Dynamic association of non-coding Y RNAs with chromatin before initiation regulates human DNA replication

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Summary

Non-coding Y RNAs are required for the initiation of chromosomal DNA replication in mammalian cells. It is unknown how they perform this function or if they associate with a nuclear structure during DNA replication. Here, we investigated the association of fluorescently labelled Y RNAs with chromatin during DNA replication in human cell nuclei and determined which replication proteins interact with immobilised Y RNAs under conditions that support chromosomal DNA replication. Our results show that Y RNAs associate with unreplicated euchromatin in late G1 phase cell nuclei before the initiation of DNA replication, but are displaced from nascent and replicated DNA present in replication foci. Endogenous Y RNAs are found associated with G1 phase nuclei and dissociated from G2 phase nuclei in intact human cells. Y RNAs interact with the origin recognition complex (ORC), the pre-replication complex (pre-RC), replication protein A (RPA) and other proteins implicated in the initiation of DNA replication, but not with proteins of the replication fork elongation complex. These data support a molecular 'catch and release' mechanism for Y RNA function during the initiation of chromosomal DNA replication, which complies with the replication licensing factor model for eukaryotic DNA replication control.

Introduction

Accurate replication of chromosomal DNA is fundamental for the propagation of the genome during the eukaryotic cell division cycle and for maintaining its integrity. A key regulation step is initiation, when two DNA replication forks are established at each replication origin (reviewed in: (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Takeda and Dutta, 2005; DePamphilis et al., 2006; Aladjem, 2007)). In this evolutionarily conserved process, the replication proteins ORC, Cdc6, Cdt1 and MCM2-7 associate in a stepwise manner with replication origins in G1 phase of the cell cycle prior to initiation, forming the pre-replicative complex (pre-RC). Initiation at these sites then depends on the activities of CDK and DDK protein kinases, and involves the recruitment of further proteins including Cdc45, GINS, MCM10, and replication protein A (RPA) to form the pre-initiation complex (pre-IC), whilst Cdc6 and Cdt1 are displaced. Finally, DNA primase and polymerases are recruited to begin DNA synthesis, marking entry into S phase. Additional levels of control have evolved in metazoan organisms (Arias and Walter, 2007), and proteins such as DUE-B, HMGA1a and the Ku autoantigen have been implicated in the initiation of DNA replication in mammalian cells (Norseen et al., 2008; Rampakakis et al., 2008; Thomae et al., 2008; Chowdhury et al., 2010).

Non-coding RNAs have emerged recently as additional factors regulating the initiation of eukaryotic DNA replication in several different species (reviewed in: (Krude, 2010)). In extracts from human somatic cells, reconstitution of chromosomal DNA replication in isolated cell nuclei requires a class of non-coding RNAs termed Y RNAs (Christov et al., 2006; Gardiner et al., 2009; Krude et al., 2009). These are a group of small structured stem-loop RNAs of about 100±15 nucleotides in length, which are conserved in vertebrate evolution (Pruijn et al., 1993; Farris et al., 1995; Mosig et al., 2007; Perreault et al., 2007). Humans have four Y RNAs (hY1, hY3, hY4 and hY5 RNAs), whilst other vertebrates have between one and four. Non-human vertebrate Y RNAs can functionally substitute for human Y RNAs in a cell-free DNA replication system (Gardiner et al., 2009), and all human Y RNAs can substitute for each other (Christov et al., 2006). This functional redundancy is due to an evolutionarily conserved double-stranded RNA domain present in all vertebrate Y RNAs, which is essential and sufficient to reconstitute DNA replication in vitro (Gardiner et al., 2009). The execution point for Y RNA function during chromosomal DNA replication has been identified as the initiation step (Krude et al., 2009). Degradation of hY3 RNA inhibits the de novo initiation of new DNA replication

forks in the mammalian cell-free system, but it does not influence progression rates of already established DNA replication forks (Krude et al., 2009).

The molecular mechanism, however, by which Y RNAs function in the initiation of mammalian chromosomal DNA replication is unclear. DNA replication is a structure-bound process in the cell nucleus (Berezney et al., 2000), yet Y RNAs are predominantly soluble and their intracellular localisation is controversial (Pruijn et al., 1997). In enucleation and cell fractionation experiments Y RNAs were found to be almost exclusively soluble and cytoplasmic in *Xenopus laevis* oocytes and mammalian cells (O'Brien et al., 1993; Peek et al., 1993; Simons et al., 1994). However, in in-situ hybridisation experiments they were detected in both the cytoplasm and the nucleus of mammalian cells (Matera et al., 1995; Farris et al., 1997). It is thus unknown if Y RNAs associate with template nuclei during DNA replication, or if they exert their function in solution. Here, we have addressed this question by studying the association of fluorescently labelled Y RNAs with template nuclei and with DNA replication proteins during DNA replication in a human cell-free system. Our results suggest a 'catch and release' mechanism for Y RNA function, by which Y RNAs associate with unreplicated chromatin prior to initiation, are essential for initiation, and then dissociate from these sites once DNA replication has initiated there.

Results

hY RNAs associate rapidly with euchromatin in late G1 phase nuclei

To enable a direct analysis of whether Y RNAs associate with nuclear structures during chromosomal DNA replication, we generated Alexa-Fluor-conjugated Y RNAs (Fig. 1). Each of the four human Y RNAs was synthesised in vitro from plasmid expression constructs using SP6 RNA polymerase (Christov et al., 2006; Gardiner et al., 2009), and these purified hY RNAs were covalently coupled at their 3' ends to an Alexa-Fluor dye (Fig. 1A). We then tested if these Alexa-Fluor-conjugated hY RNAs still reconstitute the initiation of chromosomal DNA replication in a human cell-free system (Fig. 1B). In this system, late G1 phase template nuclei initiate semi-conservative chromosomal DNA replication when incubated with with purified Y RNAs in the presence of two essential fractions from human cell extracts, termed QA and ArFT (which contain all of the soluble proteins required for the initiation of DNA replication)

(Christov et al., 2006; Gardiner et al., 2009). Each of the four Alexa-Fluor-conjugated hY RNAs increased the proportion of replicating late G1 phase template nuclei to the same extent as observed with unconjugated hY1 RNA (Fig. 1B). This increase is statistically significant (T-tests, $p<2.4x10^{-5}$, n=6-14). Therefore, conjugation of Y RNAs to Alexa-Fluor dyes at their 3' ends does not inactivate their function as DNA replication initiation factors.

In the next experiments, we investigated by confocal fluorescence microscopy whether these fluorescent hY RNAs associate with template nuclei under these in vitro conditions, which support the initiation of DNA replication (Fig. 1C, D). Alexa-Fluor-488-conjugated hY3 and hY5 RNAs associated with the late G1 phase nuclei within 1 minute of incubation in a non-uniform distribution. RGB line profiles across representative nuclei show that the amounts of chromosomal DNA (red) inversely correlated with the amounts of associated hY3 and hY5 RNAs (green; Fig. 1C, D). Areas of predominant Y RNA association therefore correspond to areas of decondensed DNA, which are characteristic for euchromatin. The pattern of nuclear association changed over time as hY RNAs were gradually lost from euchromatic sites during the 120 minutes of incubation and a proportion of hY RNA also became detected in nucleoli after 60 minutes. Alexa-Fluor-conjugated hY1 and hY4 RNA showed similar patterns (data not shown).

To control whether the association of Y RNAs with the nuclei is specific under these conditions, we also synthesised and analysed Alexa-Fluor-594-conjugated human 5S ribosomal RNA (Fig. S1A). As shown for uncoupled 5S rRNA (Christov et al., 2006), Alexa-Fluor-594-conjugated 5S rRNA did not reconstitute the initiation of chromosomal DNA replication (Fig. S1B). In contrast to hY RNAs, 5S rRNA did not detectably associate with a nuclear structure in late G1 phase template nuclei under these conditions (Fig. S1C), indicating that the observed association of Y RNAs with chromatin is specific.

In conclusion, these time-course experiments show that hY RNAs associate rapidly with euchromatin in late G1 phase template nuclei under experimental conditions which support the initiation of DNA replication in vitro.

hY1 co-localises with hY3 and hY4 RNA, but not with hY5 RNA

Next, we determined if the four different hY RNAs co-localise with each other during this reaction and co-incubated Alexa-Fluor-594-conjugated hY1 RNA with Alexa-Fluor-488-

conjugated hY3, hY4 or hY5 RNAs (Fig. 2). Both hY1 and hY3 RNAs localised to the same intranuclear sites on euchromatin at 1 min and 15 min of incubation. These sites showed variations in the hue of the yellow signal in the merged images, suggesting that the relative amounts of hY1 and hY3 RNA present at individual sites vary to some extent. hY4 RNA showed a similar co-localisation with hY1 RNA on euchromatic sites, and small additional amounts of hY4 RNA were also present in nucleoli.

In contrast, hY5 RNA did not co-localise with hY1 RNA under these conditions (Fig. 2). After 1 min of incubation, the majority of hY1 RNA associated with euchromatin whereas hY5 RNA was excluded from these sites and was enriched in the nucleoli. After 15 min of incubation, hY5 RNA was lost from the nucleus, while hY1 RNA was still present at its euchromatic sites. Therefore, the additional presence of hY1 RNA in this reaction changes the localisation of hY5 RNA (cf. Fig. 1D), suggesting that different hY RNAs have different binding affinities for euchromatin sites, and that in competition, hY1 beats hY5 for binding to these sites.

The loop domain is required for the targeting of hY RNA to euchromatin

The major difference between Y RNAs is the internal loop domain (Mosig et al., 2007; Perreault et al., 2007). This domain may therefore have a functional role by contributing to the relative binding affinities of Y RNAs to chromatin. We tested this hypothesis by analysing the association of mutant hY RNAs to chromatin (Fig. 3).

The structure of hY1 RNA with its evolutionarily conserved domains is shown in Fig. 3A as reference. The lower stem domain (LS) is formed by the pairing of the 3’ and 5’ ends of the RNA up to a central helical bulge, providing a binding site for the Ro60 protein (Chen and Wolin, 2004). The adjacent double-stranded upper stem domain is essential and sufficient for the function of Y RNAs in chromosomal DNA replication (Gardiner et al., 2009). The central loop domain (LP) is predominantly single-stranded, rich in polypyrimide tracks and contains small secondary stem loops. Finally, the single-stranded 3’-polyuridine tail provides a binding site for the La protein (Wolin and Cedervall, 2002).

Mutant ΔLS RNA contains a terminal deletion of the lower stem, ΔLP RNA an internal deletion of the loop, and the double mutant ΔLS/ΔLP RNA has deletions of both lower stem and internal loop. We synthesised these deletion mutants in vitro and following purification coupled them to Alexa-Fluor-488 (Fig. 3B). As shown for unlabelled hY1 RNA mutants (Gardiner et al., 2009),

these Alexa-Fluor-conjugated ΔLP, ΔLS or ΔLS/ΔLP RNAs supported the initiation of DNA replication in vitro (Fig. 3C).

Each of these three fluorescent mutant hY1 RNAs associated with chromatin in vitro within 1 min of incubation (Fig. 3D). The relative preference for intranuclear sites in these mutants, however, differs from that of full-length hY1 RNA. Deletion of either lower stem or internal loop resulted in a more homogeneous initial association across the nuclei, including the nucleoli. Furthermore, the double mutant had an additive phenotype, showing preferential initial binding to central heterochromatic sites including the nucleoli, in addition to their association with euchromatin. These data suggest that both lower stem and loop domains contribute to the selective association of Y RNAs with euchromatin.

To corroborate these findings independently, we co-incubated hY1 and ΔLS/ΔLP RNAs and compared their association in the same nuclei (Fig. 3E). Under these competitive conditions, hY1 RNA preferentially associated with euchromatic sites, whereas the double mutant bound chromatin without site specificity.

We conclude that the upper stem domain of Y RNAs (i.e. ΔLS/ΔLP RNA), which is essential and sufficient for Y RNA function in DNA replication (Gardiner et al., 2009), is capable of interacting with chromatin across the nucleus, albeit without specificity for particular sites. The loop and lower stem domains, which are not essential for DNA replication (Gardiner et al., 2009), contribute to the intranuclear localisation of the upper stem domain by providing selectivity for binding to euchromatin. By that molecular mechanism, hY RNAs can localise preferentially to chromosomal DNA at euchromatic sites in late G1 phase nuclei. As chromosomal DNA replication initiates at discrete euchromatic sites at the G1 to S phase transition, we went on to investigate the intranuclear localisation of hY RNAs in the context of DNA replication.

hY RNAs dissociate from replicated DNA

We tested first whether hY RNAs associate with sites of DNA replication in the cell-free system (Fig. 4). DNA replication foci were labelled by incorporation of digoxigenin-dUTP into nascent DNA and visualised by confocal immunofluorescence microscopy. Alexa-Fluor-488-conjugated hY3 RNA associated with chromatin in the late G1 phase template nuclei within 1 min of incubation, before DNA replication became detectable in vitro (Fig. 4A, top row). By 15 min, DNA replication had initiated in 28% of nuclei and by 120mins this number had increased

further to 35%. In both non-replicating and replicating nuclei, hY3 RNA remained associated but did not seem to co-localise with discrete DNA replication foci. When analysed at higher resolution (Fig. 4B), hY3 RNA was not detected at discrete DNA replication foci, , but was still concentrated at sites of unreplicated DNA. RGB line profiles confirmed that peak positions of hY3 RNA and DNA replication foci do not overlap with each other (Fig. 4B bottom panels). Identical patterns were observed for hY1, hY4 and hY5 RNA (supplementary Fig. S2). These data suggest that Y RNAs bind to unreplicated chromatin, and become displaced from these sites by DNA replication. We tested this hypothesis by measuring the amount of Alexa-Fluor-488-conjugated hY3 RNA associated with replicating and non-replicating nuclei (Fig. 4C). About half of the associated hY3 RNA dissociated from non-replicating nuclei during the first 60min of incubation. An additional, statistically significant amount of hY3 RNA dissociated from replicating nuclei, indicating that DNA replication stimulates Y RNA displacement from chromatin. We corroborated this finding by measuring the amounts of chromatin-associated hY3 RNA in the presence of the DNA replication inhibitor aphidicolin (Fig. 4D). In the presence of aphidicolin, no DNA replication foci were detectable in the nuclei (Fig. 4A, bottom row), and a significantly increased amount of hY3 RNA remained associated with the nuclei when compared to the untreated control group (Fig. 4D).

We therefore conclude that hY RNAs associate with unreplicated euchromatin in G1 phase nuclei, and once DNA replication is initiated in a Y RNA-dependent manner, DNA synthesis significantly stimulates the dissociation of Y RNA from replicating chromatin, including the discrete sites of DNA replication foci. We asked next if the lower stem and loop domains are required for the displacement of hY RNAs from replicated DNA. The three mutant RNAs ΔLS, ΔLP and ΔLS/ΔLP, which all support the initiation of DNA replication in this system, bound to unreplicated chromatin quickly, then dissociated from sites of replicated DNA, and remained detectable only on unreplicated DNA (Fig. 5). Therefore, the loop and lower stem domains are not required for the dynamic displacement of Y RNAs from replicated DNA.

Taken together, these observations suggest that Y RNAs are not a constituent of active DNA replication fork progression complexes at replication foci, nor do they remain on chromatin once it has been replicated. They bind to chromatin, however, before replication initiates, including those sites where initiation takes place. To identify a molecular link between Y RNAs and the established DNA replication machinery, we went on to investigate the interaction of human DNA replication initiation and elongation proteins with immobilised hY RNAs.

hY RNAs interact with DNA replication initiation factors

To obtain immobilised hY RNAs, we chemically coupled the oxidised 3' termini of purified hY RNAs covalently to agarose beads. These hY RNA beads were used for pull-down experiments of hY RNA-interacting proteins from human cell extracts (Fig. 6). Nuclear extract from human cells was first pre-cleared with uncoupled agarose beads and then adsorbed to affinity beads conjugated with one of the four hY RNAs. Uncoupled agarose beads were used as negative controls. Following incubation, the beads were extensively washed with physiological buffer and associated proteins were detected by Western blotting (Fig. 6).

The well-established Y RNA binding proteins Ro60 and La (Wolin and Cedervall, 2002; Chen and Wolin, 2004) interacted with all four hY RNAs under these conditions, but not to uncoupled agarose beads (Fig. 6A).

We discovered several novel interactions between hY RNAs and DNA replication initiation proteins with this approach (Fig. 6B). A subset of proteins constituting the pre-replication complex (pre-RC) associated with hY RNAs. First, the origin recognition complex ORC associated with hY RNAs. Of the tested subunits, ORC2 and ORC3 interacted with all four hY RNAs, whereas ORC4 and ORC6 showed reduced interaction with hY4 RNA when compared to the other three hY RNAs. Second, Cdt1 and Cdc6 proteins also associated with all four hY RNAs. We also detected interactions of all four hY RNAs with the DNA unwinding element binding protein DUE-B, the two subunits of the Ku autoantigen, Ku70 and Ku80, the single stranded DNA binding protein RPA and, albeit very weakly, the proliferating cell nuclear antigen PCNA. Finally, we detected interactions of HMGA1a with hY1 and hY3, but not with hY4 and hY5 RNA.

In contrast to these initiation proteins, we did not detect strong interactions between hY RNAs and proteins of the CMG and replisome complexes (Fig. 6C). Of the CMG complex, all six subunits of the MCM2-7 heterohexameric complex, CDC45 and all four human GINS subunits did not interact detectably with hY RNAs (Fig. 6C). DNA polymerases □, □ and □, MCM8, MCM10 and And-I did also not interact with hY RNAs. Several regulators of DNA replication including cyclin A, CDK2 and geminin did not interact either (Fig. 6C). However, we cannot exclude very weak interactions between hY RNAs and these latter proteins, which may have fallen short of the detection threshold for Western blotting.

Taken together, these pull-down experiments establish a novel molecular link between hY RNAs and proteins of the DNA replication initiation machinery, including the pre-RC, DUE-B and Ku, but not the elongation machinery.

hY RNAs co-localise with DNA replication initiation factors on unreplicated chromatin

In the next set of experiments, we assessed the physiological significance of these molecular interactions by investigating the intra-nuclear localisation of DNA replication initiation proteins in comparison to Y RNAs and DNA replication foci in the cell-free system (Fig. 7). Alexa-Fluor-594-conjugated hY1 RNA was incubated with late G1 phase template nuclei and fractions QA and ArFT for 1 and 60mins, respectively. After fixation, hY1 RNA was visualised directly, whereas candidate proteins and DNA replication foci were detected by indirect immunofluorescence microscopy. In pilot experiments, we discovered that the majority of available primary antibodies contained a ribonuclease activity that degraded Y RNA (Fig. S3), thus preventing any co-localisation studies. We therefore inactivated this RNAse activity with ribonuclease inhibitor for each antibody prior to immunofluorescence microscopy (Fig. S3).

The pre-RC proteins ORC2 and Cdt1 localised to many small discrete foci across the nuclei after 1min of incubation, before DNA replication initiated (Fig. 7A). At this time point, hY1 RNA generally overlapped with these proteins in the euchromatin, however, there were also discrete sites of these pre-RC proteins that did not contain hY1 RNA, predominantly located in peripheral and nucleolar heterochromatin. The Y RNA-interacting proteins DUE-B and Ku 70/80 were also concentrated at euchromatin sites that overlapped with hY1 RNA (Fig. 7A).

In addition, we analysed the localisation of hY1 RNA in comparison to the pre-RC protein MCM2 and the pre-IC protein CDC45 (Fig. 7A), even though they did not associate with hY RNAs in our pull-down assays (cf. Fig. 6). MCM2 and CDC45 were also found at discrete sites across the nuclei with a preferential enrichment in euchromatin that also overlapped with sites of hY1 RNA (Fig. 7A). Therefore, pre-RC and pre-IC proteins co-localise with hY1 RNA on euchromatin sites before DNA replication initiates.

Next, we investigated the localisation of hY1 RNA and pre-RC proteins in comparison to sites of DNA replication after it has been initiated in vitro (Fig. 7B). After an incubation of 60min, both Cdt1 and MCM2 proteins were detected with hY1 RNA at overlapping central sites in the nuclei. In contrast, DNA replication foci were detected at distinct sites in early replicating

euchromatin, which did not overlap with hY1 RNA, MCM2 or Cdt1. Therefore, hY1 RNA, MCM2 and Cdt1 proteins are all absent from sites of replicated DNA, yet they are still present at sites that have not been replicated.

Taken together, our in vitro results show that several DNA replication initiation proteins, that biochemically associate with hY RNAs, also co-localise with hY1 RNA on unreplicated chromatin, and dissociate from these sites when they are replicated. In the final set of experiments we therefore aimed to corroborate these findings in intact human cells.

Dynamic association of endogenous hY RNAs with G1 phase nuclei

To analyse the association of endogenous hY RNAs with cell nuclei before and after DNA replication in vivo, we synchronised EJ30 cells in the G1 and G2 phases of the cell cycle and measured the amounts of soluble cytosolic hY RNAs and those associated with cell nuclei (Fig. 8). As control, about 10% of total RNA was associated with cell nuclei in both G1 and G2 phase cells (Fig. 8B). As shown before (Christov et al., 2006; Christov et al., 2008), hY1 and hY3 RNA were most abundantly present in the extracts (not shown). Strikingly, between 20% and 70% of hY RNAs were associated with the nuclei in G1 phase cells (Fig. 8B). In contrast, only 10% or less of Y RNAs were still associated with G2 phase nuclei, demonstrating that hY RNAs associate with unreplicated G1 phase nuclei and become dissociated from replicated G2 phase nuclei in intact human cells.

Discussion

Y RNAs are essential for the initiation step of chromosomal DNA replication in a mammalian cell-free system (Christov et al., 2006; Gardiner et al., 2009; Krude et al., 2009). It was unknown, however, if they associate with a nuclear structure during this process. Here we have addressed this question and found that hY RNAs associate with chromatin before the initiation of DNA replication, interacting with proteins of the pre-replication complex, and dissociate from sites of replicated DNA. These data suggest a molecular mechanism for Y RNA function during DNA replication, which complies with the original licensing factor model for once-per-cell-cycle control of eukaryotic DNA replication (Laskey et al., 1981; Blow et al., 1987).

Y RNAs bind to euchromatin dynamically

Previous reports on the intracellular localisation of Y RNAs are controversial (Pruijn et al., 1997). Results of enucleation and microinjection experiments in Xenopus oocytes and mammalian cells suggested that Y RNAs are almost exclusively soluble and accumulate in the cytoplasm (O'Brien et al., 1993; Peek et al., 1993; Simons et al., 1994). Y RNAs were also detected, however, in the nucleus of asynchronous mammalian cells at the nucleolar periphery and the nucleolus by ultrastructural and fluorescence in situ hybridisation analysis (Matera et al., 1995; Farris et al., 1997). Our data show that hY RNAs associate rapidly and preferentially with euchromatin from a soluble pool before DNA replication initiates, and dissociate again from sites of replicated DNA after initiation has occurred. Consistent with the ultrastructural work, we have detected hY RNAs associated with nucleolar structures upon longer incubation in vitro. It is therefore likely that Y RNAs associate with euchromatin only transiently before the initiation of DNA replication at the G1 to S phase transition and persist longer in nucleolar heterochromatin. Such a transient interaction with euchromatin may have been missed in asynchronous cells used in the earlier studies (Matera et al., 1995; Farris et al., 1997).

All four Y RNAs bind euchromatin in late G1 phase nuclei, which is consistent with the observation that the four hY RNAs are functionally redundant for the initiation of chromosomal DNA replication in vitro (Christov et al., 2006; Gardiner et al., 2009). In co-incubation experiments, however, only hY1, hY3 and hY4 RNA show a high degree of overlap at euchromatin sites, whereas hY5 RNA preferentially binds to nucleoli. This may point towards an additional role for hY5 RNA, which is consistent with the literature. Gendron and colleagues have shown that Ro RNPs containing hY5 RNA preferentially localise to nuclei in cultured human cells (Gendron et al., 2001). More recently, Hogg and Collins have shown that hY5 RNA co-purifies with ribosomal protein L5 and its binding partner 5S rRNA (Hogg and Collins, 2007). They suggest that hY5 RNA may play a specialised role in 5S rRNA surveillance and quality control of ribosome biogenesis. As ribosome biogenesis takes place in the nucleoli, our observation would therefore be consistent with a dual function of hY5 RNA in both DNA replication and RNA surveillance.

The association of hY RNAs with nucleoli at later stages of the in vitro incubation could also be linked to their function in the initiation of DNA replication. Nucleolar DNA is replicated late in S phase and the cell-free system does not support initiation of DNA replication at these sites during a standard 3-hour incubation in vitro (Keller et al., 2002). The association of hY RNAs with nucleolar sites may thus be due to a specific association of hY RNAs with unreplicated nucleolar chromatin under these conditions, in preparation for later initiation at these sites. Systematic mutation of the domains of hY1 RNA established that both the loop and terminal stem domains confer specificity for hY1 RNA binding to euchromatin. The upper stem domain, which is essential and sufficient for the DNA replication function of vertebrate Y RNAs (Gardiner et al., 2009), binds to chromatin indiscriminately. Therefore, our experiments provide a novel and auxiliary function for the non-essential loop and lower stem domains in the context of regulating the DNA replication function of Y RNAs. Consistent with a model of modular RNP function (Hogg and Collins, 2008), its appears likely that these two domains provide a means for Y RNA diversification and their specialisation into associating with different nuclear domains. In particular, the large single-stranded poly-pyrimidine loops of hY1, hY3 and hY4 RNAs could confer the preferential interaction with euchromatin in late G1 phase nuclei. This, in turn, could facilitate the targeting of the functional determinant present in the upper stem to early replicating euchromatin sites at the G1 to S phase transition.

Y RNAs interact with initiation proteins

Despite an essential role of Y RNAs for chromosomal DNA replication in mammalian cells (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009; Krude et al., 2009; Langley et al., 2010), molecular links with the established DNA replication machinery have not been reported to date. We have shown here that all four human Y RNAs interact with DNA replication initiation proteins, including ORC, Cdt1, Cdc6, DUE-B and Ku and co-localise with these proteins on unreplicated chromatin before the initiation of chromosomal DNA replication in vitro. In contrast, replication fork proteins such the CMG complex (MCM2-7, Cdc45 and GINS), DNA polymerases and associated factors do not interact with Y RNAs at the molecular level. DNA replication forks in S phase cell nuclei are clustered into discrete replication foci (Berezney et al., 2000), and Y RNAs are not present at these foci. These molecular interactions therefore build upon our previous data showing that Y RNAs are

essential for the initiation step of DNA replication but not for chain elongation synthesis at replication forks (Krude et al., 2009).

In all eukaryotic systems, the origin recognition complex ORC is involved in the specification of DNA replication origins. It also recruits the pre-RC proteins Cdt1 and Cdc6 to these sites, which in turn recruit many copies of the MCM2-7 protein complex (Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Takeda and Dutta, 2005; DePamphilis et al., 2006; Aladjem, 2007). Our biochemical data show that Y RNAs interact with ORC, Cdt1 and Cdc6, but not with MCM2-7. We showed by confocal fluorescence microscopy that the pre-RC proteins ORC2, Cdt1 and MCM2, as well as the pre-IC protein CDC45, are present with hY RNAs at overlapping intranuclear sites on unreplicated chromatin before initiation of DNA replication in vitro. These data suggest that Y RNA function may be associated with origin specification and/or activation, pre-RC or pre-IC assembly, but not with DNA helicase activity of the GMC complex after initiation. ORC has already been shown to interact with other non-coding RNAs, which regulate initiation of DNA replication in distinct eukaryotic systems (Mohammad et al., 2007; Norseen et al., 2008; Norseen et al., 2009). Thus our data add to the concept of ORC as a target for regulation by non-coding RNAs during initiation.

The architectural chromatin protein HMGA1a has been shown to recruit ORC to viral, and possibly cellular, replication origins in an RNA-dependent manner (Norseen et al., 2008; Thomae et al., 2008). HMGA1a interacts only with hY1 and hY3, and not with hY4 or hY5 RNAs, yet all four hY RNAs are active during the initiation of DNA replication. It is therefore likely that the interaction between HMGA1a and Y RNAs is not essential for the initiation of chromosomal DNA replication in our system. It remains a possibility, however, that HMGA1a plays an auxiliary role in this process via an interaction with hY1 and hY3 RNA, perhaps at a subset of chromosomal sites.

The DUE-B protein binds to DNA unwinding elements (DUEs) at human replication origins and is implicated in the unwinding step of origin activation (Casper et al., 2005; Chowdhury et al., 2010). DUE-B binding to origins is dependent on ORC, and is in turn required for the loading of CDC45 (Chowdhury et al., 2010). Like Y RNAs, DUE-B is released from the DNA template during initiation (Chowdhury et al., 2010). Interestingly, DUE-B has structural similarity to aminoacyl-tRNA editing enzymes (Casper et al., 2005; Kemp et al., 2007), which may account for its molecular interaction with the highly structured hY RNAs. As with depletion of Y RNAs (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009), depletion of DUE-B leads to

an inhibition of DNA replication in cell-based and cell-free assays (Casper et al., 2005). A possible theory, therefore, is that the interaction between Y RNAs and DUE-B is functionally relevant for origin activation.

The abundant hetero-dimeric Ku70/80 protein is involved in many fundamental cellular processes including DNA repair, transcription and DNA replication (Tuteja and Tuteja, 2000; Downs and Jackson, 2004). In addition to its affinity for DNA ends and hairpins, Ku also binds to RNA (Yoo and Dynan, 1998; Tuteja and Tuteja, 2000), which could account for its strong molecular interaction with the hairpin-rich hY RNAs observed here. Ku has been shown to bind DNA replication origins in human cells and reduced levels of Ku result in cell growth defects and reduced rates of initiation of DNA replication (Sibani et al., 2005; Rampakakis et al., 2008). Therefore, Y RNAs and Ku may act in the same pathway regulating initiation of DNA replication in vertebrate somatic cells. However, there is a caveat to an essential role for Ku because Ku80 knockout mice are viable, despite showing growth defects (Nussenzweig et al., 1996; Zhu et al., 1996).

The single-stranded DNA binding protein RPA and the sliding clamp PCNA are both required for the initiation of human chromosomal DNA replication in vitro (Szüts et al., 2003; Szüts et al., 2005). RPA is involved in the DNA unwinding step of initiation and both proteins are required for the recruitment of DNA polymerases to activated origins in eukaryotes. Therefore, both proteins may interact with Y RNAs during initiation. However, both RPA and PCNA are also required for DNA chain elongation synthesis and consequently localise to DNA replication foci in this system (Szüts et al., 2003; Szüts et al., 2005). These dual roles for RPA and PCNA therefore necessitate further analysis to assess in how far their interaction with Y RNAs is functionally relevant for the initiation step.

A molecular mechanism for Y RNA function in DNA replication

Data presented here allow us to propose a molecular mechanism for Y RNA function during DNA replication. As shown here, soluble Y RNAs first associate with initiation-competent, unreplicated euchromatin in G1 phase. Most likely, this involves molecular interactions between Y RNAs and proteins of the initiation complex. As several initiation proteins interact with Y RNAs, it is therefore likely that Y RNAs interact with a protein complex rather than a single protein. Secondly, Y RNAs are essential for the initiation step of chromosomal DNA

replication, as shown before (Christov et al., 2006; Gardiner et al., 2009; Krude et al., 2009). This function is mediated by the upper stem domain of Y RNAs, which is evolutionarily conserved in vertebrates. Finally, once DNA synthesis is initiated in a Y RNA-dependent manner, Y RNAs dissociate from these sites when active DNA replications forks move away from the activated DNA replication origin and as shown here are dissociated from replicated chromatin and intact G2 phase nuclei. The fact that Y RNAs were not detected at replication foci and that their displacement is stimulated by DNA replication argues that they are either displaced from these sites or degraded.

Thus, Y RNAs employ a 'catch and release' mechanism for their essential function during the initiation of chromosomal DNA replication. The dynamic association of Y RNAs with unreplicated chromatin and their functional requirement for the initiation step of DNA replication mirrors that of the DNA licensing factors Cdt1 (RLF-B) and MCM2-7 (RLF-M) (Krude et al., 1996; Thommes et al., 1997; Tada et al., 2001). Their binding to unreplicated chromatin during G1 and S phase discriminates replicated from unreplicated sections of the genome and ensures that chromosomal DNA is replicated only once despite the presence of many replication origins per chromosome (Blow and Dutta, 2005; DePamphilis et al., 2006; Aladjem, 2007). Y RNAs thus comply with the 'activator' factor as proposed in the original model of replication licensing for once-per-cell-cycle control of eukaryotic DNA replication (Laskey et al., 1981; Blow et al., 1987),

Materials and Methods

Cell culture and fractionation

Human EJ30 bladder carcinoma cells were cultured and synchronised as described (Krude et al., 1997; Krude, 2000). Cytosolic and nuclear extracts of asynchronously proliferating human HeLa cells was obtained from Cilbiotech (Mons, Belgium). Fractionation of the cytosolic extract into protein fractions QA and ArFT was carried out by anion exchange chromatography on Q sepharose and arginine sepharose as described (Szüts et al., 2003; Szüts et al., 2005).

For a quantification of endogenous hY RNAs, synchronised G1 and G2 phase EJ30 cells were fractionated into nuclei and cytosolic extract by hypotonic treatment and dounce

homogenisation, followed by centrifugation at 3,000 x g (Krude et al., 1997). Nuclei were washed in hypotonic buffer and subsequently extracted in hypotonic buffer containing 0.5M NaCl (Krude et al., 1997). Total RNA was isolated from the nuclear and cytosolic extracts by phenol extraction and ethanol precipitation, and quantified by nanodrop spectrophotometry. Y RNAs in these extracts were quantified by RT-PCR as detailed previously (Christov et al., 2006), using serial dilutions of pure hY cDNA plasmids as calibrators.

Synthesis and purification of wild-type and mutant Y RNAs

Wild-type and mutant hY RNAs were synthesised in vitro as described (Christov et al., 2006; Gardiner et al., 2009). Sequences of the mutant ΔLS and ΔLP RNAs are as published (Gardiner et al., 2009), while mutant ΔLS/ΔLP is identical to USL RNA (Gardiner et al., 2009) with two additional uridine residues at its 3' terminus.

RNAs were purified by anion exchange chromatography on a MonoQ column (Amersham Biosciences). RNA was loaded in 50 mM Na-acetate, pH5.2; washed in (0.4 M NaCl, 50 mM Na-acetate pH5.2) and eluted with a linear 0.4-1 M NaCl gradient in 50 mM Na-acetate, pH5.2. The size and purity of all in vitro synthesised RNA was confirmed using 8 M urea denaturing polyacylamide gel electrophoresis and staining with SYBR Gold (Invitrogen) as described (Christov et al., 2006). Multimeric 100-nucleotide RNA molecules were used as molecular weight markers (Roche).

Coupling of hY RNAs to Alexa-Fluor fluorophores and agarose beads

Purified RNA was oxidised at the 3' end with $NaIO_4$. For coupling to fluorophores, oxidised RNA was incubated with a 5-fold molar excess of Alexa-Fluor-488 or Alexa-Fluor-594 hydrazide (Invitrogen). The coupling reaction was quenched by reduction with $NaBH_3CH$ and Alexa-Fluor-conjugated hY RNAs were isolated by ethanol precipitation. For coupling to agarose beads, oxidised RNA was incubated with adipic acid dihydrazide-agarose (Sigma) at a final concentration of 1mg/ml. This reaction was also quenched with $NaBH_3CH$.

RNA binding assays and Western blotting

Human cell extracts were pre-depleted with 6B Sepharose beads (Sigma) for 1 hour at 4˚C. Pre-depleted extracts were then incubated with RNA-coupled agarose beads or 6B Sepharose beads equilibrated in RNA binding buffer (20mM HEPES pH7.8, 100mM K-acetate, 2mM $MgCl_2$, 1mM DTT). After binding, beads were extensively washed with binding buffer and associated proteins were eluted by denaturation. Proteins were analysed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis. For Western blotting, the following primary antibodies against human proteins were used: anti-Ro60, La (both from Euro-Diagnostica); ORC2, ORC3, ORC6, HMGA1a (all from Dr A Schepers, Department of Gene Vectors, Munich, Germany); ORC4 (Santa Cruz sc-19726); Cdt1 (Abcam, ab22716 and ab83174); Cdc6 (MBL, K0069-3S); DUE-B (Dr M Leffak, Wright State University, Dayton, Ohio, USA); Ku70, Ku80 (both from Prof. S Jackson, University of Cambridge, UK); RPA (pAb-RPA1, (Szüts et al., 2003)); PCNA (PC10, Santa Cruz, sc-56); MCM2 (Abcam, ab6153); MCM7 (Santa Cruz, sc-9966); Pan-MCM (BD Pharmingen); Psf1, Psf2, Psf3, Sld5 (all from Prof. S Bell, University of Oxford, UK); Primase p58, Pol alpha p68, Pol alpha p166, Pol delta p124, CDC45 (all from Dr H-P Nasheuer, National University of Ireland, Galway); Pol epsilon p261 (Prof. F. Grosse, University of Jena, Germany); MCM8 (Santa Cruz, sc-47117); MCM10, And-I (both from Prof. A Dutta, University of Virginia, Charlottesville, USA); CDK2 (Santa Cruz, sc-163); Cyclin A (Santa Cruz, sc-53227); Geminin (Abcam, ab12147).

DNA replication in vitro and localisation assays

DNA replication reactions were performed as described (Christov et al., 2006). To study the nuclear localisation of Alexa-Fluor-conjugated hY RNAs in vitro, DNA replication reactions contained Alexa-Fluor-488 and Alexa-Fluor-594 conjugated hY RNAs at 300ng and 900ng per reaction, respectively. This difference was to compensate for a difference in signal strength between these fluorophores. To analyse DNA replication reactions, nuclei were fixed and spun onto polylysine-coated glass coverslips. Alexa-Fluor-conjugated hY RNAs were detected directly. Digoxigenin-labelled replicated DNA was detected by anti-digoxigenin fluorescein or rhodamine-conjugated F_{ab} fragments, and total DNA was counter-stained with propidium iodide as described (Krude, 2000; Szüts et al., 2003; Szüts et al., 2005). ORC2, Cdt1 and DUE-B were detected by indirect immunofluorescence using the primary antibodies described above. Ku was detected by indirect immunofluorescence using anti-Ku70/80 (Abcam, ab3108). Antibodies were pre-incubated with an experimentally determined amount of RNAse inhibitor

(Roche) prior to use (Fig. S2). Alexa-Fluor-488 or -633-conjugated goat anti-mouse and anti-rabbit IgGs and F_{ab} fragments (Invitrogen) were used as secondary antibodies. Confocal fluorescence microscopy was performed on a Leica SP1 microscope using 63x lens magnification with additional optical zoom for low- and high-resolution analysis, respectively. Individual channels were recorded sequenially, except for co-localisations of hY RNAs with DNA or replication foci, which were recorded simultaneously.

Computing and image analysis

Secondary RNA structures were calculated using the Mfold v3.2 RNA folding algorithm (web server at: http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi) under default conditions (Mathews et al., 1999; Zuker, 2003). T-tests (two-tailed, two-sample of unequal variance) and analysis of variation (ANOVA, single factor, between groups, alpha=0.05) were performed with Microsoft Excel:mac v.X software. Quantification of chromatin-associated Alexa Fluor-conjugated hY RNAswas performed by measuring the integrated pixel densities of individual nuclei on original greyscale TIFF files of single-channel confocal images with ImageJ v1.43r software (http://rsb.info.hih.gov.ij), using plugins obtained from MacBiophotonics (http://www.macbiophotonics.ca). Background subtraction was applied with 2 stdev from the mean prior to analysis. RGB line profiles were obtained from merged images using ImageJ v1.43r software using plugins from MacBiophotonics.

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Figures

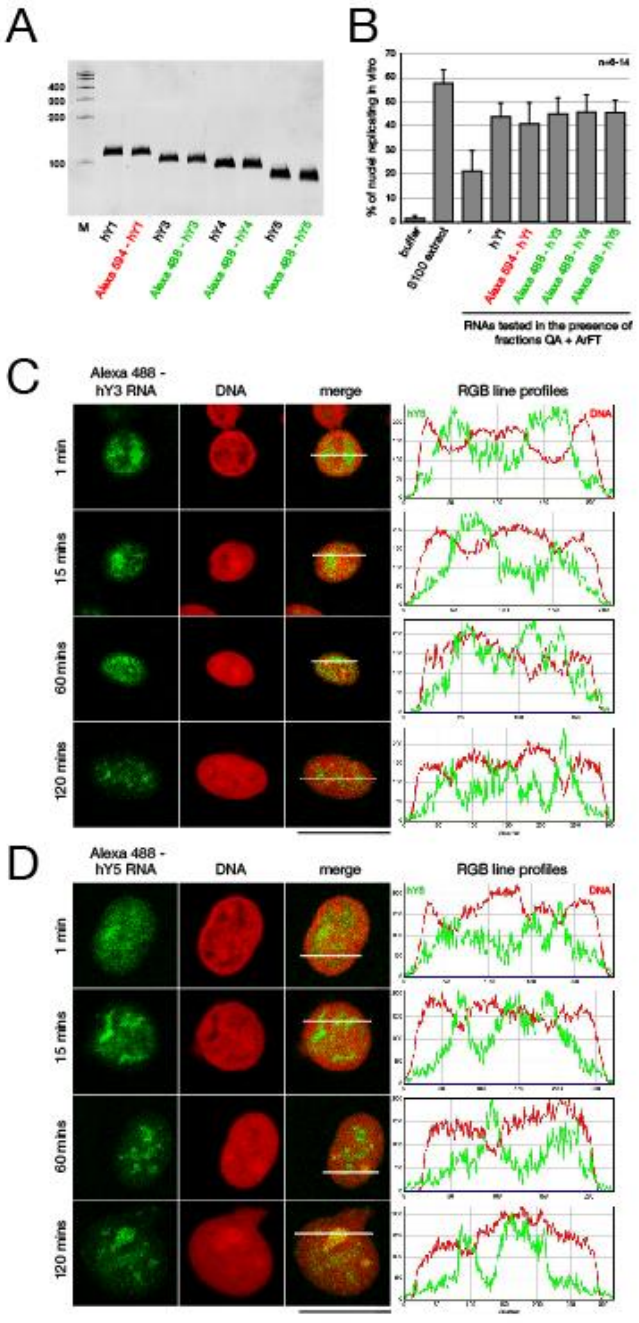

Figure 1 Human Y RNAs associate with euchromatin in vitro. *(A) Synthesis of Alexa-Fluor-conjugated hY RNAs. The four hY RNAs were synthesised by in vitro transcription, purified by anion-exchange chromatography and covalently coupled to Alexa-Fluor hydrazides at their 3' termini as indicated. RNAs were separated on a denaturing 8% polyacrylamide gel and stained with SYBR Gold. Alexa-Fluor-conjugated hY RNAs are highlighted in colour. M, 100nt nucleotide RNA ladder. (B) Alexa-Fluor-conjugated hY RNAs support DNA replication in vitro. Template nuclei from late G1-phase human cells were incubated with fractions QA and ArFT in the presence of the indicated hY RNAs (Christov et al., 2006). Replication buffer and unfractionated cytosolic extract (S100) served as negative and positive controls, respectively. Proportions of replicating nuclei were determined by immunofluorescence microscopy. Mean values and standard deviations are shown for each experiment. (C) Dynamic association of hY RNAs with late G1 phase nuclei in vitro. Alexa-Fluor-488-conjugated hY3 RNA and hY5*

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RNA were incubated with late G1 phase nuclei in the presence of fractions QA and ArFT for the indicated times. Nuclei were fixed and fluorescent hY RNA was directly visualised by confocal microscopy. DNA was counter-stained with propidium iodide. Single channels and merged images of Alexa-Fluor-488-conjugated hY3 and hY5 RNAs (green) and chromosomal DNA (red) are shown of representative nuclei. Scale bar, 10 µm. RGB line profiles across nuclei are shown on the right (green, hY RNAs; red, DNA); the analysed profiles are boxed on the corresponding merged RGB images.

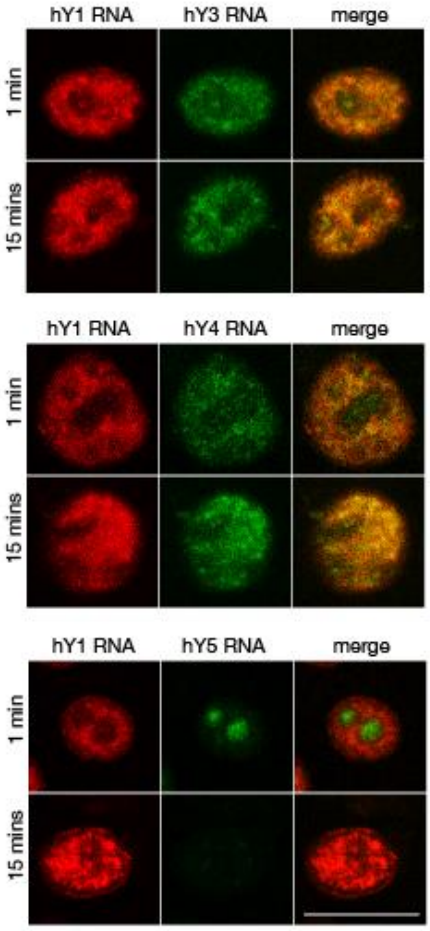

Figure 2 Co-localisation of human Y RNAs. *Alexa-Fluor-conjugated hY RNAs were co-incubated with late G1 phase nuclei in the presence of fractions QA and ArFT for 1 min and 15 min, as indicated. Nuclei were fixed and fluorescent hY RNAs were directly visualised by confocal microscopy. Single channels and merged images of Alexa-Fluor-594-conjugated hY1 RNA (red) and Alexa-Fluor-488-conjugated hY3, hY4 and hY5 RNAs (green) are shown for representative nuclei. Scale bar, 10 µm.*

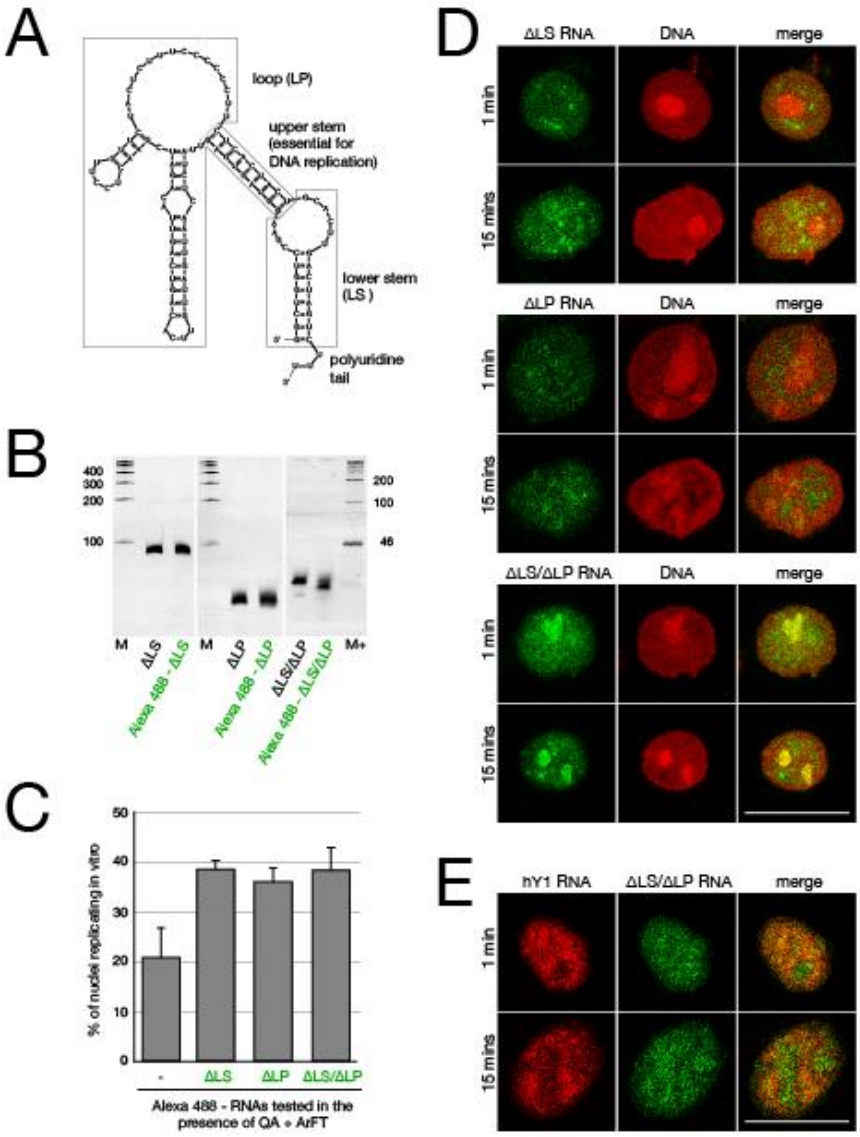

Figure 3 The loop and lower stem domains confer specificity to the association of hY RNAs with euchromatin. *(A) Domain structure of hY1 RNA. The nucleotide sequence and the most stable secondary structure of hY1 RNA is shown as determined by the Mfold algorithm. The evolutionarily conserved four domains present in all vertebrate Y RNAs are indicated. (B) Synthesis of Alexa-Fluor-conjugated hY1 RNA mutants. The three mutant hY1 RNAs were synthesised and analysed as detailed for Fig. 1. M, 100nt nucleotide RNA ladder, M+ contains an additional 46nt RNA nucleotide. (C) Alexa-Fluor-conjugated mutant hY1 RNAs support DNA replication in vitro. The indicated RNA molecules were analysed as detailed for Fig. 1. (D) Dynamic association of Alexa-Fluor-conjugated mutant hY1 RNAs with late G1 phase nuclei in vitro. Mutant hY1 RNAs were incubated for 1 and 15 mins with late G1 phase nuclei in the presence of fractions QA and ArFT. Data were analysed as detailed for Fig. 1. Scale bar, 10 µm. (E) Alexa-Fluor-594-conjugated wild-type hY1 RNA and Alexa-Fluor-488-conjugated mutant ΔLS/ΔLP RNA were co-incubated with late G1 phase nuclei in the presence of fractions QA and ArFT for 1 min and 15 min, as indicated. RNAs were directly visualised as detailed for Fig. 2. Scale bar, 10 µm.*

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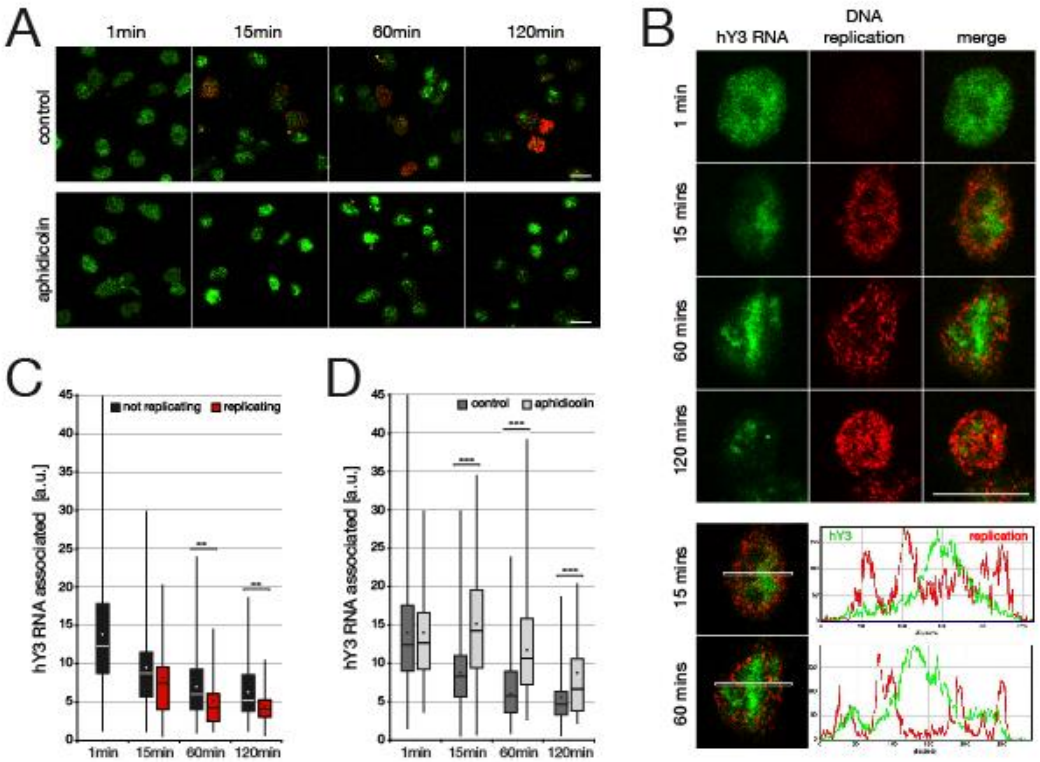

Figure 4 DNA replication stimulates displacement of hY3 RNA from chromatin. *Alexa-Fluor-488-conjugated hY3 RNA was incubated with late G1 phase template nuclei in the presence of fractions QA and ArFT for the times indicated. Nascent DNA was labelled by incorporation of digoxigenin-16-dUTP. Nuclei were fixed, fluorescent RNAs were directly visualised (green signal), and DNA replication foci were detected by anti-digoxigenin rhodamine-conjugated F_{ab} fragments (red signal). (A) Low-resolution immunofluorescence microscopy. Representative fields from reactions performed in the absence and presence of 50μM aphidicolin are shown. Scale bars, 10 μm. (B) High-resolution immunofluorescence microscopy. Representative individual nuclei are shown for each time point. Scale bar, 10 μm. RGB line profiles across selected nuclei are shown on the bottom panels; the analysed profiles are boxed on the corresponding RGB images. (C) Quantification of chromatin-associated hY3 RNA in replicating and non-replicating nuclei. For each individual nucleus of the control reaction, the amount of chromatin-associated hY3 RNA was measured as integrated fluorescence intensity as detailed under Materials and Methods. Box and whisker plots for each distribution are shown; the median is indicated by a horizontal line and the mean by an asterisk within the boxed 25-75th percentile. Statistically significant differences between matched distributions as determined by ANOVA are bracketed: two asterisks indicate $p<0.002$; with $n\geq75$ for non-replicating and $n\geq39$ for replicating nuclei. (D) Quantification of chromatin-associated hY3 RNA in the absence and presence of 50μM aphidicolin. The amount of chromatin-associated hY3 RNA is analysed and shown as for panel C; three asterisks indicate $p<0.0002$; with $n\geq116$ for the control and $n\geq75$ for the aphidicolin-treated reaction.*

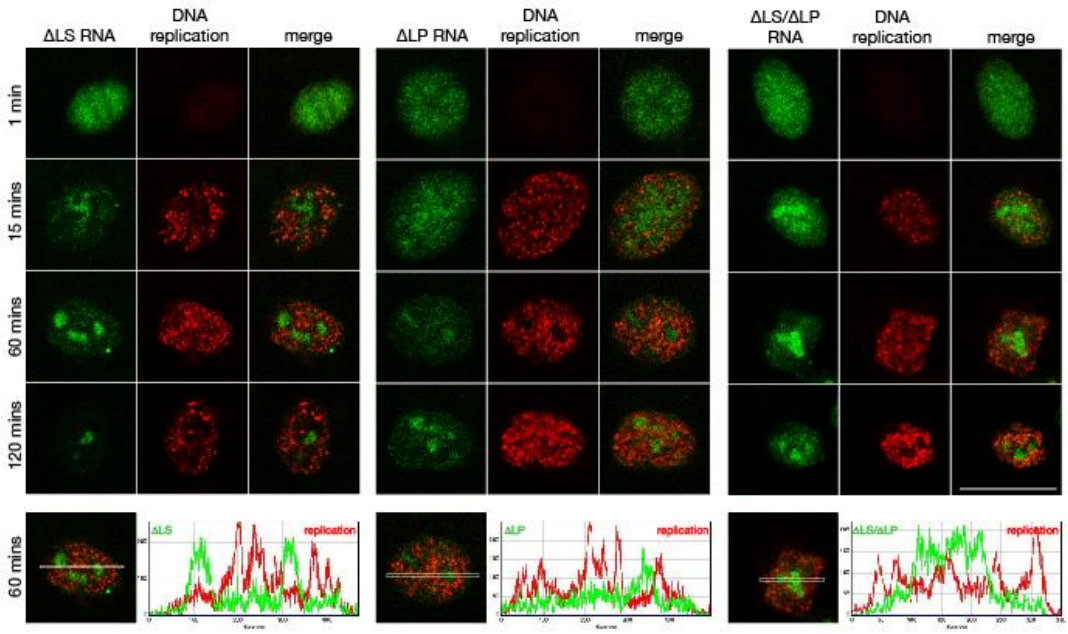

Figure 5 The loop and lower stem domains are not required for the exclusion of hY RNAs from DNA replication foci. *The three Alexa-Fluor-conjugated mutant hY1 RNAs were incubated with late G1 phase template nuclei in the presence of fractions QA and ArFT for the times indicated. Intranuclear sites of associated mutant Y RNAs and of DNA replication foci were analysed as detailed for Fig. 4B. Scale bar, 10 µm.*

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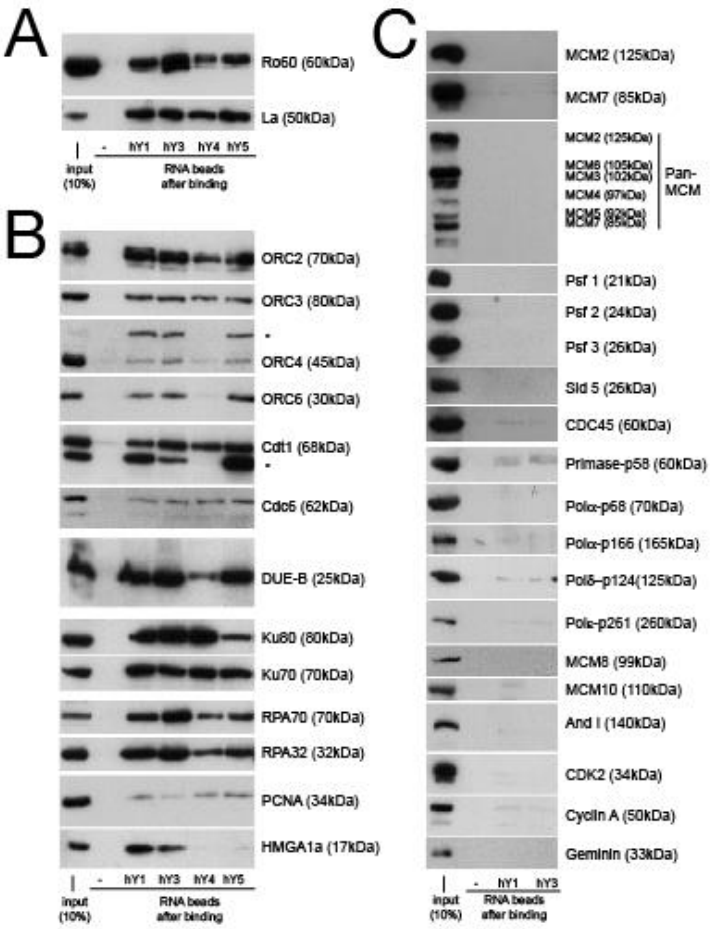

Figure 6 Interactions between DNA replication initiation proteins and hY RNA. *The four hY RNA were covalently coupled to aldipic acid dihydrazide agarose beads and incubated with HeLa cell extracts under the experimental conditions that support DNA replication in vitro. hY RNA beads were isolated, washed, and associated proteins were identified by Western blotting. 6B Sepharose beads were used as negative control (-). As reference, 10% of the input cell extract was loaded on the gel. The apparent molecular weights are given for each protein. (A) Positive controls: binding analysis of the Y RNA binding proteins Ro60 and La. To allow detection of Ro60, cytosolic extract was spiked with 10ng/µl his-tagged recombinant Ro60 protein purified from overexpressing bacteria (Belisova et al., 2005). (B) Positive results: binding analysis of DNA replication initiation proteins to immobilised hY1, hY3, hY4, and hY5 RNAs. Asterisks indicate cross-reacting bands detected by the respective antibodies. (C) Negative results: binding analysis of CMG proteins, DNA polymerases and additional DNA replication proteins to immobilised hY1 and hY3 RNAs.*

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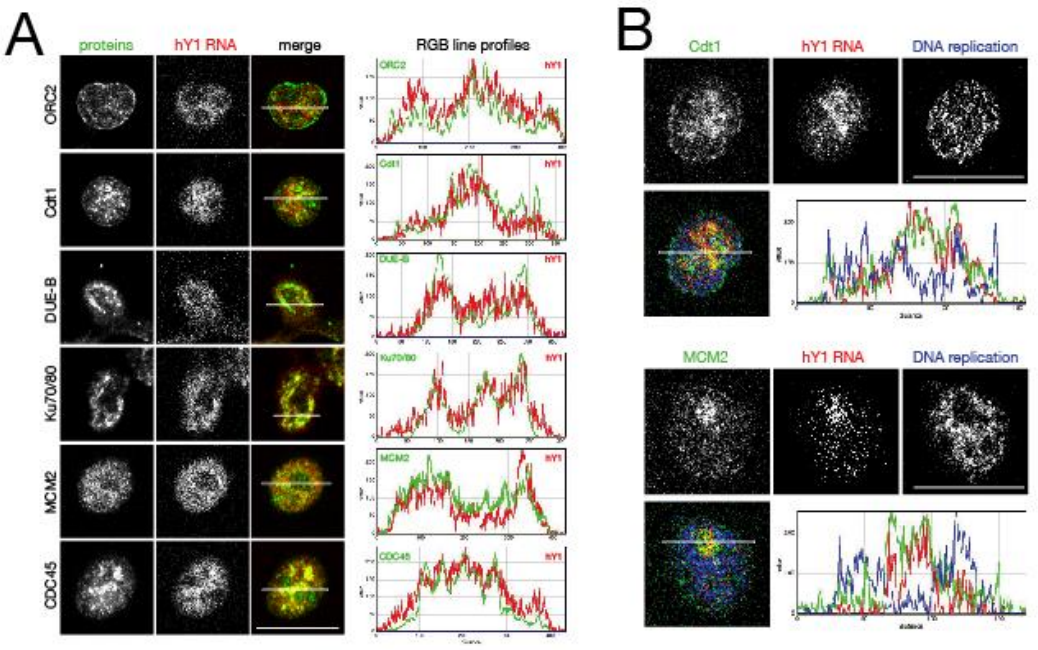


Figure 7 Co-localisation of DNA replication initiation proteins with hY1 RNA. *(A) Co-localisation analysis of hY1 RNA with initiation proteins before initiation of cghromosomal DNA replication in vitro. Alexa-Fluor-594-conjugated hY1 RNA was incubated with late G1 phase nuclei in the presence of QA and ArFT for 1min. Nuclei were fixed and fluorescent hY1 RNA was visualised directly. Nucleus-associated proteins were detected by indirect confocal immunofluorescence microscopy using Alexa-Fluor-488-conjugated secondary antibodies, as indicated. To inhibit antibody-associated ribonuclease activity (Fig. S2), primary antibodies were pre-treated with RNase inhibitor. Scale bar, 10 µm. (B) Co-localisation analysis of hY1 RNA with Cdt1 and MCM2 proteins in relation to DNA replication foci after initiation of chromosomal DNA replication in vitro. Late G1 phase nuclei were incubated with Alexa-Fluor-594-conjugated hY1 RNA, fractions QA and ArFT and digoxigenin-16-dUTP for 60 min. Individual channels of a three-colour confocal analysis are presented separately as greyscale images and as a merged RGB image. RGB line profiles across these nuclei are shown to the right (green, Cdt1 and MCM2 proteins; red, hY RNAs; blue DNA replication foci); the analysed profiles are boxed on the corresponding merged RGB images. Scale bars, 10 µm.*

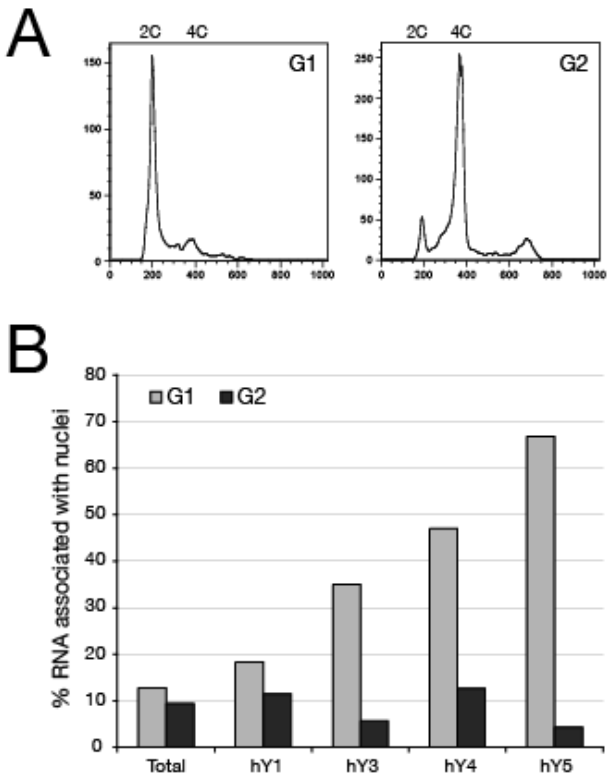
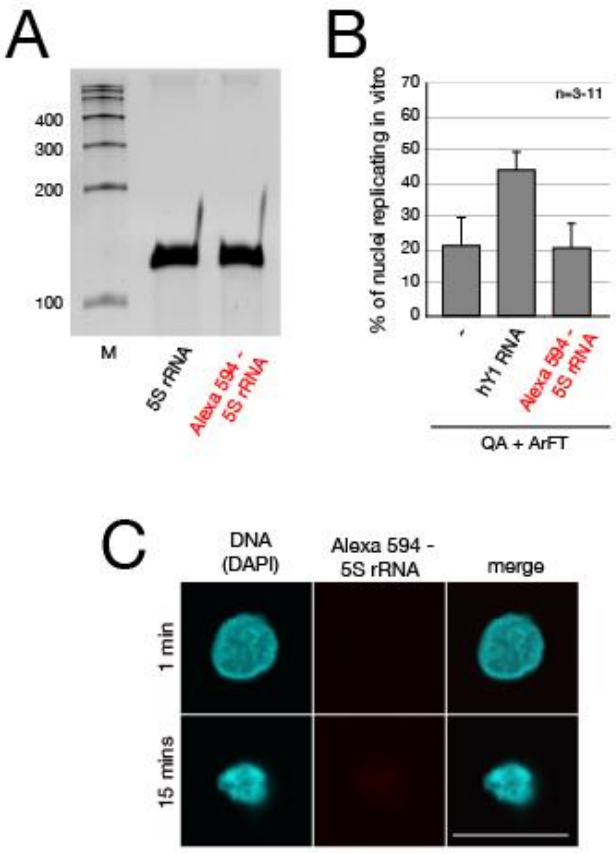


Figure 8 Endogenous hY RNAs are associated with G1 phase nuclei and dissociated from G2 phase nuclei in human cells. *EJ30 cells were synchronised in G1 and G2 phases of the cell cycle. (A) Determination of nuclear DNA content by flow cytometry. Positions of unreplicated 2C and replicated 4C DNA content are indicated for the G1 and G2 phase cell nuclei as indicated. (B) Association of endogenous hY RNAs associated with synchronised cell nuclei. Total RNA and individual hYs RNAs were quantified in cytosolic and nuclear extracts from G1 and G2 phase cells. Percentages of total RNA associated with the nuclei are shown for the indicated RNAs (mean values of 2-3 independent data acquisitions).*



Supp Figure S1 Human 5S ribosomal RNA does neither support DNA replication nor associate with chromatin in vitro. *(A) Synthesis of Alexa-Fluor-conjugated 5S rRNA. 5S rRNA was synthesised by in vitro transcription, purified by anion-exchange chromatography and covalently coupled to Alexa-Fluor-594 hydrazide at the 3' terminus. Uncoupled and coupled RNAs were separated on a denaturing 8% polyacrylamide gel and stained with SYBR Gold. RNA markers were a multimeric 100nt ladder. (B) Alexa-Fluor-conjugated 5S rRNA does not support DNA replication in vitro. Template nuclei from late G1-phase human cells were incubated with protein fractions QA and ArFT in the absence and presence of the indicated RNAs (Christov et al., 2006). hY1 RNA was used as positive control. Proportions of replicating nuclei were determined by immunofluorescence microscopy. Mean values and standard deviations are shown for each experiment. (C) 5S rRNA does not associate with late G1 phase nuclei in vitro. Alexa-Fluor-594-conjugated 5S rRNA was incubated with late G1 phase nuclei in the presence of protein fractions QA and ArFT for the indicated times. Nuclei were fixed and Alexa-Fluor-594-conjugated 5S rRNA was directly visualised by confocal microscopy (red). DNA was counter-stained with DAPI (turquoise). Single channels were recorded sequentially, which are shown together with merged images of representative nuclei. Scale bar, 10 µm.*

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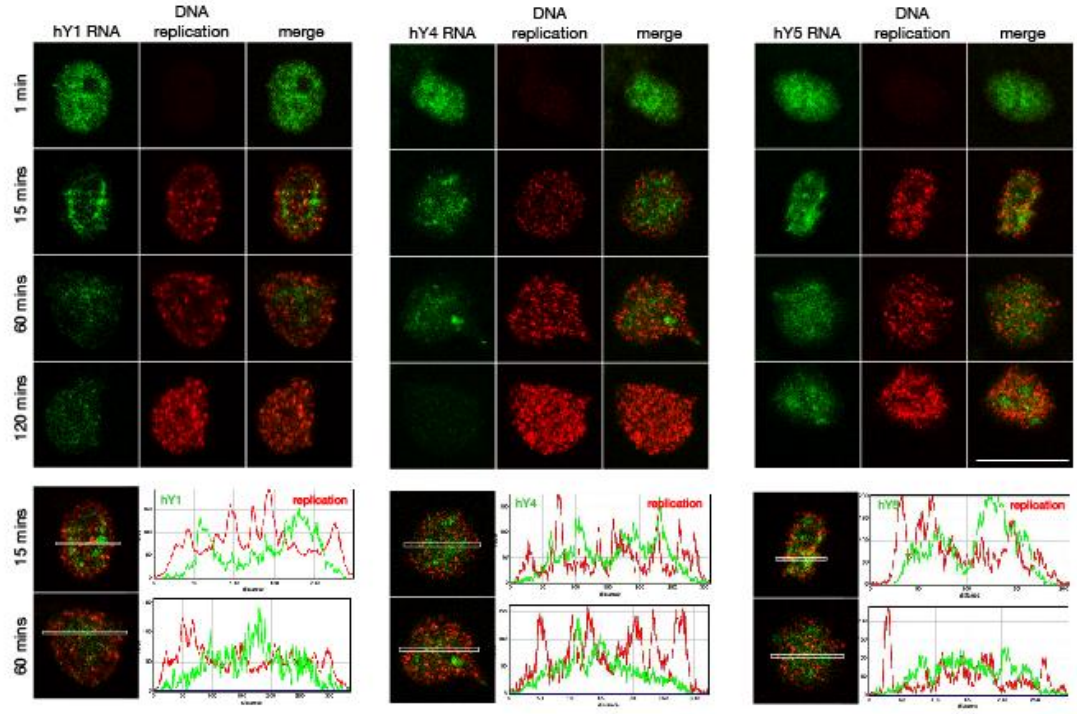
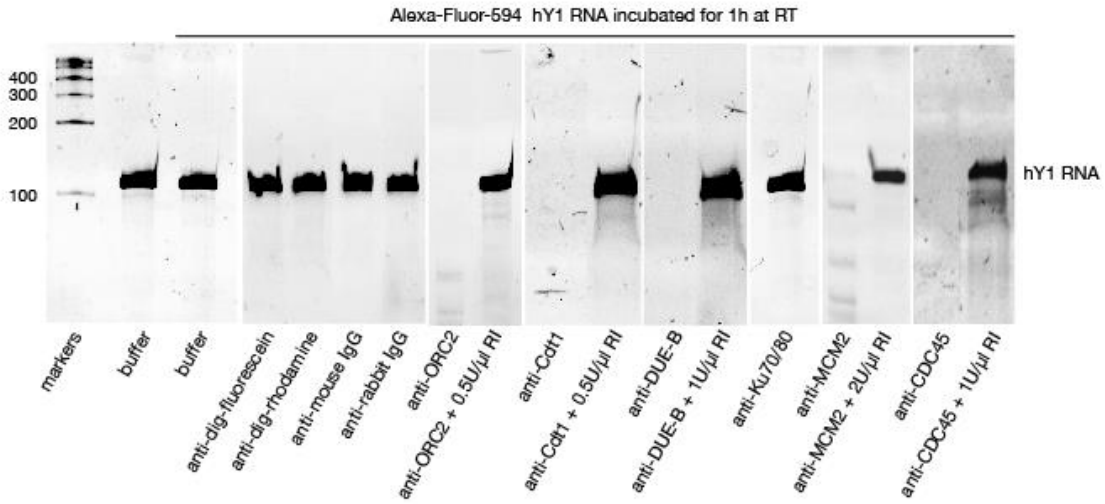
SUPPLEMENTARY FIGURES



Supp Figure S2 Human Y1, hY4 and hY5 RNAs are not associated with DNA replication foci. *Alexa-Fluor-594-conjugated hY1 RNA, Alexa-Fluor-488-conjugated hY4 and hY5 RNA were incubated with late G1 phase template nuclei in the presence of fractions QA and ArFT for the times indicated. Nascent DNA was labelled by incorporation of digoxigenin-16-dUTP. Nuclei were fixed, fluorescent RNAs were directly visualised (displayed in green channel), and DNA replication foci were detected by anti-digoxigenin F_{ab} fragments (displayed in red channel) by high-resolution confocal immunofluorescence microscopy. Representative individual nuclei are shown for each time point. Scale bar, 10 µm. RGB line profiles across selected nuclei are shown on the bottom panels; the analysed profiles are boxed on the corresponding RGB images.*



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Supp Figure S3 Analysis of antibody-associated ribonuclease activity and its inhibition by RNase inhibitor treatment. *The indicated primary and secondary antibodies were pre-incubated at the concentrations used for immunofluorescence microscopy with and without RNase inhibitor (RI) at the indicated concentrations for 1h at room temperature. 300ng of Alexa-Fluor-594-conjugated hY1 RNA was then incubated for 1h at room temperature with these pre-treated antibodies. RNA degradation was assessed by 8 M urea gel electrophoresis and staining with SYBR gold. Purified input RNA, and RNA incubated in staining buffer were used as controls.*