. See also [Figure S2](#).

downstream from the TSS, but distinct from the promoter-proximal peak (PPP) observed by RNAPII ChIP-seq ([Figure 3C](#), time = 0, see insert). It only moved into the body of genes upon drug removal, in line with the idea that DRB inhibits phosphorylation events required for the full function of the RNAPII elongation complex. The RNAPII wave front progressed 30–40 kb into genes after 25 min (min), while it reached 80–90 kb on average 40 min after DRB removal.

RECQL5 Depletion Increases Transcript Elongation Rates, Genome-wide

Having established that DRB/GRO-seq can be used to characterize transcript elongation in vivo, we now compared wild-type cells with cells lacking RECQL5 ([Figure 4](#)). Significant differences were difficult to detect at the early time points. After 40 min, however, the RNAPII-activity wave-shape was clearly altered in the RECQL5 knockdown cells, with relative depletion of polymerases in the area up to around 40 kb and with a corre-

sponding density increase in the region from 40 kb to the transcription wave front at \sim 100 kb ([Figure 4A](#)), indicating that RNAPII generally transcribed further into genes in the absence of RECQL5. [Figure 4B](#) shows a specific example in which more nascent RNAPII transcript reads were detected 80–120 kb into the *CTNNB1* gene in the absence of RECQL5 than in normal cells (more examples in [Figure S2A](#)).

We also determined the positions of the RNAPII wave fronts at 10, 25, and 40 min in appropriately long genes ([Figures 4C](#) and [S2B](#), and data not shown). These were selected only on size and other genomic parameters, and not on differential behavior in the two cell types, but RECQL5 affected transcript elongation rates at the majority of them. Indeed, with elongation rates varying dramatically from gene to gene, only those at which transcript elongation was very slow, or extremely fast, were unaffected by RECQL5 depletion ([Figure 4C](#)). Moreover, using the wave front positions, the median elongation rates could be calculated ([Figure 4D](#)). In keeping with recent results ([Danko](#)

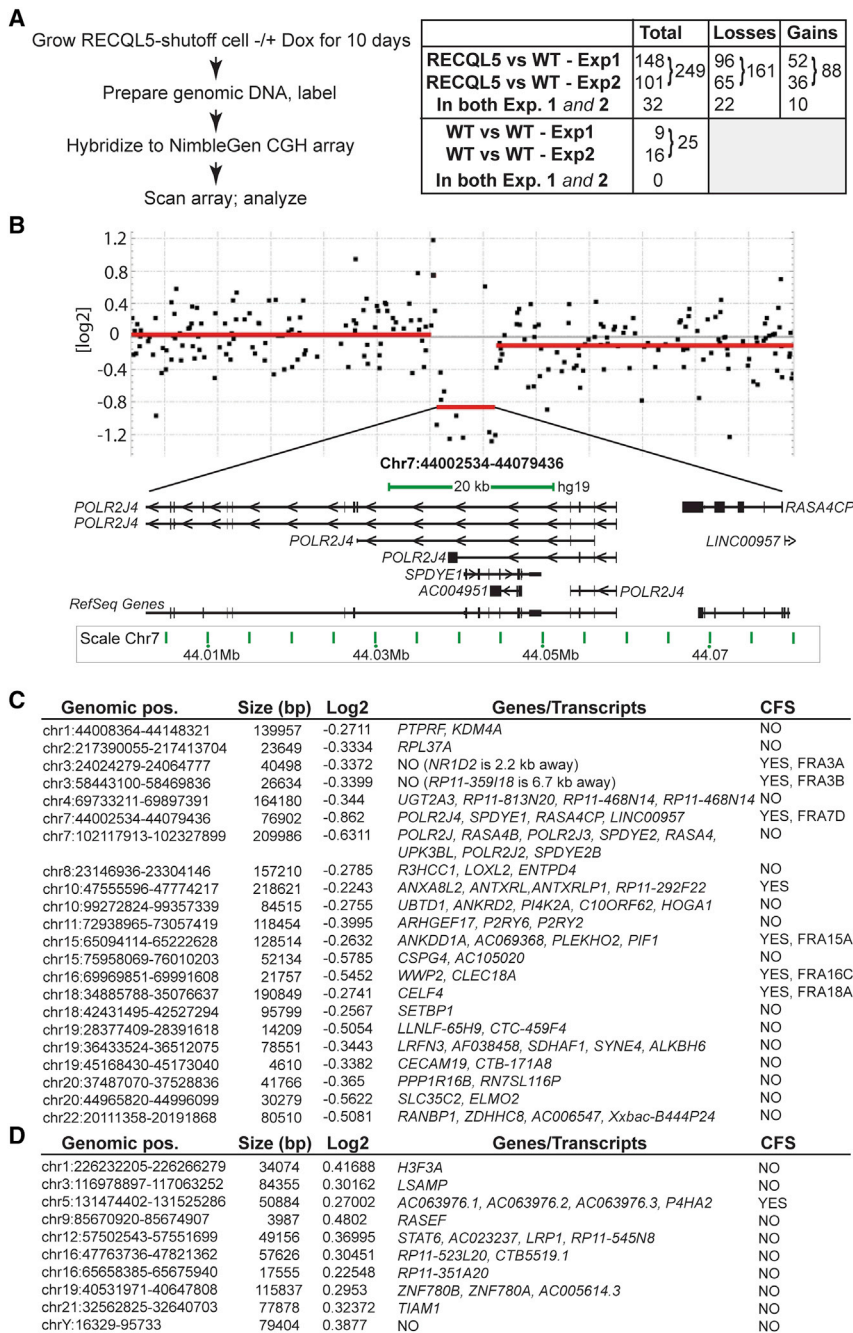


Figure 5. RECQL5 Depletion Results in Chromosomal Instability

(A) Left: outline of CGH approach. Right: gains or losses of genomic regions upon RECQL5 depletion and in WT cells.

(B) Example of gene-rich region on chromosome 7p13, lost upon RECQL5 depletion. Each black dot represents a different probe on the microarray and the red lines their mean. Expanded area depicts only some of the annotated transcripts in the lost region.

(C) Recurrent lost regions and their correlation with transcription, as well as CFSs.

(D) As in (C), but for regions of gain. See also Tables S2, S3, and S4.

among the randomly selected genes in which elongation rates could be confidently measured, 77.7% had increased elongation rates in the mutant in the 25–40 min interval (and 78.4% in the 10–40 min interval). Together, these experiments indicate that RECQL5 moderates transcript elongation rates genome-wide.

Chromosomal Rearrangements in Cells Lacking RECQL5

Previous results implicated RECQL5 in maintaining genome stability, but whether this role is connected to the effects on transcript elongation established here was unclear. We used comparative genomic hybridization (CGH) to investigate whether chromosomal rearrangements occur upon RECQL5 depletion, and, if so, whether such rearrangements were associated with transcription. CGH compares two genomic DNA samples to detect differences such as gains or losses of whole chromosomes or, more likely, chromosomal regions (Park, 2008). A batch of RECQL5-shutoff cells grown in the presence of doxycycline was split in two and grown either in the continued presence of doxycycline (to maintain RECQL5 expression [“wild-type”]), or without it to deplete RECQL5. Genomic DNA from the two cell populations was compared (Fig-

ure 5A, left; and 5B). Because these cells were identical at the outset, any change in copy number will have occurred during this short period of growth (10 days, typically 10–14 cell divisions). Strikingly, no less than 249 genomic losses and gains occurred in response to RECQL5 depletion in the two independent comparisons, using a log₂ ratio threshold of 0.2 (Figure 5A, right). This threshold signifies that more than ~15% of cells in the RECQL5-depleted cell population carry the particular rearrangement and therefore provides a conservative measure of the total number of genomic rearrangements taking place. The large

et al., 2013), transcript elongation rates close to the promoter (i.e., at the early time points) were markedly lower than during elongation further into the gene (25–40 min comparison at bottom). This also helps explain the difference between elongation rates obtained by qPCR (Figure 2C) and DRB/GRO-seq. More importantly, elongation rates were significantly higher in cells lacking RECQL5: the median elongation rate in the 25–40 min interval was 3.96 kb/min in RECQL5-depleted cells and 3.13 kb/min in wild-type cells, a rate increase of 27% in the absence of RECQL5 (p value 1.22×10^{-8}) (Figure 4D). Moreover,

number of genomic alterations (161 losses and 88 gains; see also [Table S2](#)) inspired us to check the basal level of genome instability in the wild-type cell line itself. This is relevant because in CGH, one of the samples (the wild-type) is defined as unchanging, and genomic rearrangements in the test sample are then described relative to this reference. Again, the cell culture was split in two, but with RECQL5 expression maintained in both, before the genomes of the clones were compared. In this CGH comparison, gains and losses cannot be distinguished, as the reference sample is arbitrarily set (e.g., a loss detected in the “test” sample might equally well be a gain in the “reference” sample). A total of 25 genomic alterations were detected in two independent experiments (16 and 9, respectively), 10-fold less than observed when RECQL5 was depleted ([Figure 5A](#), right; [Table S3](#)).

Interestingly, although the size of the rearrangements observed upon RECQL5 depletion was relatively small (median was 45 kb for losses, and 37 kb for chromosomal gains), we identified no less than 22 examples of loss (out of 65 and 96, respectively) and 10 gains (out of 36/52) that occurred in both independent experiments ([Figures 5A](#), right, [5C](#), and [5D](#)). So, around a third of the genomic rearrangements in one experiment were also detected in the other. It is obvious that the likelihood of such repetition happening by chance is miniscule. Indeed, the *p* value can be estimated by a permutation test and is $<3 \times 10^{-4}$. In contrast, none of the rearrangements detected when comparing WT with WT were recurring, and none of these unique events overlapped with the recurring RECQL5-related events.

We surmised that the recurring genomic rearrangements (22 examples of loss, 10 examples of gains; [Figures 5A](#), right; see also [Figures 5C](#) and [5D](#)) could be key to understanding RECQL5 function, so we initially focused on these high-confidence events. When exploring common features of the regions affected, we noted that almost a third (7 of 22; *p* value 0.033) of the loss regions reside inside one of 124 replication-dependent, common fragile sites (CFSs) mapped in human autosomes ([Fungtammasan et al., 2012](#)). Replication programs are cell-type-specific ([Letessier et al., 2011](#)), and CFSs have not been extensively mapped in HEK293 cells. The overlap of RECQL5-dependent genomic losses was thus with CFSs mapped in other cell types (typically human lymphocytes), making it all the more noteworthy. No statistically significant overlap was observed between CFSs and RECQL5-related gains (*p* value 0.319) or the events observed in wild-type cells (*p* value 0.217).

We also checked whether RECQL5-related genomic instability was associated with transcription. [Figure 5B](#) shows an example. This region (Chr7p13) is indeed gene-rich and positioned inside CFS FRA7D. Regions of recurring genomic loss generally contained genes or annotated transcripts: 20 of the 22 recurring losses encoded at least one RNAPII transcript, and the remaining two were within a few kilobases of a gene ([Figure 5C](#); [Table S2](#)). Moreover, genomic losses not only overlapped with transcription, but either one or both of their breakpoints were within an annotated transcript in 82% of cases (*p* value 4.87×10^{-5}). No statistically significant overlap with genes was observed with the breakpoints of recurring genomic gains (*p* value 0.9997) (see also [Figure 5D](#)), or with rearrangements in wild-type cells (*p* value 0.621).

We now analyzed the entire set of RECQL5-dependent genomic alterations (not only recurring events) to establish whether the associations were generally observed. Gratifyingly, the 161 RECQL5-related genomic losses were generally associated with CFSs and genes: 36 events (22%) overlapped with CFSs (*p*(permutation) 0.048), and 87% overlapped with genes (*p*(permutation) $< 10^{-4}$). In contrast, no statistically significant association was observed with RECQL5-dependent genomic gains, or with rearrangements observed in wild-type cells (*p*(permutation) 0.420 and 0.217, respectively, for CFSs; and *p* value 0.902 and 0.171, respectively, for genes). Again, either one or both of the breakpoints of the genomic losses were typically within the transcribed region of an annotated gene or transcript (in 80% of cases; *p* value 3.21×10^{-11}), while no significant general association was observed for genomic gains, or for the wild-type (*p* values of 0.902 and 0.621, respectively). We failed to detect overlap between RECQL5-dependent genome instability and so-called early replicating fragile sites (ERFSs) (*p* values 0.162 and 0.585, respectively, for losses and gains), with the caveat that these ERFSs were characterized in the mouse ([Barlow et al., 2013](#)), so that our comparisons could only be made in a limited number of syntenic regions.

Together, these data show that RECQL5 depletion gives rise to genome instability associated with common fragile sites and the transcribed region of genes.

Characteristics of Genes Containing Sites of RECQL5-Dependent Genome Instability

We now investigated the possible relationships between transcript elongation and genome instability, focusing primarily on genomic loss-associated chromosomal breaks as these were correlated with genes and CFSs. All in all, 164 well-defined genes or long noncoding transcripts with a total of 212 genomic loss-associated breakpoints in their transcribed region were suitable for such examination.

Bioinformatic analysis showed that genes with genomic loss-associated chromosomal breaks in their coding regions were inconspicuous with regard to their gene expression level (i.e., they were neither particularly highly nor lowly expressed genes), and their change in expression upon RECQL5 depletion (i.e., they did not represent a sub-group whose expression was particularly RECQL5-dependent). Moreover, the breakages observed were at varying distances from the TSS and TTS, and not, for example, enriched at either end (see [Table S4](#) for information on individual break sites in genes).

We noted that there was a tendency for genes with loss-associated chromosomal breaks in their coding regions to be long, with the mean length of such genes being 123 kb, compared to 58 kb for all human genes (median length was 74 kb, compared to 23 kb for all human genes). As an aside, although the RECQL5-dependent genomic gains were not associated with genes to a statistically significant degree (see section on CGH above), either one or both gain-associated chromosomal breakpoints were nevertheless inside the transcribed region of a gene in no less than 80% of cases. These genes were typically very long, with a median length of 105 kb and a mean length of 254 kb; indeed, 23% (15 of 64) were more than 300 kb long (against $\sim 3.4\%$ in the entire human genome). However, all else

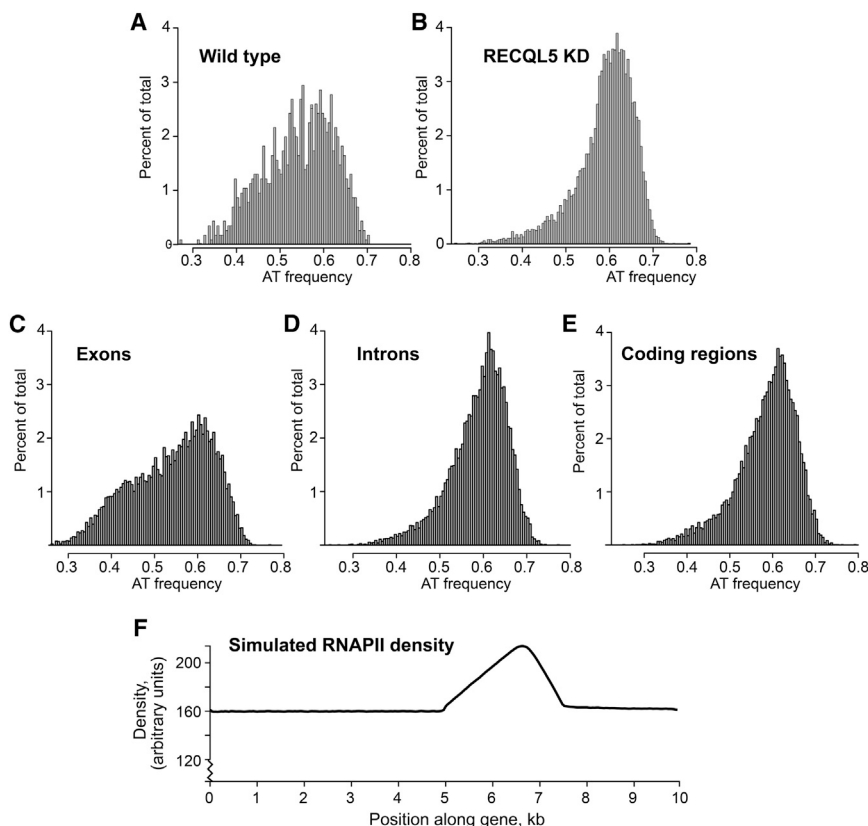


Figure 6. RECQL5 Depletion Results in Transcription Stress across the Transcribed Region

(A–E) Mean AT frequency distribution of ± 1 kb regions around RNAPII peaks in (A) wild-type, (B) RECQL5 knockdown cells (shRNA7), (C) exons, (D) introns, and (E) transcribed regions overall. See also Figure S3.

(F) CHIPMOD simulation of stochastic RNAPII pausing by 1% of the polymerase population. Parameters used, other than default: 9.99 kb gene; pause start at 5 kb; width 2.5 kb; 40% of normal (3.8 kb/min) elongation rate. Mean width of actual RNAPII ChIP-seq peaks is 2.7 kb; see Figure S4 for examples.

with those in the transcribed region of genes indicating areas of increased pausing or arrest (i.e., transcription stress) (Ehrensberger et al., 2013). Somewhat unexpectedly, individual RNAPII peak-calling using MACS (Zhang et al., 2008) uncovered a marked increase in the number of individual peaks with increasing RECQL5 knockdown (2.4- and 3.2-fold more after shRNA5 and shRNA7 knockdown, respectively). Given that RECQL5 depletion results in higher promoter-proximal peaks (Figure 1), a trivial explanation for these increases

being equal, long genes would also be more likely to be hit by a damage-event occurring at random, and although the enrichment of long genes might well be biologically meaningful, it was not statistically significant.

Most importantly, however, we were unable to find a connection between elongation speed and chromosomal breakage. In both loss- and gain-associated breakage genes where it could be estimated or calculated via DRB/GRO-seq, the elongation rate was thus close to the median rate established for all measurable genes in Figure 4 (i.e., their rate did not change in a way that distinguished them in the background of changes that occurred when RECQL5 is lost). In agreement with this, the traveling ratios of genes with chromosomal breaks were also unremarkable compared to control genes without breaks. Together, these data show that although genes with RECQL5-dependent chromosomal breaks in their coding region may be long, they do not stand out as unusual by a number of other parameters compared to the much larger group of control genes that did not display genome instability.

RECQL5-Related Transcription Stress

The absence of a clear connection between genome instability and elongation rates prompted us to investigate the role of RECQL5 in transcript elongation in more detail. Besides providing an overview of polymerase density across genes genome-wide, RNAPII ChIP-seq also uncovers local areas of increased density (peaks) within individual genes. RNAPII peaks are typically broad, often comprising several kilobases of DNA,

could be that more promoters start to display detectable promoter-proximal peaks upon RECQL5 loss. We therefore examined the 5,140 genes that already had a promoter-proximal RNAPII peak. Interestingly, instead of calling a decreased number of RNAPII ChIP-seq peaks in these genes as might have been expected from the metagene-analysis (Figures 1A and 1C), MACS called a markedly increased number of individual gene body peaks upon RECQL5-depletion (4.0- and 5.2-fold more after shRNA5 and shRNA7 knockdown, respectively; p values both $<10^{-4}$). These data suggest that although RNAPII on average traverses genes more rapidly in the absence of RECQL5, this is accompanied by incidents during which polymerase is paused or arrested in the transcribed region of genes.

To gain further insight into the nature of the intragenic RNAPII peaks, we aligned the underlying sequences in the hope of uncovering similarities. Neither sequence consensus elements, nor strongly over- or under-represented oligonucleotides given the background frequencies were uncovered (Figure S3A; data not shown). However, regions affected by RECQL5 depletion had a distinct mononucleotide frequency distribution, with the peaks in RECQL5-depleted cells generally being more AT-rich than those in wild-type cells (Figure 6, compare A and B). In general, exons are significantly more GC-rich than introns and also—because introns are typically 10-fold longer than exons—than transcribed regions in general (Louie et al., 2003) (Figures 6C–6E). In apparent agreement with the finding that RNAPII tends to pause at intron-exon junctions (Kwak et al., 2013), the sequence-signature of RNAPII-peaks in wild-type cells looked

more similar to that of exons than introns (Figure 6, compare A with C and D). In contrast, RNAPII peaks in RECQL5-depleted cells had an AT-signature more similar to that of introns or transcribed regions in general (Figure 6, compare B with C, D and E), suggesting that the increased transcription stress observed upon loss of RECQL5 is distributed across transcribed regions.

It is worth noting that genomic areas containing an RNAPII peak in the wild-type cells also typically harbored increased RNAPII density after RECQL5 depletion. The opposite was less obvious, but still noticeable (Figure S3B). The much higher frequency of detectable RNAPII pausing and arrest in RECQL5-depleted cell therefore does not imply that transcription stress *only* occurs in RECQL5-depleted cells, nor that RNAPII *only* pauses at intron-exon junctions in wild-type cells. Rather, it suggests that while the same causes underlie pausing and arrest in RECQL5-depleted cells, wild-type cells can better buffer impediments to elongation.

It may appear counter-intuitive that RECQL5 depletion results in both elevated transcription stress *and* faster transcript elongation. However, computer modeling by an updated version of CHIPMOD (Ehrensberger et al., 2013), which can now also simulate stochastic and rare polymerase behavior, supports the idea that transcription stress can be detected even if such events are very rare. Indeed, even if merely 1% percent of transcribing polymerases experience modest pausing through, or transcriptional arrest in, a gene region, this has a striking effect on the predicted RNAPII density profile (Figure 6F, data not shown).

Together, these results suggest that RECQL5-depleted cells suffer from substantial transcription stress, so that although elongation rates are higher, this is accompanied by a more interrupted transcript elongation process: events during which elongating RNAPIIs stochastically pause or arrest.

Evidence that RECQL5-Related Transcription Stress Causes Genome Instability

We finally investigated whether RECQL5-dependent genome instability might be connected to transcription stress. In an ideal, imaginary experiment, transcription stress would be measured at positions of chromosomal gene breakage, but immediately *prior* to these events taking place in the individual cells that react to RECQL5 depletion. This is obviously not practically feasible. We were instead restricted to comparing the position of RNAPII transcription stress events detected by ChIP-seq with the position of chromosomal breakages detected by CGH (Examples of such events are shown in Figure S4). As a control, computer-generated chromosome breaks were made, at random, across the genome (100 trials) in a quantity that yielded random breaks in genes in numbers that were similar, on average, to those observed in the actual experiments (see [Extended Experimental Procedures](#) for details).

We initially focused on genomic breaks in the transcribed region of the 5,140 genes containing a clear promoter-proximal peak. Moreover, only RNAPII peaks that were in the transcribed region of the same gene as the loss-associated chromosomal break site were considered. This left 26 individual loss-associated chromosome breaks for the analysis. Remarkably, when compared to the computer-generated, random breakpoints in genes, the actual breakpoints were clearly positioned very close

to the RNAPII peaks detected by ChIP-seq (Figure 7A), indicating a connection between transcription stress and genome instability in RECQL5-depleted cells.

We note that regions of transcription stress are not ever-present. Indeed, even upon RECQL5 loss, less than half of the annotated genes in the human genome had one or more RNAPII peaks called in their transcribed region. This was true whether they had breakpoints or not, and similar observations were made for genomic loss- and gain-associated breaks. When corrected for gene length (relevant since long genes would be expected to have more RNAPII peaks), genes with breakpoints actually had somewhat fewer RNAPII peaks than control genes (~2-fold fewer), indicating that breakpoint genes were not general hotbeds of transcription stress.

We also expanded the analysis to all chromosomal loss-associated break sites and the accompanying nearest area of RNAPII transcription stress, irrespective of origin. This is relevant because RNAPII transcription is pervasive and also produces noncoding RNA, for example in enhancers and downstream from the coding region of genes. We here compiled a list of the distances between genomic loss-associated chromosomal breaks and their nearest RNAPII peak, in order of increasing distance (observed distances). A similar list was compiled for the random computer-generated chromosomal breaks (expected distances). These two distance lists were then plotted together in a scatter plot, from the shortest to the longest distance-pair (Figure 7B). In this analysis, if the distance between observed chromosomal loss-associated break sites to an RNAPII peak were no closer than would be expected by chance, the distance-pairs would lie on the diagonal line of the plot. Instead, the points were consistently below the diagonal, showing that RNAPII peaks were closer to the observed chromosomal loss-associated break sites than would be predicted by chance (Figure 7B). Intriguingly, the subgroup of genomic *gain*-associated chromosomal breakpoints that occurred inside genes were also closer to RNAPII ChIP-seq peaks than would have been expected (Figure S5), suggesting that RECQL5-dependent genome instability in genes is generally associated with areas of increased RNAPII transcription stress.

We also noted that not only the RNAPII peaks detected upon RECQL5 knockdown (Figure 7B, blue and green spheres), but also those observed in wild-type cells (red spheres) were closer to the chromosomal breakpoints than would have been expected by chance. Somewhat surprisingly, it actually appeared that the latter, much less abundant RNAPII peaks were even closer to sites of chromosomal breakage than those arising in RECQL5-depleted cells. Remarkably, however, these wild-type peaks simply represented highly consistent sites of RNAPII transcription stress, also observed after RECQL5 loss: out of the 51 RNAPII peaks plotted for wild-type cells, 49 were thus also called as peaks and plotted in either one or both the RECQL5 shRNA knockdown samples. This result is important as it implies that sites of transcription stress in wild-type cells may also give rise to genome instability. In the absence of RECQL5, however, there is both a great increase in transcription stress, and an inability to suppress the deleterious consequence for genome stability of such stress.

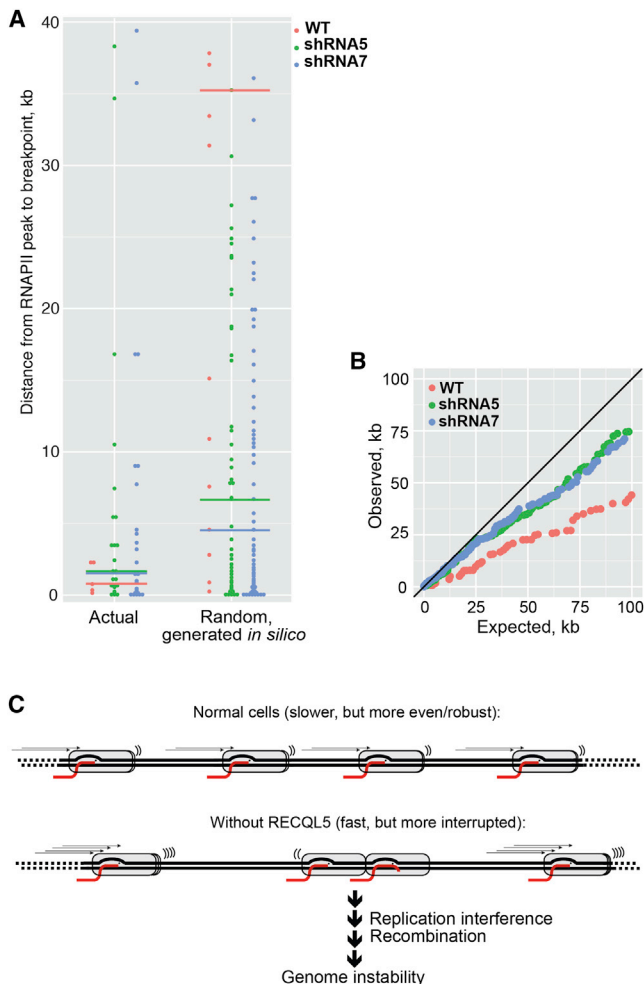


Figure 7. RECQL5-Dependent Genome Instability Occurs in Areas of Elevated Transcription Stress

(A) Plot of distances from genomic loss-associated chromosomal breakpoints to the nearest RNAPII peak in the same gene. Left: breakpoints observed in the RECQL5 shut-off experiments. Right: simulated examples (100 independent trials, averaged by rank) of computer-generated breakpoints in genes across the human genome (as represented on the Nimblegen chip). Note that there will be fluctuation in the number of computer-generated distances due to the frequency of simulated breakpoints landing in genes with RNAPII peaks varying from simulation to simulation. Horizontal lines indicate median values (of the observed distances on the left, of the averaged randomized on the right).

(B) Plot of distances from chromosomal breakpoints (observed breakpoints on y axis and random computer-generated breakpoints on x axis) to their nearest RNAPII peak, regardless of either being in a gene.

(C) Model for the role of RECQL5 at the interface between transcription and the maintenance of genome stability. See text for details.

DISCUSSION

The data presented here provide evidence that RECQL5 plays a general role in the control of transcript elongation in human cells. In its absence, transcript elongation rates increase, the distribution profile of RNAPII is markedly altered across the genome, and higher levels of RNAPII pausing or arrest (i.e., transcription

stress) are detected. Cells lacking RECQL5 also exhibit increased genome instability in the transcribed region of genes and at common fragile sites, and this is correlated with an increase in transcription stress in these particular regions.

RECQL5, a General RNAPII Elongation Factor

A large number of cofactors are required for normal transcript elongation by RNAPII (reviewed by Selth et al., 2010). However, most of these factors do not appear to affect the rate of transcript elongation in vivo. For example, rate determination in yeast failed to detect an effect of factors such as Set1, Set2, Elongator, Chd1, Elongin (*ELA1*), transcription-coupled repair factor Rad26, the Paf1 and THO complexes, Spt4, TFIIS, and Bye1 (Mason and Struhl, 2005). This in no way implies that these factors are not important for transcription, but forms a basis for appreciating the surprising discovery that RECQL5 affects transcript elongation rates at the vast majority of human genes. Moreover, using this effect as criterion, RECQL5 is a negative elongation factor: its depletion has the surprising effect of increasing RNAPII elongation rate compared to wild-type cells. Nevertheless, RECQL5 does also have a significant positive effect on transcript elongation in that it decreases transcription stress and the negative effects thereof. We propose that RECQL5 may “smoothen” or “buffer” transcript elongation, resulting in a somewhat slower, but more robust and less interrupted transcription process (Figure 7C). In any case, RECQL5 represents a new type of general RNAPII elongation factor.

We note that the genome-wide analysis suggests that RECQL5 also affects not only promoter- (Figure 1), but also terminator-proximal transcription events (to be published elsewhere), but how this relates to its elongation effects remains to be investigated. It is, however, an obvious possibility that the underlying biochemical mechanism is the same.

RECQL5 and the Maintenance of Genome Integrity

The results presented here indicate that genomic rearrangements in RECQL5-depleted cells are correlated with regions of elevated transcription stress, implying that suppression of such stress and its immediate consequence is crucial to maintain genome stability. As indicated above, it seems possible, even likely, that the involvement of RECQL5 in suppressing transcription stress (and thus maintaining genome stability) in effect comes at the cost of reducing average elongation speeds (Figure 7C).

It is worth emphasizing that transcription stress is not, in itself, genome-destabilizing. Indeed, RNAPII ChIP-seq experiments detect RNAPII pausing and arrest throughout the human genome, but this only infrequently results in genome rearrangements. Importantly, there is compelling evidence that transcription-associated genome instability requires clashes between RNAPII transcription and DNA replication (Aguilera and Gómez-González, 2008; Helmrich et al., 2013). Our results suggest that it is not transcription per se, but more specifically transcriptional pausing and arrest, which represents a challenge to genome stability during DNA replication. Recent results, albeit in bacteria (Dutta et al., 2011), support the idea that transcription stress, in this case specifically RNAP arrest and backtracking, can give rise to replication-derived genome instability.

Common fragile sites (CFSs) are genomic regions, frequently deleted in human cancer, which are predisposed to breakage when exposing cultured cells to replication stress (Debatisse et al., 2012). Recent results argue for a connection between CFSs and transcription-replication collision in long human genes (Helmrich et al., 2011; Le Tallec et al., 2013), with CFSs often situated in areas of high AT content that contain long genes (Durkin and Glover, 2007; Debatissse et al., 2012). They are also typically located in regions depleted of DNA replication origins, making replication rely on long-traveling forks, the breakdown of which cannot be compensated for by other origin firing (Letessier et al., 2011). However, the role played by transcription in CFS generation has remained unclear. Our striking finding RECQL5-dependent genomic rearrangements overlap with both CFSs and the transcribed region of long RNAPII genes points to a role for RECQL5 in suppressing breakage at CFSs. It is an obvious possibility that at least a subset of CFSs is associated with, or a direct consequence of, transcription stress. Indeed, we note that transcriptional pausing and arrest may largely be stochastic, and that it does not necessarily increase with rising gene expression levels, potentially helping to explain why the occurrence of CFSs, although typically occurring in genes, is not correlated with their transcription level (Le Tallec et al., 2013).

The biochemical mechanisms underlying DNA recombination are starting to emerge, but the possible explanations for the difference between chromosomal losses and gains remain theoretical (Art et al., 2012). With that in mind, we do not presently understand why RECQL5-driven genomic losses overlap much better than genomic gains with CFSs and genes. We note, however, that although the overlap between genomic gains and genes did not satisfy typical statistical confidence levels, a very large proportion of these events (80%) did occur inside genes. It is potentially important that the genes involved were typically very long and harbored regions of transcription stress near the chromosomal breakpoints, reinforcing the impression that RECQL5-dependent genomic rearrangements typically occur as a consequence of transcription-associated events. Work in chicken DT40 cells indicate that mutation of RECQL5's RNAPII-interaction domain does not have the same severe phenotypic effect as a complete RECQL5 knockout, suggesting that RECQL5 also has a transcription-independent function in suppressing genome instability (Islam et al., 2010), which might help explain the remaining, apparently transcription-unrelated genome instability events we observed.

We note that compounds such as camptothecin and psoralen, and even endogenous DNA lesions would affect transcript elongation and result in considerable transcription stress, likely resulting in a need for the transcription function of RECQL5 described here. This might explain the intriguing connection between RECQL5 and these causes of DNA damage (see, for example, Li et al., 2011; Tadokoro et al., 2012; Ramamoorthy et al., 2013). Finally, our data potentially shed light not only on the function of RECQL5, but also on the mammalian RECQ family as a whole. These proteins evolved to enable the suppression of genome instability, including that derived from transcription-associated DNA recombination during replication. In outline, such suppression might occur by modifying the behavior of either of the proteins governing these

processes, namely the replicating DNA polymerase(s), the recombination proteins, or RNA polymerase II. At this simplified, conceptual level, factors such as RECQ4 and BLM would suppress undesirable activity by DNA replication- and recombination factors, respectively (Liu, 2010; Larsen and Hickson, 2013), while RECQL5 would control elongating RNAPII, helping explain the additive or sometimes synergistic effects on genome instability levels attained when the function of RECQL5 and another RECQ gene is eliminated concomitantly (Hu et al., 2005; Popuri et al., 2013).

EXPERIMENTAL PROCEDURES

Cell Lines

HEK293, HEK293T-Rex, and derived cell lines were grown under standard conditions. RECQL5 shRNAs were from Thermo Scientific. For generating a stable cell line in which endogenous RECQL5 was depleted so that virtually all RECQL5 was from a doxycycline-regulated gene, a RECQL5-expressing plasmid was mutagenized to make the produced mRNA shRNA-resistant. This RECQL5 form was stably expressed in HEK293T-Rex cells that were subsequently infected with lentiviral particles carrying the RECQL5 shRNA construct depleting endogenous RECQL5. Single colonies were selected with puromycin in the presence of 0.2 ng/ml of doxycycline to allow physiologic expression level of the shRNA-resistant version of RECQL5.

Gene-Specific and Genome-wide Analysis of Transcription

RNAPII ChIP-seq was performed, with sequencing on the Illumina platform. DRB/GRO-seq integrates DRB inhibition to measure transcript elongation (Singh and Padgett, 2009) with GRO-seq (Core et al., 2008). Cells were treated with DRB for 3.5 hr, then released into DRB-free medium, with aliquots taken for GRO-seq at different time points.

Comparative Genomic Hybridization

RECQL5 shut-off cells were grown for 10 days, either with or without doxycycline. Genomic DNA from the compared clones was isolated, labeled, hybridized to 3 × 720K human arrays, and washed according to the manufacturer's recommendations (Nimblegen-Roche). The array was scanned by the Nimblegen MS200 micro-array scanning system. Primary data extraction, analysis, and visualization were performed using the DEVA software (Roche).

Bioinformatic Analysis

Reads were aligned to the reference genome using Bowtie (Langmead et al., 2009) and BWA (Li and Durbin, 2009) for ChIP-seq and GRO-seq, respectively. Mapped reads were sorted and indexed using SAMtools (Li et al., 2009). RNAPII peaks were called using MACS v1.4.2 (Zhang et al., 2008) and underlying sequence comparison done with XXmotif (Hartmann et al., 2013). Generation and normalization of read depth profiles was performed in Bioconductor (Gentleman et al., 2004).

For further details, please see [Extended Experimental Procedures](#).

ACCESSION NUMBERS

The GEO accession number for the genome-wide data-sets reported in this paper is GSE49134.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.03.048>.

AUTHOR CONTRIBUTIONS

M.S. performed the experiments, with contributions from T.K. (CGH) and H.W. R.M., G.P.K., and A.S. performed most bioinformatics and statistical analysis,

but ChIP-seq peak sequence analysis was done by M.H. and J.S. J.Q.S. wrote the manuscript, with help from all coauthors.

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