# Y RNA functions at the initiation step of mammalian chromosomal DNA replication

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## Summary

**Non-coding Y RNAs have recently been identified as essential novel factors for chromosomal DNA replication in mammalian cell nuclei, but mechanistic details of their function have not been defined. Here, we identify the execution point for Y RNA function during chromosomal DNA replication in a mammalian cell-free system. We determined the effect of degradation of Y3 RNA on replication origin activation and on fork progression rates at single-molecule resolution by DNA combing and nascent-strand analysis. Degradation of Y3 RNA inhibits the establishment of new DNA replication forks at the G1- to S-phase transition and during S phase. This inhibition is negated by addition of exogenous Y1 RNA. By contrast, progression rates of DNA replication forks are not affected by degradation of Y3 RNA or supplementation with exogenous Y1 RNA. These data indicate that Y RNAs are required for the establishment, but not for the elongation, of chromosomal DNA replication forks in mammalian cell nuclei. We conclude that the execution point for non-coding Y RNA function is the activation of chromosomal DNA replication origins.**

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Key words: DNA replication, Non-coding RNA, Initiation, DNA combing

## Introduction

Replication of chromosomal DNA in eukaryotes is regulated predominantly at the level of origin activation, which leads to the establishment of DNA replication forks during S phase of the cell-division cycle (reviewed by Costa and Blow, 2007; Gilbert, 2001; Machida et al., 2005). This pathway is controlled through the sequential interaction of DNA-replication licensing proteins ORC, Cdc6, Cdt1 and MCM2-MCM7 with chromosomal origins to form pre-replicative complexes (pre-RCs) upon transition from mitosis into G1 phase (reviewed by Bell and Dutta, 2002; DePamphilis et al., 2006; Takeda and Dutta, 2005). Origin activation depends on the cyclin- and Dbf4-dependent protein kinase activities of Cdk2 and Cdc7, respectively, which convert pre-RCs into active DNA replication forks via the recruitment of DNA polymerases and additional replication factors. This regulation network is evolutionarily conserved in eukaryotes from yeast to human; however, additional levels of control have evolved in metazoan organisms (reviewed by Arias and Walter, 2007).

The identification of new essential factors for chromosomal DNA replication in mammalian somatic cells has become possible with the development of cell-free systems that support the establishment and elongation of chromosomal DNA replication forks (reviewed by Krude, 2006). Replication of chromosomal DNA is initiated in template nuclei from G1-phase cells upon incubation in extracts from either asynchronously proliferating human cells or from cells synchronised in S phase (Krude, 2000; Krude et al., 1997; Stoeber et al., 1998). In fractionation and reconstitution experiments using human cell extracts, we have recently identified the small non-coding Y RNAs as novel factors that are essential for chromosomal DNA replication in isolated G1-phase nuclei (Christov et al., 2006).

Y RNAs were originally discovered in the early 1980s as an RNA component of Ro ribonucleoprotein particles (Ro RNPs), detected by sera from patients suffering from systemic lupus erythematosis (Lerner et al., 1981). The associated major antigen is the Ro60 protein, which is functionally implicated in RNA quality control, ribosome biogenesis and cellular responses to stress (Chen and Wolin, 2004; Fuchs et al., 2006; Stein et al., 2005). Y RNAs are transcribed by RNA polymerase III (Hendrick et al., 1981; Maraia et al., 1996; Maraia et al., 1994; Matera et al., 1995). They fold into characteristic stem-loop structures (Farris et al., 1999; Teunissen et al., 2000). Both the structure and nucleotide sequence elements of Y RNAs are evolutionarily conserved within vertebrates (Mosig et al., 2007; Perreault et al., 2007).

In humans, four Y RNAs are expressed (hY1, hY3, hY4 and hY5 RNA), and they are present in the soluble cell extracts used in cell-free DNA-replication systems (Christov et al., 2006). Loss-of-function experiments have established that specific degradation of either hY1 or hY3 RNA in these extracts by ribonuclease-H activity, mediated by a treatment with anti-sense DNA, results in an inhibition of DNA replication in G1-phase template nuclei incubated in the treated extracts in vitro (Christov et al., 2006). Re-addition of different non-degraded hY RNAs negates this inhibition of DNA replication, indicating that Y RNAs are functionally redundant in this system.

A functional requirement of Y RNAs for DNA replication has also been found in cell-based systems. Degradation of hY1 or hY3 RNAs in proliferating cultured human cells by RNA interference significantly reduces the proportion of S-phase cells in the treated cell population and leads to a cytostatic inhibition of cell proliferation (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009). Conversely, the expression of hY RNAs is significantly

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upregulated in solid human tumours, compared with non-malignant normal tissues (Christov et al., 2008).

The mechanistic execution point for Y RNA function during chromosomal DNA replication is unknown. The published data (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009) are consistent with a function of Y RNA in the activation of DNA replication origins leading to the establishment of new replication forks, in DNA chain elongation at progressing DNA replication forks, or a combination of both. To discriminate between these possibilities, we used Y RNA degradation and re-addition experiments in the mammalian cell-free system and investigated the consequences on chromosomal DNA replication by DNA combing and nascent DNA strand analysis. The results define a mechanistic execution point for Y RNA function in the establishment of new DNA replication forks.

## Results

### Y RNAs are required for DNA replication in G1-phase nuclei in vitro

Late-G1-phase template nuclei initiate chromosomal DNA replication in a mammalian cell-free DNA-replication system upon incubation in extracts from human S-phase cells (reviewed by Krude, 2006). S-phase nuclei support run-on DNA replication at existing replication forks in this system, but initiation of DNA replication at mid- to late-firing origins cannot formally be excluded. We used this system to define the execution point of Y RNA function during mammalian chromosomal DNA replication by the single-molecule approach of DNA combing and by nascent-strand analysis. Template nuclei for the initiation and elongation of chromosomal DNA replication in vitro were isolated from mouse NIH3T3 cells, synchronised by releasing quiescent cells into the cell cycle for 16 and 24 hours (Stoeber et al., 1998). This synchronisation protocol avoids the formation of DNA breaks as result of chemical synchronisation protocols (Szüts and Krude, 2004), which would interfere with DNA-combing analysis. Importantly, these late-G1-phase template nuclei require Y RNA for chromosomal DNA replication in vitro (Christov et al., 2006).

At 16 and 24 hours after release from quiescence, 7±1% and 56±7% of NIH3T3 cells incorporated bromo-deoxyuridine (BrdU), respectively (Fig. 1A), indicating synchronisation in G1 and S phase. The proportion of 16-hour template nuclei replicating in S-phase extract during the initial 15-minute interval corresponded to the proportion of BrdU-labelled nuclei that had already entered S phase in vivo (Fig. 1B). The proportion of replicating nuclei gradually increased from 11±3% to 29±7% during the first 120 minutes of the incubation and did not change after that. This increase was significant (*t*-test: $P$=0.0004). By contrast, the proportion of 24-hour nuclei replicating in S-phase extract vitro did not change significantly during the 180-minute incubation and corresponded to the proportion of BrdU-labelled S-phase nuclei. We conclude that about 20% of the G1-phase template nuclei initiate chromosomal DNA replication in vitro asynchronously after an initial lag period, whereas the proportion of replicating S-phase nuclei does not change upon incubation in vitro.

Semi-conservative DNA replication, which has been initiated in mammalian G1-phase nuclei in vitro, depends on the presence of non-coding Y RNAs (Christov et al., 2006). Specific and efficient degradation of Y RNAs in cytosolic extracts can be achieved by targeting endogenous RNAse-H activity to the Y RNAs via complementary antisense DNA oligonucleotides (Christov et al., 2006; Matera et al., 1995). In the following experiments, we used

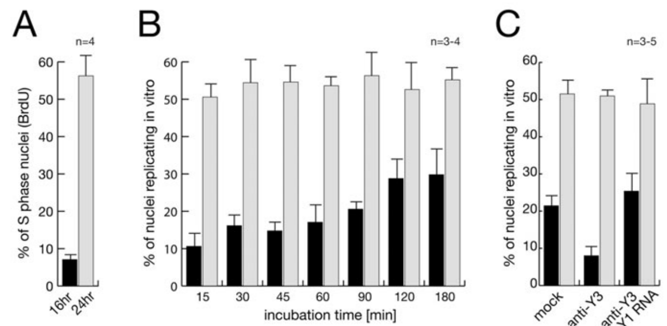


**Fig. 1.** Replication of chromosomal DNA in isolated G1- and S-phase nuclei. Mouse NIH3T3 cells were synchronised in late-G1 phase and in S phase by a release from quiescence for 16 or 24 hours, respectively, and their nuclei were isolated and used as templates for chromosomal DNA replication in vitro (Stoeber et al., 1998). Black and grey histogram bars represent data obtained with 16- and 24-hour nuclei, respectively. (A) S-phase proportions of template nuclei. The S-phase index of cells released for 16 or 24 hours was determined by incorporation of BrdU at the time of preparation. (B) Time course of DNA replication in vitro. Template nuclei were incubated for the indicated times in cytosolic extract from human S-phase cells and proportions of nuclei replicating in vitro were determined by incorporation of digoxigenin-dUTP. (C) Chromosomal DNA replication in G1-phase nuclei depends on Y RNAs. 16- and 24-hour nuclei were incubated for 180 minutes in S-phase cytosolic extracts, pre-treated as indicated. Mock, pre-treatment with standard T3 sequencing primer; anti-Y3, pre-treatment with an anti-sense DNA oligonucleotides to target endogenous ribonuclease H to Y3 RNA (Christov et al., 2006); +hY1, incubated in the additional presence of excess non-targeted hY1 RNA. The antisense DNA oligonucleotide treatment depletes the amount of hY3 RNA in human cytosolic extracts by more than 99% (Christov et al., 2006). Proportions of replicating nuclei were determined by confocal fluorescence microscopy. For examples of original micrographs see supplementary material Fig. S1. Mean values, standard deviations and the number of independent experiments ($n$) are given for each panel.

a DNA antisense oligonucleotide sequence (Christov et al., 2006) that targets both human hY3 and mouse mY3 RNA for degradation. Degradation of Y3 RNA reduced the proportion of 16-hour nuclei replicating in vitro down to the proportion of BrdU-labelled S-phase nuclei (Fig. 1C; supplementary material Fig. S1). This inhibition was significant (*t*-test: $P$=0.0005). Addition of functionally redundant hY1 RNA, which is refractory to ongoing degradation of Y3 RNA in this approach, fully negated this inhibition (Fig. 1C; supplementary material Fig. S1), as reported before (Christov et al., 2006). By contrast, neither Y3 RNA degradation nor supplementation with hY1 RNA significantly changed the proportion of replicating S-phase template nuclei under these conditions (Fig. 1C; supplementary material Fig. S1). However, Y3 RNA degradation resulted in an apparently weaker incorporation of digoxigenin-dUTP into these nuclei in vitro (supplementary material Fig. S1).

These data suggest that Y RNAs are required for the initiation of DNA replication in G1-phase nuclei. They further suggest that S-phase nuclei continue to replicate DNA after Y3 RNA degradation in vitro, but the total amount of DNA replication in these nuclei might also be sensitive to Y RNA degradation. This experimental approach cannot unanimously discriminate between potential requirements of Y RNAs for run-on replication at existing forks or for initiation at late-firing origins in S-phase nuclei. In the next experiments, therefore, we used DNA combing to analyse at single-molecule resolution whether Y RNAs are required for the initiation and/or elongation steps of chromosomal DNA replication.

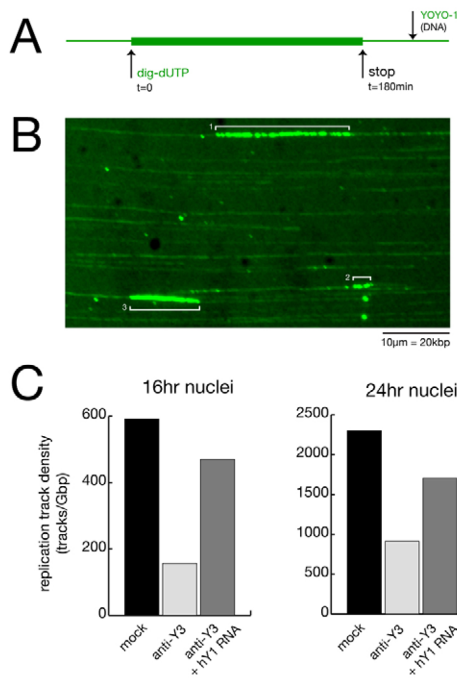


**Fig. 2.** Y RNA degradation reduces the density of replication tracks. (A) Labelling scheme for DNA-combing analysis. DNA replicated during the 180-minute incubation is labelled by incorporation of digoxigenin-dUTP (dig-dUTP), which is detected by fluorescent antibodies after DNA combing. Non-replicating DNA fibres are labelled by YOYO-1. (B) Visualisation of replication tracks on single chromosomal DNA fibres after DNA combing and fluorescence microscopy. Scale bar: 10 μm; equivalent to 20 kbp. The position and length of replication tracks present on this panel are indicated by white brackets numbered arbitrarily. (C) Overall replication-track densities. The overall length of all YOYO-1 stained DNA fibres was measured on randomly chosen micrographs (>60 and >30 Mbp of chromosomal DNA fibres were measured per reaction for 16- and 24-hour nuclei, respectively). The numbers of all individual replication tracks present on these fibres were counted and divided by the overall DNA length. Data were normalised as tracks per Gbp. Results are shown for template nuclei incubated in S-phase extracts pre-treated as indicated.

### Y RNAs are required for the initiation of DNA replication tracks

DNA combing aligns DNA fibres on glass coverslips in a parallel fashion (Bensimon et al., 1994; Michalet et al., 1997) and individual tracks of replicated DNA can be visualised on combed DNA by fluorescent microscopy (Herrick et al., 2000; Marheineke and Hyrien, 2001). We labelled individual replication tracks in NIH3T3 cell nuclei in vitro with digoxigenin-dUTP for 180 minutes (Fig. 2A). After DNA combing, digoxigenin was detected by a green-fluorescent probe and non-replicating DNA fibres were counterstained with the dye YOYO-1 (Fig. 2A). Digoxigenin and YOYO-1 were visualised in the same fluorescence channel, but could be discriminated based on their relative fluorescence intensity (Fig. 2B).

We investigated by DNA combing whether the density of replication tracks changed upon degradation of Y3 RNA in 16- and 24-hour nuclei (Fig. 2C). DNA combing reproducibly stretches individual DNA fibres with a constant stretching factor of 2 kbp/μm (Bensimon et al., 1994; Michalet et al., 1997). We measured the length of all YOYO-1-stained DNA fibres in randomly chosen micrographs, counted the number of individual replication tracks present and deduced the densities of replication tracks (Fig. 2C). The resolution for the identification of individual replication tracks was limited to 0.45 μm by the size of non-specific fluorescent particles present in the microscopic fields (Fig. 2B). Therefore, replication tracks of ≤0.45 μm (0.9 kbp) were excluded from this analysis. It is important to keep in mind that the replication-track densities per in vitro reaction are influenced by both the number of nuclei replicating and the number of replication forks present per nucleus. It is not a direct measure of fork density per nucleus.

Degradation of Y3 RNA resulted in a fourfold reduction of the overall replication-track density from 600 to 150 tracks/Gbp in 16-hour nuclei (Fig. 2C, left panel). Supplementation of non-targeted hY1 RNA to this reaction negated this reduction, suggesting that the establishment of new replication tracks in late-G1-phase nuclei depends on Y RNA. In 24-hour nuclei, degradation of Y3 RNA resulted in a 2.5-fold reduction of the replication-track density, which was negated upon addition of hY1 RNA (Fig. 2C, right panel). We conclude that the density of replication tracks is sensitive to degradation of Y3 RNA, not only in G1, but also in S-phase nuclei.

The use of two distinct labels in DNA combing allows detection of replication origin activation and quantification of individual replication fork progression rates in vitro (Herrick et al., 2000; Marheineke et al., 2009; Marheineke and Hyrien, 2001; Marheineke and Hyrien, 2004; Marheineke et al., 2005). We therefore added digoxigenin-dUTP at the beginning of an in vitro replication reaction, and biotin-dUTP after 60 minutes, and visualised the resulting replication tracks after DNA combing (Fig. 3). Digoxigenin was detected by a green-fluorescent probe and biotin by a red-fluorescent probe. Therefore, DNA replicated within the first 60 minutes of incubation was visualised on individual DNA fibres as a bright-green track, and DNA replicated between 60 and 180 minutes as a bright-yellow track because of the overlap of green and red signals (Fig. 3A). Non-replicating DNA fibres were counterstained with YOYO-1 (Fig. 3A).

This dual-labelling approach allows the determination of five different replication track patterns, integrating timing and directionality of individual replication forks (Marheineke et al., 2009; Marheineke et al., 2005). Examples are shown in Fig. 3B. Type-I patterns arise from initiation of two divergent replication forks from a single origin during the first labelling interval in vitro (0-60 minutes). Type-II patterns arise from initiation of one or two forks during the second labelling interval in vitro (60-180 minutes). Type-III patterns arise from unidirectional movement of one fork, or from two forks where one fork has stalled before the addition of the second label. Type-IV patterns arise from termination or fork stalling during the first labelling interval. Finally, type-V patterns arise from termination of two convergent forks in vitro during the second labelling interval. Whereas types I and II unequivocally indicate initiation events in vitro, the other three types (III-V) indicate initiation events that might have taken place either in vivo, prior to preparation of template nuclei, or in vitro, during the first labelling interval.

In 16-hour nuclei, early bidirectional initiation patterns of type I were rarely observed (less than 2%), whereas late initiation patterns of type II were the most abundant ones with a frequency of 40% (Fig. 3C, top panel). We tested whether a delayed addition of the second label at 120 minutes would lead to an increased occurrence of type-I patterns, at the expense of type-II patterns. This was not the case; instead, we observed a reduction of type-II patterns from 40% down to 12% and a compensating increase of both pattern types III and IV (data not shown). Therefore, the low abundance of type-I patterns can be explained by a predominance of unidirectional initiation events in this cell-free system, by

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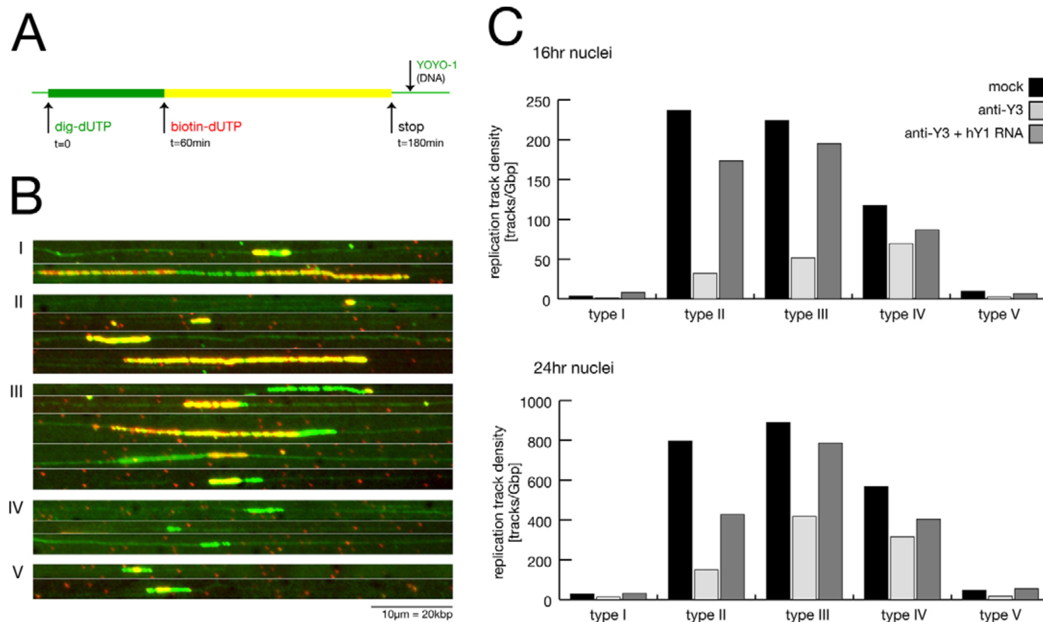


**Fig. 3.** Initiation of DNA replication tracks depends on Y RNA. (A) Labelling scheme. DNA replicated during the first 60-minute interval of the incubation is highlighted in green (dig-dUTP incorporation only) and DNA replicated during the second 60- to 180-minute interval is highlighted in yellow because of a merge of green (dig-dUTP) and red (biotin-dUTP) signal. Non-replicating DNA fibres are labelled by YOYO-1. (B) Classification of replication tracks. Replication tracks on chromosomal DNA were visualised by fluorescence microscopy on combed chromosomal DNA via incorporation of digoxigenin-UTP and biotin-dUTP, and non-replicating DNA was counterstained with YOYO-1 (Marheineke et al., 2005). For the definition of pattern types I-V see the Results section. A selection of tracks is presented for each type. Scale bar: 10 μm; equivalent to 20 kbp. (C) Densities of specific replication track patterns. The frequencies of individual replication track patterns I-V were determined for the indicated reactions as detailed in the legend to Fig. 2. A total of 362 (251), 322 (214) and 282 (219) tracks were scored for mock, anti-Y3 and anti-Y3+hY1 RNA reactions in 16-hour (24-hour) nuclei, respectively. These raw frequencies were normalised as the number of tracks per Gbp using the overall track abundance values from Fig. 2.

asymmetric fork movement or by fork stalling. Unidirectional initiation and/or asymmetric fork movement after bidirectional initiation have been reported before in the human cell-free DNA-replication system (Marheineke et al., 2005).

Importantly, the density of type-II initiation patterns (detected by early addition of the second label, at 60 minutes) was reduced 7.4-fold upon degradation of Y3 RNA (Fig. 3C, top panel), demonstrating a requirement of Y RNA for initiation of chromosomal DNA replication in vitro. Type-III patterns were detected at a frequency of 38% and degradation of Y3 RNA led to a 4.1-fold reduction of their density (Fig. 3C, top panel). This observation is consistent with a requirement of Y RNA also for unidirectional initiation and/or bidirectional initiation, in which stalling of one fork during the first 60 minutes leads to asymmetric fork movement. Type-IV patterns were observed at a lower frequency of 20%, and bidirectional termination patterns (type V) were rarely observed. In contrast to initiation patterns, the densities of these termination patterns (type IV and V) were reduced by less than twofold upon Y3 RNA degradation. In all cases, supplementation with non-targeted hY1 RNA negated the reduction of replication-track density obtained by Y3 RNA degradation (Fig. 3C, top panel). These data show that the density of initiation-specific replication tracks decreases in 16-hour nuclei after degradation of Y3 RNA.

In 24-hour nuclei, the densities of initiation-specific type-II replication tracks were reduced 5.5-fold upon Y3 RNA degradation, and were reconstituted upon supplementation with hY1 RNA (Fig. 3C, bottom panel). This observation establishes two important points regarding DNA replication in S-phase nuclei in vitro. First, initiation of new replication forks in vitro is not restricted to G1-phase template nuclei, but it occurs in S-phase nuclei as well. It is worth noting here that S-phase nuclei replicating in vitro therefore contain a mix of replication forks, namely those that had been initiated in vivo prior to the preparation of the nuclei and those that were initiated in vitro. This heterogeneity shows that S-phase nuclei synthesise DNA in the absence of initiation factors through chain elongation at pre-existing forks (Krude, 2006) (Fig. 1) and, on top of this, that they initiate new forks in the presence of initiation factors (Figs 2 and 3). Second, non-coding Y RNAs are required for the initiation of new DNA replication forks not only at the G1- to S-phase transition, but also during later stages of S phase.

We conclude that degradation of Y RNAs inhibits the initiation of new replication forks in late-G1-phase and in S-phase nuclei in vitro.

### Chain elongation does not depend on Y RNA

Dual labelling and DNA combing also allows measurements of the progression rates of individual replication forks (Herrick et al., 2000; Marheineke et al., 2009; Marheineke and Hyrien, 2001; Marheineke and Hyrien, 2004; Marheineke et al., 2005). We therefore investigated whether Y RNA depletion has any effect on the elongation stage of DNA replication. Individual replication tracks of pattern types I and V arise from two replication forks, whereas pattern types II, III and IV can arise from either one or two forks. To allow an unbiased interpretation, we refer to the rate at which any given replication track is synthesised as the 'replication track extension rate', keeping in mind that it might be due to one or two replication forks.

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We determined the rates of replication track extension by measuring the track length in kbp for each of the two abundant track patterns (II and III) and divided this value by the labelling time (2 hours of biotin for type II; 3 hours of digoxigenin for type III). We did not analyse pattern types I and V because of the small sample size nor pattern type IV because of unspecified termination or stalling during the labelling time. Individual replication track extension rates varied largely between 10 and >400 bp/minute for both pattern types and reaction conditions in 16- and 24-hour nuclei (Fig. 4). Rate distributions were asymmetric, indicating heterogeneity of replication-fork progression rates.

In 16-hour nuclei, the rate distributions were very similar (Fig. 4A). However, a small reduction was seen after Y3 RNA degradation compared with the mock-treated and reconstituted reaction conditions for both pattern types. In 24-hour nuclei, all rate distributions appeared very similar (Fig. 4B). Importantly, none of these small variations in the rates of replication track extension between the three reaction conditions were significant (ANOVA: $P$=0.44 for type II, $P$=0.09 for type III in 16-hour nuclei; $P$=0.53 for type II, $P$=0.49 for type III in 24-hour nuclei; $\alpha$=0.05). We conclude that replication-fork progression rates are not affected by the degradation of Y3 RNA.

Finally, we compared replication-track extension rates in the double-labelled type-III patterns in the first 60 minutes with those in the subsequent 120 minutes. The average rate dropped from 140.9±114.8 bp/minute during the first labelling interval to 106.5±99.2 bp/minute during the second, consistent with a slowing down of fork progression over time and/or a limited extent of replication-fork stalling after longer incubation in vitro. Importantly, average rates of replication track extension during the second labelling interval also did not vary significantly after mock-treatment, Y3 RNA degradation or supplementation with hY1 RNA (ANOVA: $P$=0.28 for 16-hour nuclei, and $P$=0.98 for 24-hour nuclei; $\alpha$=0.05). We therefore conclude that neither the chain elongation of chromosomal DNA replication nor the stability of the DNA replication fork are dependent on Y RNAs.

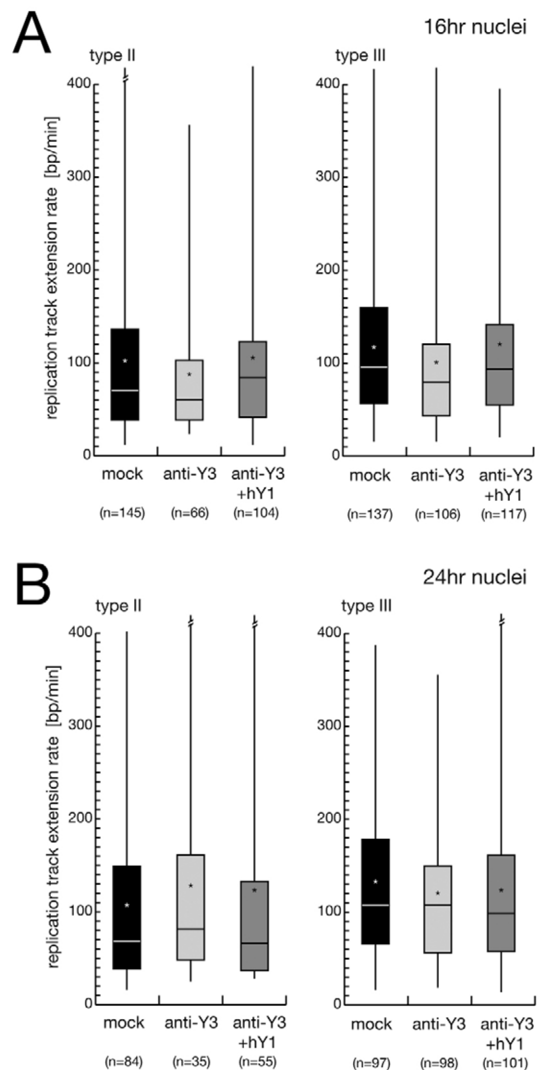


**Fig. 4.** Y RNA degradation does not affect replication track extension rates. 16- and 24-hour template nuclei were incubated for 180 minutes in cytosolic S-phase extracts pre-treated as indicated. Replication tracks were labelled and visualised by DNA combing as detailed in Fig. 3A. The length of individual tracks was determined, and their extension rates were calculated as bp per minute of labelling. Type-II patterns were allocated 2 hours of biotin labelling time and type-III patterns 3 hours of digoxigenin labelling time. Total numbers ($n$) of independent replication tracks measured are given for each analysis. (A) Distribution of replication track extension rates for type-II and -III patterns in 16-hour nuclei incubated in treated cytosol as indicated. Box-and-whisker plots are shown with thin vertical lines indicating the range, boxes the 25th-75th percentile, black horizontal lines the median and asterisks the mean for each distribution. (B) Distribution of replication track extension rates for type-II and -III patterns in 24-hour nuclei as indicated.

## Y RNAs are required for the synthesis of nascent DNA strands

In order to consolidate the conclusions drawn from DNA combing and fibre fluorescence by independent experiments, we performed nascent-DNA-strand analysis. Initiation of new DNA replication forks results in the synthesis of single-stranded nascent DNA strands that are not covalently bound to the parental DNA strands. By contrast, chain extension synthesis during the elongation stage is characterised by the covalent attachment of nucleotides or short Okazaki fragments to the growing high-molecular-weight DNA strands. We therefore prepared nascent DNA strands and used alkaline gel electrophoresis to determine whether Y RNAs are required for nascent-strand synthesis, or for chain elongation.

Incubation of 16-hour and 24-hour template nuclei in S-phase extract for 15 minutes resulted in the synthesis of nascent strands over a wide size range from a hundred up to several thousand nucleotides (Fig. 5A). This pattern did not change upon longer incubation times; however, high-molecular-weight DNA accumulated at later time points (Fig. 5A). Therefore, nascent-strand synthesis is initiated asynchronously throughout the in vitro reaction in both G1- and S-phase nuclei. Furthermore, the resolution limit of nascent-strand analysis was about tenfold higher than DNA combing, because nascent strands of about 100 nucleotides could be detected on alkaline gels, in contrast to a resolution limit of about 900 nucleotides in DNA combing (see above; Fig. 2B; Fig. 3B).

We investigated by alkaline gel electrophoresis (Fig. 5B) and quantitative phosphorimaging whether initiation or elongation of nascent DNA strands depends on the presence of Y RNAs. Degradation of Y3 RNA led to significantly reduced amounts of single-stranded nascent DNA in the 0.1- to 10-kb range down to 53±7% of that of the mock-treated sample in 16-hour nuclei ($t$-test: $P$=0.0001, $n$=5), and to 61±11% in 24-hour nuclei ($P$=0.0003, $n$=6). Addition of non-degraded hY1 RNA negated this reduction, back up to 92±8% in 16-hour nuclei ($t$-test: $P$=0.00005, $n$=5), and to 109±18% in 24-hour nuclei ($P$=0.0005, $n$=6). In control

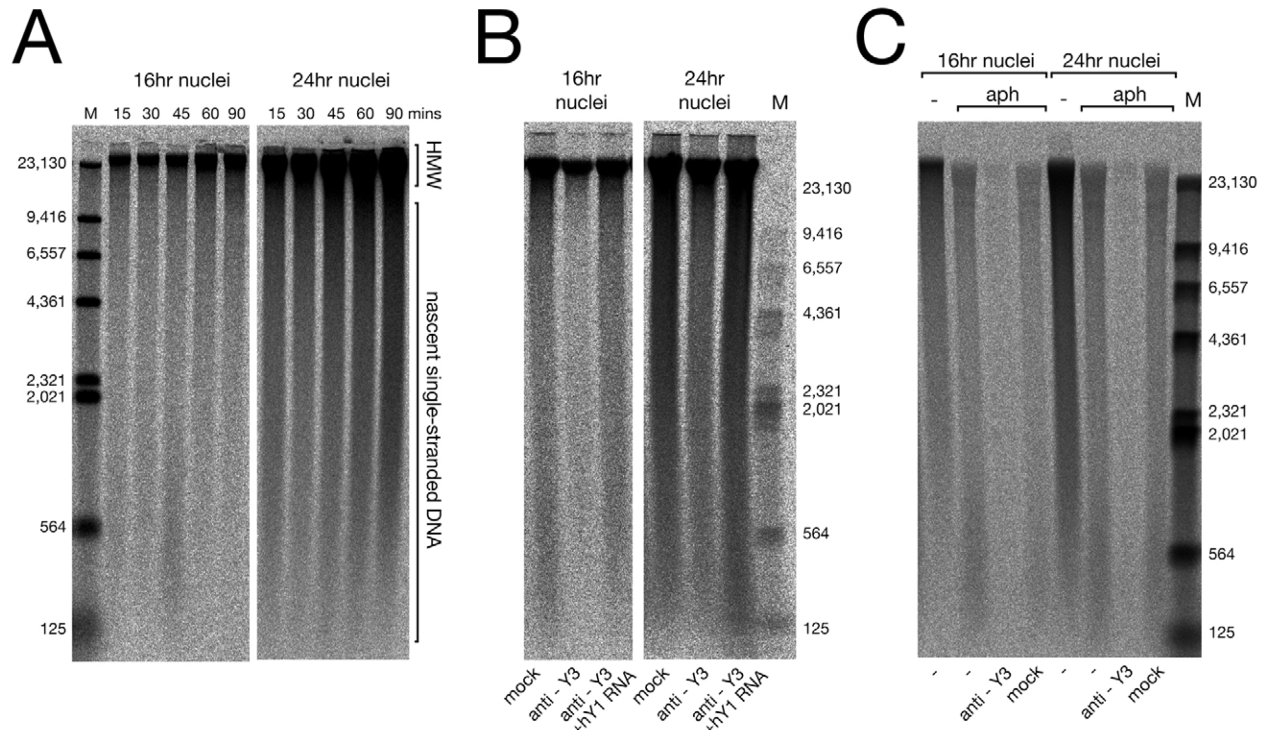


**Fig. 5.** Nascent single-stranded DNA synthesis depends on Y RNAs. (A) Time course of nascent-strand synthesis in vitro. 16- and 24-hour template nuclei were incubated in cytosolic S-phase extract in the presence of [$\alpha$-$^{33}$P]-dCTP for the indicated times. DNA was isolated, denatured, separated on an alkaline agarose gel and visualised by phosphorimaging. DNA from the same number of template nuclei was loaded per lane. Positions of end-labelled *Hin*dIII-digested phage $\lambda$ DNA (M) fragments, high-molecular-weight DNA chains (HMW) and nascent single-stranded DNA are indicated. Both panels are from the same gel. (B) Abundance of nascent strand DNA after Y3 RNA degradation. 16- and 24-hour template nuclei were incubated for 120 minutes in pre-treated cytosolic S-phase extracts as indicated. Nascent DNA was visualised by phosphorimaging. Both panels are from the same representative gel. (C) Initiation of short nascent strands in the presence of the elongation inhibitor aphidicolin depends on Y3 RNA. 16- and 24-hour template nuclei were incubated for 120 minutes in the absence or presence of 40 μM aphidicolin (aph) in pre-treated cytosolic S-phase extracts as indicated. Nascent DNA was visualised by phosphorimaging.

experiments, addition of roscovitine or olomoucine, which competitively inhibit the initiation step of DNA replication by blocking cyclin-Cdk complexes (Meijer et al., 1997), led to a similar reduction of nascent-DNA-strand abundance (not shown). By contrast, addition of aphidicolin, which competitively inhibits chain elongation by blocking DNA polymerases, resulted in the accumulation of short nascent strands of 0.1-2.0 kb (Fig. 5C). Importantly, Y3 RNA degradation abolished the synthesis of these small DNA nascent strands (Fig. 5C).

In summary, Y3 RNA degradation leads to an overall reduction of nascent strands independent of their size, and does not lead to an accumulation of short strands. We therefore conclude that the initiation step of nascent-strand synthesis rather than the chain-elongation step is inhibited after Y3 RNA degradation in both G1- and S-phase nuclei, thereby corroborating the conclusions drawn from the DNA-combing experiments.

### Elongation of nascent DNA strands does not depend on Y RNAs

Finally, we investigated whether degradation of Y3 RNA affects nascent-strand elongation by determining the average rate at which nascent strands are converted into high-molecular-weight DNA in vitro (Fig. 6). S-phase nuclei were pre-labelled in vitro, transferred into corresponding fresh extracts and incubated further without radiolabel. The size distribution of nascent strands was analysed after 0, 15 and 60 minutes of chase time by alkaline gel electrophoresis (Fig. 6A). Short nascent strands disappeared over time whether or not Y RNA had been degraded, whereas higher-molecular-weight DNA remained (Fig. 6A). We quantified the relative abundance of short (0.1-2.0 kb), intermediate (2-10 kb) and long (<10 kb) nascent DNA strands by phosphorimaging and plotted the relative abundances of these size classes after 0, 15 and 60 minutes of chase time for each of the three reactions (Fig. 6B). This relative quantification controls for variations in gel loading and for differences in total amounts of labelled DNA per reaction. Importantly, short nascent strands were chased into longer strands over time, whether or not Y RNA had been degraded (Fig. 6B). Importantly, small variations in chase rates between the three reaction conditions were not significant for any size class (ANOVA: $P$=0.3 for 0- to 15-minute chase time, $P$=0.15 for 15-60 minutes in the >10-kb size class; $P$=0.34 for 0-15 minutes, $P$=0.74 for 15-60 minutes in the 2- to 10-kb size class; $P$=0.06 for 0-15 minutes, $P$=0.43 for 15-60 minutes in the 0.1- to 2-kb size class; $\alpha$=0.05). These data indicate that the elongation stage of nascent DNA chain synthesis in mammalian cell nuclei in vitro does not depend on the presence of Y RNAs, corroborating the results from the DNA-combing experiments (see Fig. 4).

In conclusion, independent results from both DNA combing and nascent-strand analysis entirely agree with each other, and provide compelling evidence in favour of a requirement of non-coding Y RNAs for the initiation step of chromosomal DNA replication, but not for chain elongation. Therefore, the execution point of Y RNA

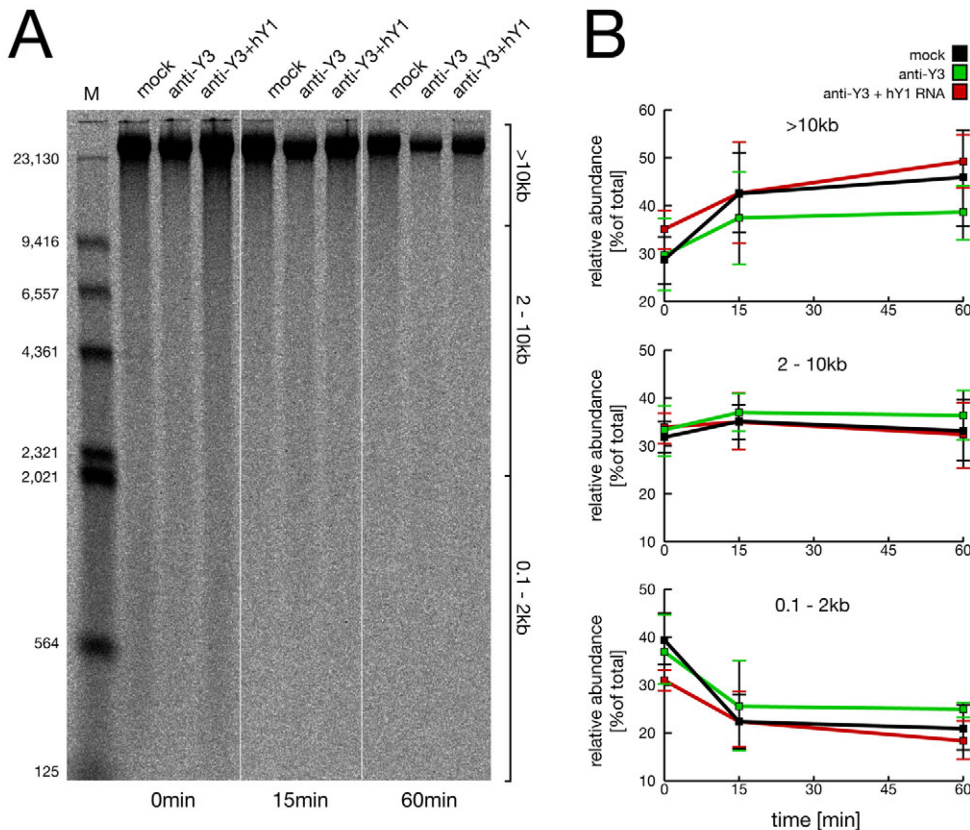


**Fig. 6.** Nascent-strand extension synthesis is not dependent on Y RNA. 24-hour nuclei were incubated in treated extracts for 120 minutes as indicated in the presence of [α-$^{33}$P]-dCTP, transferred into corresponding fresh extracts without [α-$^{33}$P]-dCTP, and incubated further for 0, 15 and 60 minutes. (A) Visualisation of nascent strands. Radiolabelled DNA was isolated, separated on an alkaline agarose gel and visualised by phosphorimaging. DNA from the same number of template nuclei was loaded per lane. Thin white lines are superimposed to aid visualisation of the three incubation times. Data presentation is as in Fig. 5; nascent-strand size classes of 0.1-2 kb, 2-10 kb and >10 kb are indicated. A representative gel is shown. (B) Quantitative analysis of the relative abundance of nascent strands. The abundance of radiolabelled nascent strands in size classes of 0.1-2 kb, 2-10 kb and >10 kb were determined by phosphorimaging for the indicated time points and reaction conditions. Data are expressed as the percentage of radiolabel in each indicated size class; the 100% value is the cumulative total radiolabel for each reaction (total size class >0.1 kb). Mean values and standard deviations of three independent experiments are shown.

function in mammalian chromosomal DNA replication is the initiation, and not the chain elongation, step.

## Discussion

In this study, we addressed the mechanistic execution point for Y RNA function during DNA replication in mammalian somatic cell nuclei at single-molecule resolution by molecular combing and DNA fibre fluorescence microscopy, and by nascent-strand analysis. We observed that the establishment of new DNA replication forks and the initiation of nascent-DNA-strand synthesis were inhibited when Y3 RNA was degraded. This inhibition was negated by the addition of hY1 RNA. By contrast, rates of replication-fork progression and nascent-strand elongation did not change upon Y3 RNA degradation. We therefore conclude that the execution point for non-coding Y RNA function during chromosomal DNA replication is not DNA chain elongation, but the activation of chromosomal DNA replication origins, leading to the initiation of new DNA replication forks (Fig. 7).

### Y RNAs are required for the initiation step of DNA replication

Here, we have provided several lines of evidence to support a requirement for Y RNAs during the initiation step of chromosomal DNA replication. First, G1-phase nuclei initiated DNA replication in vitro only when Y3 RNA was not degraded, consistent with our previous study (Christov et al., 2006). Second, the density of newly initiated DNA replication tracks in vitro was significantly reduced upon Y3 RNA degradation. An independent confirmation that the inhibition of an initiation factor leads to the reduction of replication initiation track densities, as measured by DNA combing, has been published recently. Santocanale and co-workers have shown that specific inhibition of Cdc7 kinase by the compound PHA-767491 in mammalian cells results in a marked decrease of the density of activated origins (Montagnoli et al., 2008). And third, we have seen here that the abundance of single-stranded nascent DNA was significantly reduced when Y3 RNA was degraded. Importantly, exogenous hY1 RNA negated all these inhibitions obtained by degradation of endogenous Y3 RNA. These data therefore establish that Y RNAs are required specifically for the initiation step of DNA replication, leading to the establishment of new replication forks.

The requirement of Y RNAs reported here for origin activation in vitro can explain mechanistically the inhibition of DNA replication and cell proliferation upon knocking down Y RNA expression levels in asynchronously proliferating intact human cells by RNA interference (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009). Transfection of small interfering RNAs (siRNAs) against hY1 and hY3 RNAs leads to a reduced proportion of actively replicating cells and to a cytostatic inhibition of cell proliferation (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009). An inhibition of origin firing upon Y RNA degradation in vivo on the one hand would prevent G1-phase cells from entering S phase. On the other hand, the percentage of actively replicating cells in S phase would be reduced over time because, in the absence of new initiation events, existing DNA replication forks would initially continue to synthesise DNA strands, but would eventually terminate or stall with partially replicated DNA.

A functional role for human Y RNAs as essential initiation factors for chromosomal DNA replication in mammalian cell nuclei might at first glance be difficult to reconcile with knockout experiments of Ro60 protein in mice. Wolin and co-workers have reported that a knockout of the this Y RNA-interacting protein leads to reduced levels of mY1 and mY3 RNA in adult brain tissue and embryonic stem (ES) cells (Chen et al., 2003; Xue et al., 2003). Ro60 knockout

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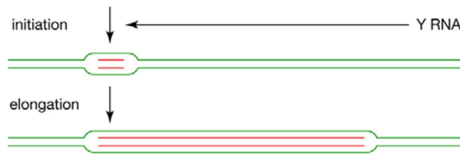


**Fig. 7.** The execution point for Y RNA function during mammalian chromosomal DNA replication. Y RNAs are required for the initiation of new DNA replication forks (top), but not for their elongation synthesis after their establishment (bottom). Parental high-molecular-weight DNA strands are shown in green; newly synthesised nascent strands are shown in red.

mice are viable and show no major proliferation defect, despite a reduction of mouse Y RNA levels. This paradox might be resolved by considering how Y RNAs are degraded in these different experiments. Because Ro60 acts as a nuclear-export factor for Y RNAs (Rutjes et al., 2001; Simons et al., 1996), one prediction would be that deletion of Ro60 protein actually increases nuclear concentrations of newly transcribed Y RNAs, which would suffice for initiating DNA replication despite reduced levels of the overall soluble, predominantly cytoplasmic pool of Y RNAs. By contrast, continuous ribonucleolytic degradation as used in our studies would deplete the targeted Y RNA pools in cell nuclei and cytoplasm, thus inhibiting initiation of DNA replication. Furthermore, deletion of the Ro60 binding site on hY1 RNA does not inhibit its DNA-replication function (Christov et al., 2006; Gardiner et al., 2009), showing that Ro60 binding and initiation of DNA replication are two independent functions of Y RNAs.

In the experiments reported here, degradation of Y3 RNA led to a significant reduction in both the density of DNA replication initiation tracks and abundance of nascent DNA strands, but not to their total disappearance. In nuclear replication assays, depletion of Y3 RNA reduced the percentage of replicating G1-phase nuclei right down to the percentage of contaminating true S-phase nuclei, whereas S-phase nuclei continued replicating their DNA at already established forks under these conditions. These nuclear assays suggest that degradation of Y3 RNA is sufficient to completely inactivate the initiation activity of the cell extract required for stimulating the entry of a G1-phase nucleus into a replicating S-phase-like state in vitro. In assays of DNA combing and nascent strand abundance, however, Y3 RNA degradation led to a significant reduction of new initiation events in both G1- and S-phase nuclei, while a background of some new replication tracks and nascent DNA strands were still initiated. The data allow the conclusion that a residual initiation activity is still present in the system after Y3 RNA degradation. It is therefore possible that this residual initiation activity might be so low that only a few isolated replication events are initiated in G1-phase nuclei, which would be detected in DNA combing and nascent-strand assays, but that the majority of these G1-phase nuclei would not incorporate sufficient biotin- or digoxigenin-dUTP to be scored as positive in nuclear replication assays. Alternatively, this residual initiation activity might be restricted to S-phase nuclei. The residual activity might be due to non-degraded Y RNAs remaining in the experimental system (i.e. mY1, hY1, hY4 and hY5). Alternatively, the template nuclei might contribute a limited amount of mY3 RNA to the reaction, which would have not been pre-degraded by the RNAse-H activity. These observations could point towards a possible functional specialisation of different Y RNAs in origin activation. In one scenario, some chromosomal origins might require a specific Y RNA for their activation (i.e. a potential Y1-RNA-dependent origin would still fire when Y3 RNA is degraded). Alternatively, different origins might require different overall levels of Y RNAs for their activation (i.e. degradation of Y3 RNA would be sufficient to inactivate a sensitive origin but degradation of additional Y RNAs would be required for inactivation of a less-sensitive origin). Finally, some chromosomal origins might not require Y RNAs for their activation at all.

These models could be tested in a detailed study of differential Y RNA requirement for individual origin activation on a genomic scale; unfortunately, this is still hampered by our very limited current knowledge of chromosomal origins in mammalian cells (reviewed by Gilbert, 2001; Machida et al., 2005). However, recent developments of new efficient mapping techniques are currently expanding our knowledge on mammalian chromosomal DNA-replication origins (Cadoret et al., 2008; Gomez and Antequera, 2008; Lucas et al., 2007) so that investigations into how Y RNAs regulate their specific activation might become feasible in the foreseeable future.

The DNA-combing data reported here indicated that DNA replication initiates in the mammalian cell-free system predominantly in an apparently unidirectional manner. Unidirectional initiation, or bidirectional initiation leading to asymmetric fork movements due to stalling of one fork, has also been detected by DNA combing in the human cell-free replication system (Marheineke et al., 2005). By contrast, most DNA-combing studies performed with DNA replicated in intact cells have provided evidence for bidirectional initiation (Anglana et al., 2003; Conti et al., 2007; Lebofsky and Bensimon, 2005); however, unidirectional initiation in vivo has also been documented, for instance in human rDNA loci (Lebofsky and Bensimon, 2005). Predominant unidirectional initiation in the mammalian cell-free replication system can be explained trivially by inefficient reaction conditions due to the use of diluted cell extracts, compared with the situation in the intact cell nucleus (Marheineke et al., 2005). In addition, a new mode of asymmetric bidirectional initiation has recently been described for the human DBF4 origin (Romero and Lee, 2008), whereby two unidirectional forks are initiated in a staggered manner from two adjacent chromosomal sites.

In any case, the efficient establishment of new DNA replication forks by either a uni- or bidirectional mode of initiation in the mammalian cell-free system requires the presence of non-coding Y RNAs.

### DNA-replication-fork progression does not depend on Y RNAs

The mammalian cell-free DNA replication system showed highly variable replication-fork progression speeds, as seen in the human cell-free system (Fig. 4) (Marheineke et al., 2005). This high variability has also been observed in intact mammalian cells in vivo (Anglana et al., 2003; Conti et al., 2007; Daboussi et al., 2008; Lebofsky and Bensimon, 2005; Petermann et al., 2006). This heterogeneity is therefore not an in vitro artefact and might be explained by dynamic regulation of fork progression rates through different chromatin domains within a given nucleus, or between different cells.

Individual fork progression rates in mammalian cell-free DNA replication systems in vitro are about one order of magnitude lower than in corresponding intact cells. Average genome-wide fork progression rates have been determined in mammalian cells in vivo by DNA-fibre-labelling techniques to be in the range of 0.9-2.0 kbp/minute (Anglana et al., 2003; Conti et al., 2007; Daboussi et

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al., 2008; Jackson and Pombo, 1998; Lebofsky and Bensimon, 2005; Petermann et al., 2006). By contrast, when human cell nuclei were incubated in human proliferating cell extracts, individual forks progressed at about 300±150 bp/minute (Marheineke et al., 2005). Replication forks in 16-hour and 24-hour mouse nuclei incubated in untreated human S-phase cell extract progressed with rates of 160±120 and 152±107 bp/minute, respectively. The reduced fork progression rates between cell-based and cell-free systems are most likely due to the artificially low concentration of replication factors in dilute mammalian cell extracts, as discussed before (Marheineke et al., 2005). In any case, density substitution analysis has established unequivocally that Y RNA-dependent DNA synthesis in the cell-free system is due to semiconservative DNA replication, and not DNA repair (Christov et al., 2006).

We used single-stranded DNA antisense oligonucleotides for targeting RNAse-H activity to complementary Y3 RNA, leading to its specific degradation in vitro (Christov et al., 2006; Matera et al., 1995). Treatment of cell extracts with single-stranded DNA oligonucleotides of any sequence, including the negative control, reduced mean DNA-replication-fork progression rates from about 150 bp/minute to about 120 bp/minute. This inhibition of fork progression is non-specific and might possibly be due to sequestration of DNA replication factors, such as the single-stranded-DNA-binding protein RPA.

Importantly, Y RNA degradation did not change replication-fork progression speeds significantly in either G1- or S-phase nuclei. Additionally, Y RNA degradation did not significantly change the conversion rates of short nascent strands into high-molecular-weight DNA. Therefore, DNA strand elongation at replication forks does not require Y RNA. Consistent with these observations, the elongation steps of DNA replication in DNA tumour virus systems can be reconstituted entirely from purified human host-cell proteins in the absence of non-coding Y RNAs (Challberg and Kelly, 1989; Waga and Stillman, 1998).

### The execution point for Y RNA function in chromosomal DNA replication

In summary, data presented here have established that Y RNAs are required for the initiation step of mammalian chromosomal DNA replication, but not for DNA chain elongation (Fig. 7). We can therefore conclude that the execution point of Y RNA function during mammalian chromosomal DNA replication is the initiation step, leading to the activation of chromosomal DNA replication origins and the establishment of new DNA replication forks. This execution point for Y RNA function can be mediated directly or indirectly through molecular interactions between Y RNAs and proteins of the replication-initiation machinery.

Interestingly, roles for different non-coding RNAs in the initiation of eukaryotic DNA replication have been described recently. In the ciliate *Tetrahymena*, 26T RNA, a fragment of 26S rRNA, has been described as an integral component of the initiator complex ORC. 26T RNA is required for targeting ORC to a replication origin in the ribosomal DNA via RNA-DNA hybridisation (Mohammad et al., 2007). In human cells, a structured G-rich RNA has been shown to mediate the recruitment of ORC to the Epstein-Barr virus origin of plasmid replication (OriP) via the viral protein EBNA1 (Norseen et al., 2008). A similar G-rich RNA-dependent mechanism has also been suggested for ORC recruitment to some cellular origins involving the RNA-binding protein HMGA1a (Norseen et al., 2008).

At present, we do not know the molecular interactions by which Y RNAs mediate the initiation of chromosomal DNA replication in mammalian somatic cell nuclei. However, the identification of the execution point for Y RNA function as the initiation step of DNA replication is an essential step towards dissecting the underlying molecular regulation network. It seems reasonable to suggest as a current working model that Y RNAs, similar to the other unrelated non-coding RNAs involved in DNA replication initiation in different model systems, could provide an additional level of control in vertebrate somatic cells over the conserved eukaryotic protein machinery for the initiation of chromosomal DNA replication. Future experiments will focus on the identification and characterisation of molecular interactions between Y RNAs and constituents of the replication-initiation machinery.

## Materials and Methods

### Cell culture and synchronisation

Mouse NIH3T3 cells were cultured as monolayers and synchronised by release from quiescence as described (Stoeber et al., 1998). Release times were 16 hours for synchronisation in late-G1 phase and 24 hours for S phase. Human HeLa cells were synchronised in S phase by a 24-hour treatment with 2 mM thymidine followed by a release into fresh medium for 2 hours, as described (Krude et al., 1997).

### DNA-synthesis reactions and labelling of reaction products

Template nuclei and human S-phase cytosolic extract were prepared exactly as described previously (Krude, 2000; Krude et al., 1997; Stoeber et al., 1998). Endogenous Y3 RNA was degraded by a pre-treatment of S-phase HeLa cytosolic extract with specific anti-sense DNA oligonucleotides as described (Christov et al., 2006).

Standard DNA-replication-initiation reactions contained the following components: S-phase HeLa cytosolic extract (38 μl), a buffered mix of rNTPs and dNTPs (10 μl), and $2 \times 10^5$ nuclei (2 μl) from synchronised NIH3T3 cells (Stoeber et al., 1998). Incubation time was 3 hours unless indicated otherwise. Recombinant hY1 RNA was synthesised by in vitro transcription (Christov et al., 2006) and added to DNA replication reactions at 300 ng/reaction as indicated. Nascent DNA was labelled for molecular combing and fluorescence microscopy by addition at the indicated times of 10 μM digoxigenin-11-dUTP and/or biotin-16-dUTP (Roche), and for alkaline gel electrophoresis and phosphorimaging by addition of [$\alpha$-$^{33}$P]-dCTP (Perkin-Elmer).

Stock solutions of the elongation inhibitor aphidicolin (Sigma) were prepared in DMSO (Sigma) and added to DNA replication reactions at a final concentration of 40 μM. Control reactions were supplemented with corresponding volumes of DMSO.

For nuclear transfer, nuclei were spun down after a 120-minute labelling incubation at 2000 ***g*** for 5 minutes, gently resuspended and washed with 1 ml SuNaSpBSA solution (250 mM sucrose, 75 mM NaCl, 0.5 mM spermine trihydrochloride, 0.15 mM spermidine tetrahydrochloride, 3% bovine serum albumin), spun down again, resuspended in a 50 μl volume of a corresponding new full reaction mix lacking only [$\alpha$-$^{33}$P]-dCTP, and incubated further for up to 60 minutes at 37°C.

### Molecular combing and fluorescence microscopy

For a preparation of chromosomal DNA fibres for DNA combing, the standard reactions were scaled-up fourfold and high-molecular-weight chromosomal DNA was purified after encapsulation in neutral LMP-agarose blocks as described previously (Marheineke et al., 2009; Marheineke and Hyrien, 2001; Marheineke et al., 2005). DNA was combed on silanised glass coverslips, and labelled replication tracks were visualised by fluorescence microscopy and analysed as detailed previously (Marheineke et al., 2009; Marheineke and Hyrien, 2001; Marheineke et al., 2005). Silanised coverslips for DNA combing were prepared as described (Labit et al., 2008).

Proportions of replicating nuclei were determined separately by fluorescence confocal microscopy (Krude, 2000; Szüts et al., 2003).

### Alkaline agarose gels and phosphorimaging

DNA replication reactions were stopped at the indicated times and radiolabelled nascent single-stranded DNA strands were analysed by alkaline gel electrophoresis exactly as published (Luciani et al., 2004). Nascent strands were visualised by phosphorimaging of the dried gels. Nascent-strand abundance was quantified with ImageJ software (v1.38; http://rsb.info.nih.gov/ij), using uncompressed phosphorimager files in .TIFF format.

### Statistics

Student's *t*-tests (two-tailed, two-sample of unequal variance) and analysis of variation (ANOVA; single factor, between groups) were performed with Microsoft Excel software.

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