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The chromatin remodeling complex NoRC controls replication timing of rRNA genes

Junwei Li[1], Raffaella Santoro[1], Karel Koberna[2,3] and Ingrid Grummt[1,*]

[1]Division of Molecular Biology of the Cell II, German Cancer Research Center, Heidelberg, Germany, [2]Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Charles University, Prague, Czech Republic and [3]Institute of Cellular Biology and Pathology, 1st Faculty of Medicine, Charles University, Prague, Czech Republic

The ATP-dependent chromatin remodeling complex NoRC silences a fraction of mammalian ribosomal RNA genes (rDNA) by establishing heterochromatic structures at the rDNA promoter. Here we show that NoRC also plays a role in replication timing of rDNA. rDNA is replicated in a biphasic manner, active genes (~60%) replicating early and silent ones (~40%) replicating late in S-phase. The chromatin structure that marks active and silent rDNA repeats is propagated during cell division. To examine the function of NoRC in epigenetic inheritance and replication timing, we have monitored the chromatin structure, transcriptional activity and replication timing of rDNA in a cell line that moderately overexpresses NoRC. NoRC is exclusively associated with late-replicating rDNA arrays. Overexpression of NoRC silences rDNA transcription, reduces the size and number of nucleoli, impairs cell proliferation and resets replication timing from early to late. The results demonstrate that NoRC is an important determinant of replication timing and epigenetic marks are heritably maintained through DNA replication.

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Introduction

In eukaryotes, the tandem ribosomal RNA genes (rDNA) repeats exist in two distinct types of chromatin, an 'open' one that is permissive to transcription and a 'closed' one that is transcriptionally refractive. Though the mechanisms that establish and maintain the active and silent state of the rDNA locus are poorly understood, recent experiments have shown that the promoter of active rRNA genes is unmethylated and associated with histones that are highly acetylated. The opposite pattern is predominant among silent genes (Santoro *et al*, 2002). Thus, active and inactive rRNA genes are demarcated both by their pattern of DNA methylation and by specific modifications of their associated histones, a finding that links the 'histone code' (Strahl and Allis, 2000) to the 'cytosine methylation code' (reviewed by Bird, 2002).

The concept of the epigenetic code implies that the information of histone modifications and DNA methylation is memorized, that is, transmitted from one cell generation to the next, although the DNA must be unpackaged during replication. Thus, the events occurring during S-phase provide a 'window of opportunity' for either changing or maintaining existing epigenetic states. This raises the question of how the active and silent states of chromatin are established anew after each round of replication. In mammals, the replication of chromatin is temporally compartmentalized. Housekeeping genes undergo replication in the first half of S-phase in all cell types, whereas the replication of many tissue-specific genes is developmentally controlled, being late in most tissues but early in the tissue of expression (reviewed by Gilbert, 2002; Goren and Cedar, 2003). Despite the intriguing relationship between the eu- or heterochromatic features and the timing of DNA replication, the mechanisms that connect chromatin structure, transcriptional activity and replication timing have not yet been fully elucidated. One possibility is that genes undergoing replication in early S-phase are exposed to factors that are required for the formation of euchromatin and active transcription complexes, whereas genes that replicate late in S-phase experience a different nuclear environment that favors the generation of a repressive chromatin structure. Alternatively, the structure of heterochromatin may delay the initiation of replication.

With regard to rDNA, we have previously shown that NoRC, an ATP-dependent chromatin remodeling complex that has been identified in mouse and human cells, plays a key role in the establishment of the silent state of rDNA (Strohner *et al*, 2001; Santoro *et al*, 2002). NoRC consists of two subunits, SNF2h, the mammalian homolog of *Drosophila* ISWI, and TIP5, a >200 kDa protein that shares several domains with the largest subunits of human ATP-dependent chromatin remodeling complexes ACF, WCRF, CHRAC and WICH (Ito *et al*, 1999; Bochar *et al*, 2000; LeRoy *et al*, 2000; Poot *et al*, 2000; Bozhenok *et al*, 2002). NoRC silences rDNA transcription through recruitment of histone-modifying and DNA-methylating activities to the rDNA promoter, thereby establishing and/or maintaining a repressive higher-order chromatin structure (Santoro *et al*, 2002; Zhou *et al*, 2002). The results suggest that NoRC serves as a scaffold that coordinates the activities of several macromolecular complexes that modify histones, methylate DNA and establish a 'closed' chromatin state.

Given that the time of replication origin firing correlates with the heritably active or repressed state of a gene locus, it is reasonable to predict that late-replicating rRNA genes are constitutively silent. If this was the case, the remodeling complex NoRC could be a direct determinant of late origin

*Corresponding author. Division of Molecular Biology of the Cell II, German Cancer Research Center, DKFZ, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. Tel.: +49 6221 423441; Fax: +49 6221 423404; E-mail: I.Grummt@dkfz-heidelberg.de

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firing. To address this issue, we have isolated nascent DNA molecules from NIH3T3 cells at different times during S-phase and monitored histone acetylation, transcriptional activity, CpG methylation and NoRC occupancy at newly replicated rDNA. The results reveal that rDNA repeats replicate in a biphasic manner, euchromatic active genes replicating early and heterochromatic silent ones replicating late in S-phase. Consistent with NoRC serving an essential role in rDNA heterochromatin formation, NoRC is exclusively associated with late-replicating rDNA copies. Moreover, we show that moderate overexpression of NoRC reduces the fraction of active rDNA transcription units, decreases the number of nucleoli and impairs cell proliferation. Notably, overexpression of NoRC shifts replication timing from early to late, demonstrating that NoRC is an important determinant of replication timing. The results reveal a functional interplay and mechanistic connection between the activity of a chromatin remodeling complex, replication timing and rDNA transcription, and contribute to our understanding how epigenetic states are established and maintained *in vivo*.

Results

NoRC is associated with late-replicating rDNA

To investigate the relationship between rDNA transcription and replication timing, NIH3T3 cells were synchronized at the G1/S border with aphidicolin, released from the aphidicolin block and nascent DNA was pulse-labeled with bromodeoxyuridine (BrdU) at different times during S-phase. To monitor replication timing, newly replicated DNA was isolated by immunoprecipitation with anti-BrdU antibody and the relative abundance of rDNA in each sample was analyzed by PCR. To ensure reproducibility, three independent experiments were performed and nascent DNA was analyzed by either semiquantitative PCR or real-time PCR. To control replication timing, an early-replicating marker gene (α-globin) and X141, a late-replicating locus of constitutive heterochromatin (Azuara *et al*, 2003), were included in these experiments (Figure 1). As expected, α-globin was abundant in early-replicating fractions, whereas the abundance of X141 peaked in late S-phase. Significantly, rDNA was found to be replicated in two distinct phases, approximately 60% being replicated early (~3 h after release from the aphidicolin block) and 40% late in S-phase (6–7 h after release).

Owing to the established correlation between replication timing, chromatin structure and transcriptional activity, it is reasonable to assume that the early-replicating fraction contains actively transcribed ribosomal gene copies, while the late-replicating fraction is enriched in silent rRNA genes. Active and silent rRNA genes can be distinguished by the degree of DNA methylation, active genes being unmethylated and sensitive to digestion by *Hpa*II, whereas silent genes are methylated, that is, resistant to *Hpa*II digestion (Santoro and Grummt, 2001; Santoro *et al*, 2002). To compare the methylation state of early- and late-replicating rDNA, BrdU-labeled DNA from cells that were harvested 3 and 7 h after release from the aphidicolin block was digested with *Hpa*II or *Msp*I and subjected to PCR amplification using forward primers that map upstream or downstream of the CCGG sequence upstream of the transcription start site. If the promoter is

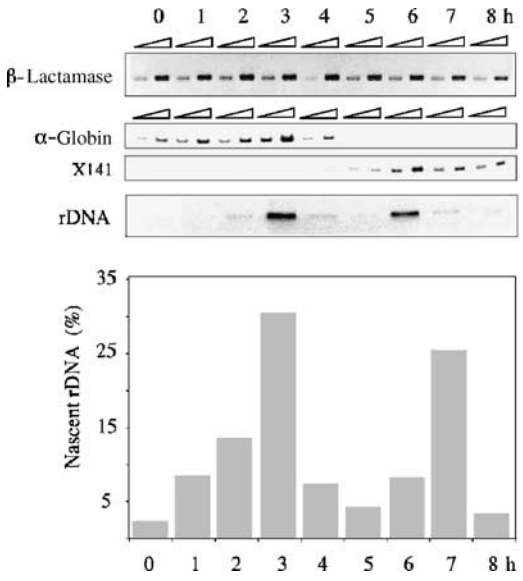

Figure 1 Biphasic replication of rDNA in synchronized cells. Synchronized NIH3T3 cells were pulse-labeled (30 min) with BrdU in 1 h intervals and nascent DNA was immunoprecipitated with anti-BrdU antibody. To calibrate for DNA recovery during immunoprecipitation, BrdU-labeled *E. coli* DNA was added to the reactions and immunoprecipitated DNA was analyzed by PCR amplification of a fragment of the β-lactamase gene. The triangles indicate that two amounts of DNA were analyzed. To control S-phase progression, a known early-replicating gene (α-globin) and late-replicating locus (X141, Azuara *et al*, 2003) was amplified as well (lower panels). Nascent rDNA was analyzed by semiquantitative PCR using primers that are specific for the 5′-terminal part of rDNA. The histogram shows a similar experiment in which nascent rDNA was quantified by real-time PCR. In the experiment above, the peak of late-replicating rDNA was observed after 6 h, while in the experiment below and in three other experiments late replication of rDNA occurred after 7 h.

methylated, amplification with the upstream primer will yield a 295 nucleotide (nt) fragment, whereas lack of DNA methylation or cleavage with *Msp*I will yield no PCR product. As demonstrated in Figure 2A, early-replicating rDNA was digested by *Hpa*II (lanes 1, 2), while late-replicating rDNA was resistant to *Hpa*II digestion (lanes 5, 6), demonstrating that methylated rRNA genes replicate late in S-phase.

Active and inactive rRNA genes exhibit a different chromatin structure and are characterized by different histone modifications. To examine histone acetylation and NoRC occupancy at early- and late-replicating rDNA, sequential chromatin immunoprecipitation (ChIP) assays were performed. Cells were pulse-labeled with BrdU in early or late S-phase, crosslinked with formaldehyde, and chromatin was precipitated with anti-AcH4 or anti-TIP5 antibodies. After digestion with proteinase K, nascent DNA was immunoprecipitated with anti-BrdU antibody and co-precipitated rDNA was quantified by real-time PCR (Figure 2B). This analysis revealed that the promoter of early-replicating, BrdU-labeled rRNA genes was enriched in acetylated histone H4, whereas histones at late-replicating rDNA were hypoacetylated. Notably, NoRC was exclusively associated with late-replicating rDNA. The preferential association of TIP5 with late-replicating rDNA underscores the role of NoRC in the establishment and propagation of the silent state of rDNA.

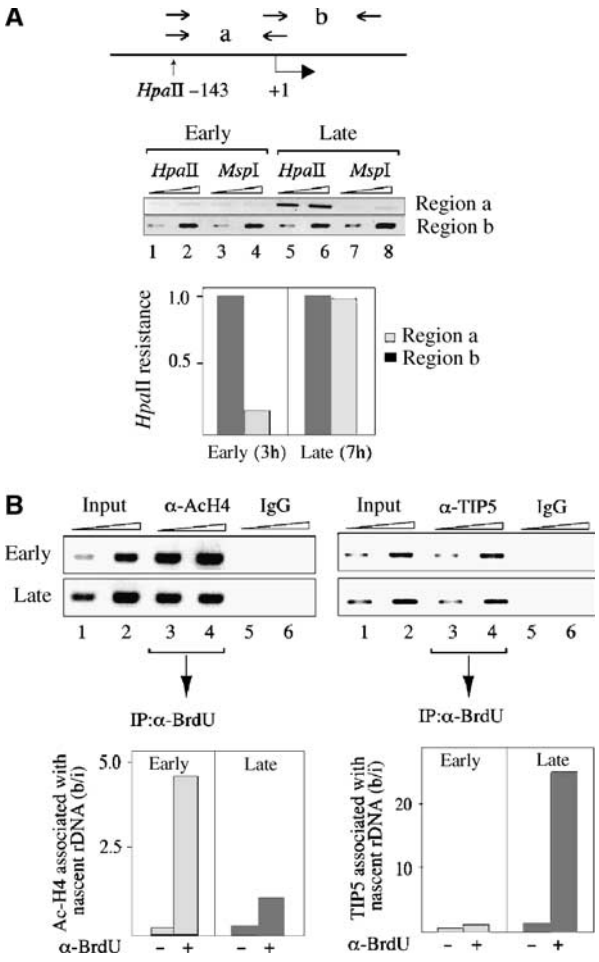

Figure 2 Late-replicating rDNA exhibits heterochromatic features. (**A**) Late-replicating rDNA is methylated at CpG residues. Immunoprecipitated early- (3 h) and late- (7 h) replicating nascent rDNA was digested with *Hpa*II or *Msp*I and analyzed by PCR using primers that map upstream or downstream of the *Hpa*II site at −143 as indicated. The bar diagram below shows the relative amount of *Hpa*II-resistant nascent rDNA as determined by real-time PCR. (**B**) NoRC is associated with late-replicating rDNA that contains hypoacetylated histone H4. Synchronized NIH3T3 cells were pulse-labeled with BrdU during early (3 h) and late (7 h) S-phase, and crosslinked chromatin was immunoprecipitated with anti-AcH4 (Upstate) or anti-TIP5 antibodies (Strohner *et al*, 2001). Precipitated rDNA was assayed by PCR amplification of 5′-terminal rDNA sequences (upper panels). After deproteinization, nascent DNA was immunoprecipitated with anti-BrdU antibody and analyzed by real-time PCR using primers that amplify rDNA sequences from −165 to +16. The bar diagrams show the average of rDNA associated with AcH4 and TIP5, respectively, estimated from two independent experiments.

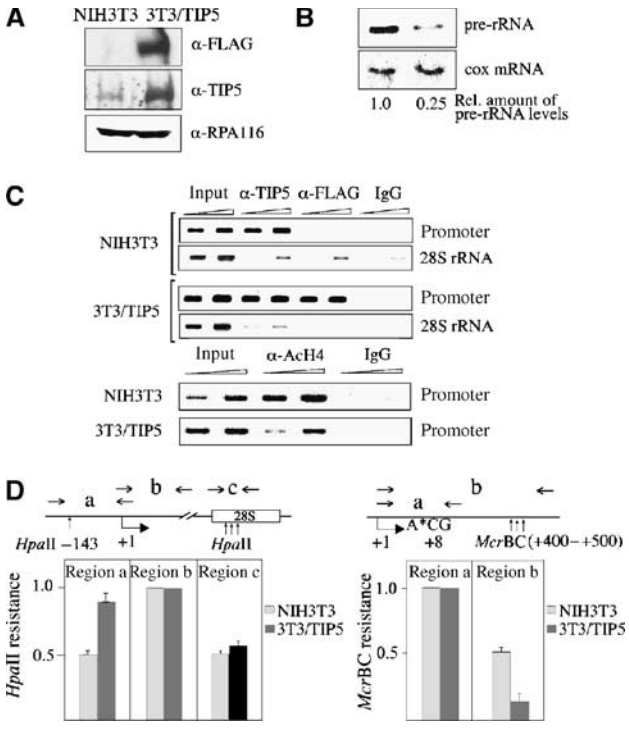

Figure 3 Characterization of 3T3/TIP5, a cell line that overexpresses FLAG-tagged TIP5. (**A**) Overexpression of FLAG-tagged TIP5 in NIH3T3 cells. Comparable amounts of lysates from NIH3T3 and 3T3/TIP5 (clone #6) cells were analyzed on immunoblots using anti-FLAG or anti-TIP5 antibodies. To normalize for protein loading, the level of PolI was monitored with anti-RPA116 antibodies. Quantitation of the anti-TIP5 immunoblot revealed that the expression level of TIP5 was 2.6-fold higher in 3T3/TIP5 compared to NIH3T3 cells. (**B**) Pre-rRNA synthesis is inhibited in 3T3/TIP5 cells. RNA (8 μg) from NIH3T3 and 3T3/TIP5 cells was analyzed on Northern blots using a riboprobe that hybridizes to the extreme 5′ end of 45S pre-rRNA. To normalize for variations of RNA loading, the blot was subsequently hybridized to a probe complementary to cytochrome C oxidase (cox) mRNA (lower panel). Quantitation of pre-rRNA levels by RT–PCR and normalized to GADPH mRNA revealed a 75% decrease of pre-rRNA in 3T3/TIP5 compared to NIH3T3 cells. (**C**) FLAG-tagged TIP5 is associated with the rDNA promoter. Crosslinked chromatin from NIH3T3 and 3T3/TIP5 cells was incubated with anti-FLAG antibodies, anti-rabbit IgGs (upper panels) or anti-AcH4 antibodies (lower panels). Two amounts of precipitated rDNA were analyzed by PCR using primers that amplify 5′-terminal rDNA sequences (from −165 to +16) or part of the 28S rRNA coding region, and were compared with 1 and 3% of input chromatin. (**D**) The rDNA promoter is hypermethylated in 3T3/TIP5 cells. Genomic DNA from NIH3T3 and 3T3/TIP5 cells was digested with *Hpa*II (left panel) or *McrBC* (right panel). The relative levels of *Hpa*II- and *McrBC*-resistant rDNA (5′-terminal sequences and 28S rRNA coding region) were estimated by real-time PCR using primer pairs that amplify the indicated regions of the rDNA repeat. *Hpa*II-resistant DNA of the promoter and 28S rRNA coding region (regions a and c) was normalized to the amount of DNA from region b. *McrBC*-resistant rDNA was estimated by real-time PCR comparing sequences from −7 to +581 (region b) with sequences from −7 to +87 (region a). The diagram shows the position of primers used and the *McrBC* cleavage sites.

Epigenetic changes at the rDNA promoter in cells overexpressing TIP5

The specific association of TIP5 with late-replicating rDNA raised the question whether NoRC plays an active role in replication timing. To assess the role of NoRC in setting up late-replicating rDNA, we established a stable cell line, termed 3T3/TIP5, that expresses FLAG-tagged TIP5. Quantitative Western blots revealed that expression levels of tagged TIP5 in 3T3/TIP5 cells were 2.6-fold higher than the amount of endogenous TIP5 in parental NIH3T3 cells (Figure 3A). Consistent with NoRC mediating transcriptional silencing, the steady-state level of 45S pre-rRNA was strongly reduced in 3T3/TIP5 cells (Figure 3B). ChIP experiments revealed that FLAG-tagged TIP5 was associated with the 5′ end of the rDNA transcription unit and histone H4 associated with this region was hypoacetylated (Figure 3C). The observation that both recombinant TIP5 and endogenous NoRC were associated with the rDNA promoter but not the coding

region indicates that NoRC remains bound to the promoter and does not track with PolI.

Moreover, the level of methylated rDNA was increased in 3T3/TIP5 cells (Figure 3D). While in NIH3T3 cells ~50% of rDNA was methylated, that is, resistant to *Hpa*II digestion, the fraction of methylated rDNA repeats was increased to 80–90% in 3T3/TIP5 cells. Alterations in rDNA methylation were restricted to the CpG residues within the 5′-terminal region and did not spread into 28S rRNA coding sequences. Hypermethylation of the rDNA promoter was also demonstrated by digestion of DNA from 3T3/TIP5 cells with *McrBC*, an enzyme that digests DNA only if it is methylated. Again, in NIH3T3 cells ~50% of rDNA was resistant to *McrBC* digestion, whereas in 3T3/TIP5 cells this fraction was decreased to 10–20% (Figure 3D, right panel). Together, the results demonstrate that moderate overexpression of TIP5 inhibits pre-rRNA synthesis by inducing a heterochromatic structure at previously 'open' rDNA repeats. The finding that NoRC-mediated *de novo* methylation was restricted to the 5′-terminal part of rDNA implies that interactions between TIP5 and TTF-I recruit NoRC to the rDNA promoter (Santoro *et al*, 2002; Nemeth *et al*, 2004). Once targeted to rDNA, NoRC recruits DNA methyltransferase that methylates a critical CpG residue within the upstream control element (UCE) of the rDNA promoter and this modification is sufficient for gene silencing (Santoro and Grummt, 2001). Methylation of the coding region, on the other hand, appears to require additional steps that are independent of NoRC.

Overexpression of TIP5 alters cell morphology and impairs cell growth

Given that rRNA synthesis and hence ribosome biogenesis is tightly linked to cellular metabolic activity, one would expect that cell proliferation was inhibited in cells overexpressing TIP5. Indeed, 3T3/TIP5 cells grow two-fold more slowly than parental NIH3T3 cells (Figure 4A). In addition, the morphology of 3T3/TIP5 cells was drastically altered. 3T3/TIP5 cells lost the character of typical polygonal NIH3T3 cells, exhibiting a slender, spindle-like shape with little cytoplasm (Figure 4B). Thus, enhanced levels of NoRC did not only decrease pre-rRNA levels, but also affected cell morphology and cell proliferation.

It is noteworthy that both the size and number of nucleoli were substantially reduced in 3T3/TIP5 cells. The size of nucleoli decreased from $40.4 \pm 11.6\,\mu m^2$ in NIH3T3 cells to $15 \pm 5.2\,\mu m^2$ in 3T3/TIP5 cells. Statistical evaluation of the number of nucleoli revealed an average of 3.9 ± 1.3 nucleoli in NIH3T3 compared to 2.5 ± 1.2 in 3T3/TIP5 cells (Figure 4C). If the cells were cultured in the presence of trichostatin A (TSA), an inhibitor of histone deacetylases, the number of nucleoli in 3T3/TIP5 increased from 2.5 ± 1.2 to 3.7 ± 1.4, while both the size and number of nucleoli in NIH3T3 cells remained unaffected by TSA treatment. The increased number of nucleoli in 3T3/TIP5 cells after inhibition of histone deacetylation indicates that TSA treatment has activated rDNA repeats that were previously silent. In support of this, TSA treatment increased pre-rRNA synthesis in 3T3/TIP5 cells to levels observed in NIH3T3 cells (Figure 4D). This result supports previous studies showing that one of the first steps in NoRC-mediated heterochromatin formation is recruitment of HDAC1 and histone deacetylation to the rDNA promoter (Zhou *et al*, 2002).

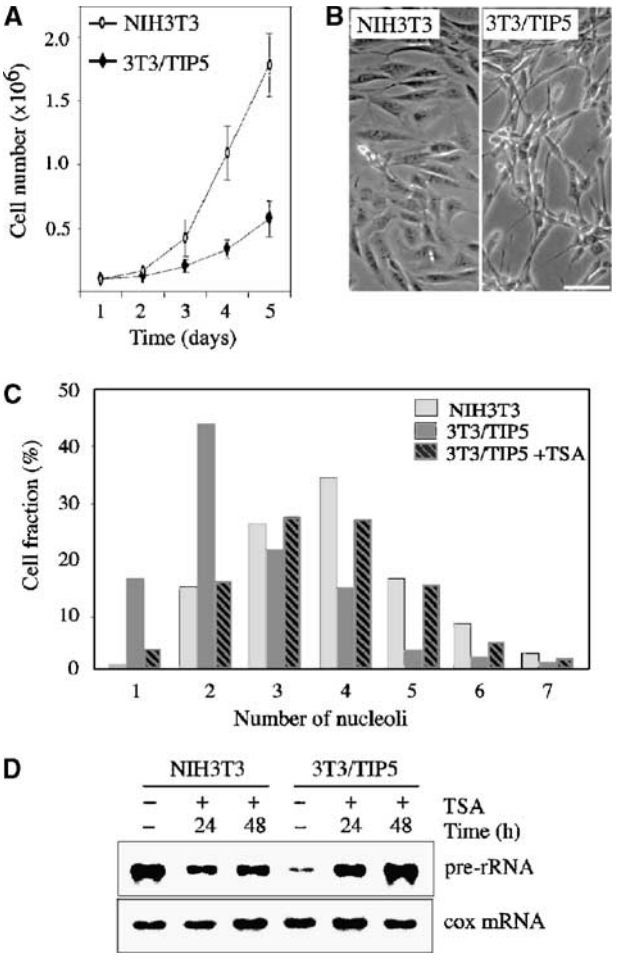

Figure 4 Overexpression of NoRC impairs cell growth and decreases the size and number of nucleoli. (**A**) Growth is retarded in 3T3/TIP5 cells. 1×10^5 NIH3T3 and 3T3/TIP5 cells were plated in triplicate and the cell number was counted for 5 days. (**B**) Overexpression of TIP5 alters the morphology of NIH3T3 cells. Phase-contrast images of NIH3T3 and 3T3/TIP5 cells are shown (bar: 100 μm). 3T3/TIP5 cells are distinguished from parental NIH3T3 cells by a narrow rim of cytoplasm around the nuclei and long cytoplasmatic protrusions. (**C**) The number of nucleoli is reduced in 3T3/TIP5 cells. The histogram depicts the average number of nucleoli in NIH3T3 and 3T3/TIP5 cells. The hatched bars mark the number of nucleoli of 3T3/TIP5 cells that were cultured for 3 days in the presence of 30 nM TSA. Evaluation of the number of nucleoli was performed using analySIS software (Soft Imaging System GmbH). The average of three independent experiments, each involving 300 scored cells, is shown. (**D**) TSA treatment enhances pre-rRNA synthesis in 3T3/TIP5 cells. NIH3T3 and 3T3/TIP5 cells were cultured for 24 and 48 h in the presence of 30 nM TSA and pre-rRNA was monitored on Northern blots.

Overexpression of NoRC shifts rDNA replication timing from early to late

The finding that NoRC plays an active role in the establishment of heterochromatic features raises the question whether NoRC controls replication timing of rDNA. Owing to the striking correlation between transcriptional activity and replication timing, one would intuitively predict that the proportion of late-replicating rRNA genes was increased in cells overexpressing TIP5. To assess the ratio of early- and late-replicating rDNA, NIH3T3 and 3T3/TIP5 cells were labeled with BrdU at defined times during S-phase and immunopurified rDNA was analyzed by PCR amplification. Again, a

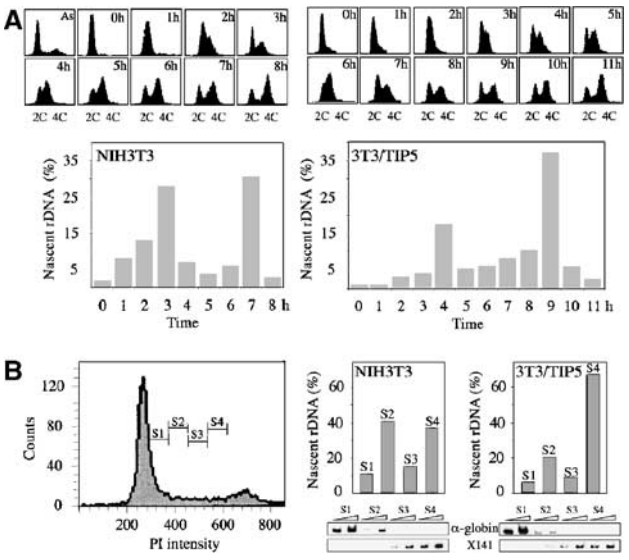

Figure 5 NoRC sets replication timing of rDNA from early to late. (**A**) Replication timing of rDNA in NIH3T3 and 3T3/TIP5 cells. NIH3T3 and 3T3/TIP5 cells were synchronized by aphidicolin treatment, released into S-phase, and labeled for 30 min with BrdU at the indicated times. Nascent DNA was immunoprecipitated with an antibody specific for BrdU and analyzed by real-time PCR using primers that amplify 5′-terminal rDNA sequences. To calculate losses of nascent DNA during immunoprecipitation, BrdU-substituted *E. coli* DNA was added to each of the samples and immunoprecipitated DNA was analyzed by amplification of a part of the β-lactamase gene. The bar diagram shows the amount of nascent rDNA normalized to co-immunoprecipitated BrdU-labeled *E. coli* DNA. A FACS analysis demonstrating S-phase progression of the two cell lines is shown above. (**B**) Biphasic replication of rDNA in asynchronous cells. NIH3T3 and 3T3/TIP5 cells were pulse-labeled with BrdU and separated by cell sorting into four cell cycle fractions during S-phase (S1–S4). A typical FACS profile of propidium iodide intensity is shown at the right. Nascent DNA was immunoprecipitated from each fraction with anti-BrdU antibody and the abundance of rDNA in each fraction was determined by real-time PCR. The relative abundance of nascent rDNA was normalized to co-precipitated BrdU-labeled *E. coli* DNA. PCR products of early-replicating (α-globin) and late-replicating loci (X141) are shown below.

bimodal replication pattern of rDNA was observed. However, both cell lines differed both in the overall length of S-phase and the ratio of early- to late-replicating rRNA genes. The S-phase was prolonged in 3T3/TIP5 cells, lasting about 11 h as compared to 8 h in NIH3T3 cells (Figure 5A). Second, and most importantly, the replication timing of rRNA genes was shifted toward late S-phase. The ratio of early- to late-replicating rRNA genes decreased from 60:40% in NIH3T3 cells to 30:70% in 3T3/TIP5 cells.

The shift of rDNA replication timing from early to late was also observed in nonsynchronized cells. In the experiment in Figure 5B, exponentially growing NIH3T3 and 3T3/TIP5 cells were labeled with BrdU, sorted on the basis of total DNA content by FACS, and nascent DNA was precipitated from different fractions (S1–S4) of S-phase. Amplification of precipitated DNA revealed a ratio of early- to late-replicating rRNA genes of 50:50% in NIH3T3 cells and 20:80% in 3T3/TIP5 cells. The fact that overexpression of TIP5 caused the majority of rDNA to replicate late suggests that NoRC establishes a heterochromatic state that inhibits replication origin firing in early S-phase. Hence, NoRC occupancy at silent rDNA copies provides a mechanism to establish specific epigenetic marks that are maintained after progression of the replication fork.

Early and late replication timing is inherited during S-phase

Studies in yeast have shown that active and silent rRNA gene copies are not clustered and all genes have the same probability of being active or silent (Dammann *et al*, 1995). In eukaryotes, however, the rDNA arrays are distributed on several nucleolar organizer regions (NORs), and the coordinated silencing of whole NORs in nucleolar dominance indicates that there are regulatory mechanisms that act on a scale much larger than single rRNA genes (Reeder, 1985; Pikaard, 2000). This raises the question whether the active and silent states at individual rDNA clusters are inherited from one generation to the next. To address this issue, we have labeled nascent rDNA either early or late in S-phase and monitored the chromatin status of rDNA after two cell division cycles by measuring CpG methylation of BrdU-labeled DNA. We reasoned that if the epigenetic status was inherited, the methylation level of early- and late-replicating labeled rDNA should remain unchanged. If, however, the chromatin structure was not inherited, that is, has to be re-established anew after each round of replication by NoRC-mediated silencing events, a significant portion of early-replicating BrdU-labeled DNA should become methylated during the next replication cycle. The result in Figure 6 supports the concept of stable propagation of the active and silent states of rRNA genes during cell division. The majority of rDNA that has been labeled during early or late S-phase, respectively, maintains the methylation status throughout two cell divisions. That is, rDNA repeats that replicate late in one cell will replicate late in the next cell cycle too. This demonstrates that the transcriptional competence of individual rDNA clusters is inherited from one generation to the next and implies that, once established, the chromatin structure of active or silent rRNA genes is inherited to the daughter cells.

Discussion

In mammalian cells, replication timing is linked to transcriptional control through its liaison with chromatin structure. The mechanisms that establish the link between transcriptional activity, chromatin structure and the temporal order of DNA replication remained elusive. In this communication, we have investigated the role of the chromatin remodeling complex NoRC in replication timing of rDNA. In cycling eukaryotic cells, only a fraction of rRNA genes displays characteristics of a typical 'housekeeping' gene, that is, the locus is unmethylated, exhibits euchromatic features and is transcriptionally active. Other rDNA arrays are in a heterochromatic conformation and are transcriptionally silent. The simultaneous existence of both active and silent rRNA gene copies facilitates studies on the molecular mechanisms that link chromatin structure to transcription, replication and nucleolar morphology. Consistent with previous studies showing that rDNA is replicated in a biphasic manner (Berger *et al*, 1997), we found that transcriptionally active rRNA genes replicate early, whereas silent rDNA arrays replicate late. The difference in replication timing was ~4 h, with S-phase lasting about ~8 h. BrdU-labeled early-replicating

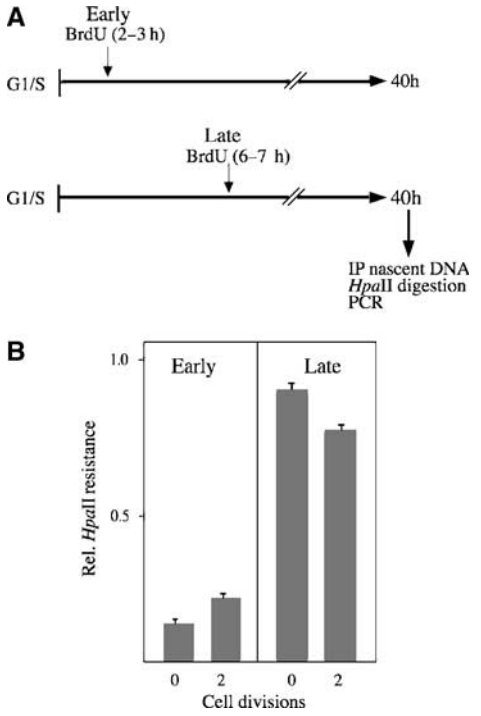

Figure 6 Epigenetic marks are preserved after cell division. (**A**) Experimental strategy to measure the propagation of rDNA methylation. (**B**) NIH3T3 cells were synchronized and released into S-phase. During S-phase progression, cells were labeled with BrdU either at the early (2–3 h) or late (6–7 h) stages of S-phase. Cells were then cultured in fresh medium for 40 h (two cell division cycles). DNA was purified, nascent DNA was enriched by immunoprecipitation, digested with *Hpa*II or *Msp*I and analyzed by real-time PCR using the primers shown in Figure 2A. The bar diagram shows the relative amount of *Hpa*II-resistant nascent rDNA which was isolated both immediately after BrdU labeling and after two cell division cycles (40 h).

rDNA was enriched for acetylated histone H4 (AcH4) and devoid of NoRC. In contrast, NoRC was exclusively associated with late-replicating rDNA that was depleted of AcH4 and methylated at CpG residues. Given that NoRC is the key determinant of heterochromatin formation and transcriptional silencing, this finding suggests that the association of NoRC with rDNA and subsequent recruitment of co-repressors provide a mechanism to establish a chromatin structure that is replicated late in S-phase and propagates the silent state through cell division. In support of this, overexpression of TIP5 shifted replication of a significant fraction of rRNA genes from early to late S-phase. Thus, NoRC plays an active role in setting the clock for silent rRNA genes replicating late.

The correlation between chromatin structure and replication timing suggests that the replication fork is exposed to a different nuclear environment during early and late S-phase, which favors either the formation of active transcription complexes or the generation of repressive chromatin structures. In the latter case, the availability of chromatin-modifying repressors would be restricted to late S-phase. Indeed, Dnmt1 was found to colocalize with HDAC2 at replication foci only during late stages of S-phase, when transcriptionally repressed chromatin was replicated (Rountree *et al*, 2000). This indicates that histone deacetylase and DNA methyltransferase activities were targeted to the replication fork to secure the inheritance of epigenetic marks through mitotic cell division. Likewise, NoRC has been shown to silence rDNA transcription by recruiting the SIN3 co-repressor and DNA methyltransferases Dnmt1 and 3b to the rDNA promoter to alter chromatin from an 'open' euchromatic to a 'closed' heterochromatic structure (Santoro *et al*, 2002; Zhou *et al*, 2002). The finding that NoRC is exclusively associated with late-replicating rRNA genes supports the view that NoRC directly recruits HDAC1 and Dnmt1 to the replication fork during the second half of S-phase, thereby propagating existing epigenetic marks and reinforcing the inheritance of silent chromatin.

Much remains to be learned about the mechanisms that mediate spreading of heterochromatin over large domains and transmit silent chromatin states through DNA replication. With regard to spreading of epigenetic marks at the rDNA locus, it is noteworthy that NoRC-mediated increase in rDNA methylation was restricted to CpG residues within the promoter region and did not spread into the transcribed region. This indicates that TIP5-mediated *de novo* methylation and histone modification are sufficient for establishing heterochromatic features at the rDNA promoter and for transcriptional silencing of previously 'open' genes, but not for spreading of heterochromatic marks into the transcribed region. This result is similar to studies in rat cells showing that CpG methylation was concentrated in regulatory sequences of rDNA, and that methylation of one single *Hpa*II site in the promoter region strongly correlated with transcriptional silencing (Stancheva *et al*, 1997).

The results of this communication reveal marked differences between yeast and higher eukaryotes in the propagation of the active and silent state of rDNA. Yeast, which lacks CpG methylation, can modulate the number of actively transcribed rRNA genes depending on the growth conditions (Dammann *et al*, 1993; Sandmeier *et al*, 2002). Moreover, psoralen crosslinking studies in yeast have demonstrated that active rRNA gene copies are not clustered but rather randomly distributed within the single NOR (Dammann *et al*, 1995; French *et al*, 2003). Although it is not known how yeast cells select the ribosomal genes to be active or inactive, it seems likely that disruption of the chromatin structure at active genes is mediated by PolI molecules advancing through the rDNA template (Lucchini and Sogo, 1995). In mammalian cells, however, our data suggest that the presence of stable epigenetic marks imparts a 'memory' on certain transcription units that is inherited through cell division. While in yeast all rRNA genes have the same probability of being potentially active or silent, different location of active and inactive genes has been observed in mammals. Silent rDNA arrays are located in the extranucleolar space, frequently associated with the perinucleolar heterochromatin. Active rDNA repeats, on the other hand, are located inside the nucleolus within the dense fibrillar components (Mosgöller, 2004). Our observation that the number of nucleoli is reduced in a cell line that overexpresses TIP5, and this reduction is overcome by TSA treatment, suggests that arrays of rDNA repeats rather than single rRNA genes are silenced. In support of this, coordinated silencing of whole NORs is evident in genetic hybrids displaying nucleolar dominance, the situation whereby the rRNA genes inherited from one parent are transcribed, and assemble one or more nucleoli, but the rRNA genes of the other parent are inactive (Reeder, 1985; Pikaard, 2000). Moreover, chicken cells that

are either di-, tri- or tetrasomic for the NOR-bearing chromosome display the same level of rRNA transcription regardless of the copy number of rRNA genes (Muscarella *et al*, 1985), indicating that there are regulatory mechanisms that act on a scale much larger than single rRNA genes. However, the nature of such putative large-scale NOR regulatory mechanisms is not yet known.

Materials and methods

Cell synchronization, BrdU labeling, and isolation of nascent DNA

NIH3T3 cells were maintained at confluence in DMEM/10% FCS for 2 days before reseeding to 60–80% confluence and culturing for 18–20 h in medium containing 2 μg/ml aphidicolin (Sigma) to arrest cells at the G1/S-phase boundary. After release from the aphidicolin block, nascent DNA was pulse-labeled at defined time points by incubating the cells for 30 min with 30 μM 5′-BrdU (Sigma). Cellular DNA was isolated, sonicated to ~800 bp fragments, heat-denatured and 2 μg of genomic DNA was incubated at room temperature for 30 min with 1.25 μg of anti-BrdU antibody (Roche) in 0.14 M NaCl, 10 mM phosphate buffer (pH 7.2), 0.05% Triton X-100. DNA–antibody complexes were incubated with 40 μg of anti-mouse IgGs (Sigma) for 30 min, the precipitates were collected by centrifugation and dissolved in 50 mM Tris–HCl (pH 8.0), 0.5% SDS, 1 M NaCl and 10 mM EDTA. Proteins were digested overnight with 0.25 mg/ml of proteinase K and DNA was purified by phenol–chloroform extractions and ethanol precipitation. To calculate losses of nascent DNA during the purification procedure, 20 ng of BrdU-substituted *Escherichia coli* DNA was added to each sample before sonification.

FACS sorting of S-phase cells

DNA of 3×10^7 logarithmically growing cells was labeled with 50 μM BrdU for 90 min. After fixation with 70% ethanol, cells were resuspended in 40 mM Tris–HCl (pH 7.4), 0.8% NaCl, 21 mM $MgCl_2$, 0.05% NP-40, propidium iodine (50 μg/ml) and RNase A (0.5 mg/ml) and sorted by FACS into S-phase fractions on the basis of DNA content. Cells were sorted with a Becton Dickinson FACS Vantage sorter. In all, 20 000 cells were collected from different fractions (S1–4) of S-phase directly into lysis buffer (1 M NaCl, 10 mM EDTA, 50 mM Tris–HCl (pH 8.0), 0.5% SDS, 0.2 mg/ml proteinase K). Nascent DNA was purified, sonicated, denatured and immunoprecipitated with a monoclonal antibody specific for BrdU.

Establishment of a cell line that overexpresses TIP5

NIH3T3 cells expressing FLAG-tagged TIP5 were generated by transfection with pcDNA-FLAG-TIP5 (Santoro *et al*, 2002), and expanding individual colonies after culturing for 3 weeks in the presence of G-418 (1 mg/ml). Clones expressing tagged TIP5 were identified on immunoblots using anti-FLAG antibodies (Sigma) or anti-TIP5 antibodies raised against amino acids 323–723 (Strohner *et al*, 2001).

RNA analysis

45S pre-rRNA levels in NIH3T3 and 3T3/TIP5 cells were monitored on Northern blots by hybridization to antisense RNA complementary to the first 155 nt of unprocessed 45S pre-RNA (Voit *et al*, 1999). To normalize for differences in RNA loading, the filter was also hybridized with a probe that is complementary to cytochrome C oxidase (cox) mRNA. Alternatively, pre-rRNA was analyzed by reverse transcription followed by RT–PCR.

ChIP and PCR amplification

Chromatin from crosslinked cells was sheared by sonication, incubated overnight with specific antibodies and precipitated proteins were captured with protein G-sepharose saturated with salmon sperm DNA. After elution and reversion of crosslinks by heating for 6 h at 65°C, 1 and 3% of precipitated DNA were amplified by PCR with 30 cycles (30 s 95°C, 40 s 55°C, 40 s 72°C) in the presence of 1.5 mM $MgCl_2$, 200 μM dNTPs and 10 pmol of primers. The sequence of primers used is available on request. PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Quantitative real-time PCR was performed with the same primers using a LightCycler (Roche) and the SYBR Green detection system.

Methylation sensitivity assays

Genomic DNA was prepared from NIH3T3 and 3T3/TIP5 cells, digested with the methylation-sensitive enzyme *Hpa*II and analyzed by PCR using either of two forward primers (−165/−145 or −7/+16) and reverse primers (−7/+16 or +111/+130) to amplify 5′-terminal rDNA sequences, or a pair of primers that amplifies part of the 28S rRNA coding sequence (+8124 to +8549). The level of *Hpa*II-resistant rDNA was normalized to the amount of amplified rDNA encompassing sequences from −7 to +130. Alternatively, DNA was digested with *McrBC* and analyzed by PCR using a forward primer (−7/+16) and either of two reverse primers (+68/+87 or +562/581). During restriction enzyme digestion, 200 ng of plasmid DNA was added to an aliquot of each sample to monitor DNA cleavage on ethidium bromide-stained agarose gels.

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