

# Intergenic transcripts originating from a subclass of ribosomal DNA repeats silence ribosomal RNA genes in *trans*

Raffaella Santoro<sup>‡</sup>, Kerstin-Maike Schmitz, Juan Sandoval & Ingrid Grummt<sup>†</sup>

Division of Molecular Biology of the Cell II, German Cancer Research Center, DKFZ–ZMBH–Alliance, Heidelberg, Germany

**Epigenetic silencing of a fraction of ribosomal DNA (rDNA) requires association of the nucleolar chromatin-remodelling complex NoRC to 150–250 nucleotide RNAs (pRNA) that originate from an RNA polymerase I promoter located in the intergenic spacer separating rDNA repeats. Here, we show that NoRC-associated pRNA is transcribed from a sub-fraction of hypomethylated rRNA genes during mid S phase, acting in *trans* to inherit DNA methylation and transcriptional repression of late-replicating silent rDNA copies. The results reveal variability between individual rDNA clusters with distinct functional consequences.**

Keywords: epigenetic silencing; NoRC; non-coding RNA; rDNA; spacer promoter

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## INTRODUCTION

Mammalian ribosomal RNA (rRNA) genes are alternating modules of intergenic spacers (IGSs) and transcribed regions that encode rRNA precursors (pre-rRNA). In rats, mice, *Drosophila* and *Xenopus*, the IGS contains one or more RNA polymerase I (Pol I) promoters with high homology to the core region of the main ribosomal DNA (rDNA) promoter (Labhart & Reeder, 1984; de Winter & Moss, 1986; Kuhn & Grummt, 1987; Grimaldi & di Nocera, 1988). Transcripts originating from spacer promoters are co-directional with pre-rRNA synthesis and have been shown to enhance transcription from the main rDNA promoter, possibly by delivering Pol I (Grimaldi & di Nocera, 1988; Putnam & Pikaard, 1992). Furthermore, intergenic spacer rRNA (IGS rRNA) has been shown to have a crucial function in rDNA silencing. In mice, intergenic transcripts originating from a promoter located approximately 2 kb upstream from the pre-rRNA start site are processed into a heterogeneous population of 150–250 nucleotide RNAs,

dubbed promoter RNA (pRNA) as their sequence matches the rDNA promoter (Mayer *et al*, 2006). The pRNA associates with nucleolar remodelling complex (NoRC), a sucrose non-fermenting protein 2 homologue (SNF2h)-containing chromatin-remodelling complex, which induces long-term transcriptional silencing of a fraction of rRNA genes (Strohner *et al*, 2001; Santoro *et al*, 2002; Santoro & Grummt, 2005). NoRC-dependent heterochromatin formation and rDNA silencing depends on the association of NoRC with pRNA (Mayer *et al*, 2006, 2008).

As the steady-state level of IGS rRNA is very low, we considered which mechanisms might account for the under-representation of IGS transcripts. Several models are conceivable, which are not necessarily exclusive: the spacer promoter could be extremely weak, IGS transcripts could be rapidly degraded, the synthesis of IGS rRNA could be restricted to a small fraction of rDNA repeats, or IGS rRNA could be synthesized during a defined time during the cell cycle. Here, we show that spacer transcripts are synthesized from a fraction of unmethylated rRNA genes and mediate CpG methylation and heterochromatin formation at late-replicating, silent rDNA repeats. The results support the idea that mammalian rDNA arrays are functionally distinct and intergenic transcripts from a specific subclass of rDNA repeats regulate the epigenetic state of rRNA genes in *trans*.

## RESULTS

### Spacer transcripts and pre-rRNA are differently regulated

Previous studies have demonstrated that silencing of a fraction of rDNA repeats requires the association of NoRC with RNA from a Pol I promoter that is located in the IGS approximately 2 kb upstream from the pre-rRNA transcription start site (Mayer *et al*, 2006). To elucidate the mechanisms that regulate transcripts originating from the spacer promoter, we compared the activity of the spacer promoter with the main rRNA gene promoter. In cell-free transcription assays, templates harbouring the spacer promoter (pMr–2175) promoted the synthesis of specific run-off transcripts, although with lower efficiency than templates comprising the pre-rRNA promoter (pMr–168; Fig 1A). A similar difference in transcription activity was observed in transient transfection experiments with artificial minigenes harbouring the main rDNA promoter (pMr–1922/+130-BH) or the spacer

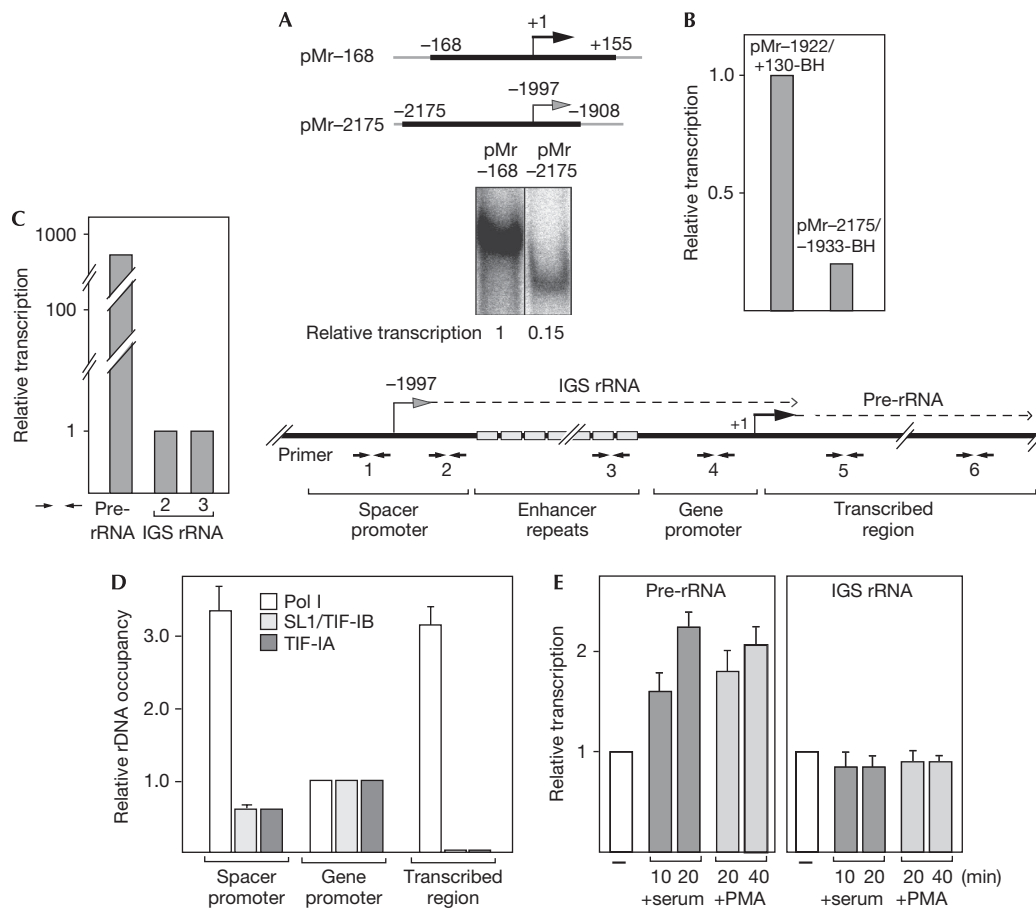
Division of Molecular Biology of the Cell II, German Cancer Research Center, DKFZ–ZMBH–Alliance, INF 581, 69120 Heidelberg, Germany

<sup>‡</sup>Present address: Institute of Veterinary Biochemistry and Molecular Biology, University of Zurich, Winterthurerstrasse 190, Zürich CH-8057, Switzerland

<sup>†</sup>Corresponding author. Tel: +49 6221 423423; Fax: +49 6221 423404;

E-mail: i.grummt@dkfz.de

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**Fig 1** | The steady-state level of IGS rRNA is under-represented in cellular RNA. (A) *In vitro* transcription. Run-off transcripts from pMr-168 and pMr-2175 linearized with *NdeI*. The scheme depicts the regions of rDNA contained in pMr-168 and pMr-2175. (B) Transcription of reporter genes pMr-1922/+130-BH and pMr-2175/-1933-BH in NIH3T3 cells. Values were normalized to expression levels of a co-transfected luciferase reporter gene and to *GAPDH* mRNA. (C) Steady-state levels of IGS rRNA and pre-rRNA. RNA from exponentially growing NIH3T3 cells was subjected to RT-qPCR; values were normalized to *GAPDH* mRNA. The scheme depicts the structural organization of the 5'-terminal part of murine rDNA, including the transcription start sites of IGS rRNA and pre-rRNA (dotted lines), and the primer pairs used for qPCR amplification (numbered arrows). The light grey boxes are the variable number of enhancer repeats between the spacer promoter and the main gene promoter. (D) Chromatin immunoprecipitation. Polyclonal antibodies against Pol I or the respective transcription factors were used to show the association of Pol I (RPA116), SL1/TIF-IB (TAF<sub>110</sub>) and TIF-IA with different regions of rDNA. DNA was amplified with the indicated primer pairs. Data show the ratio of rDNA in the immunoprecipitates and input rDNA (b/i). Error bars indicate  $\pm$  s.d. values of three experiments. (E) RT-qPCR of IGS rRNA and pre-rRNA after mitogenic stimulation of serum-starved NIH3T3 cells by serum or PMA (0.1  $\mu$ M) for the indicated times. Values were normalized to *GAPDH* mRNA. Error bars indicate  $\pm$  s.d. values of three independent experiments. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; IGS, intergenic spacer; mRNA, messenger RNA; Pol I, RNA polymerase I; PMA, phorbol 12-myristate 13-acetate; pre-rRNA, rRNA precursor; rRNA, ribosomal RNA; RT-qPCR, reverse transcriptase quantitative PCR; TAF, TATA box-associated factor; TIF, transcription initiation factor.

promoter (pMr-2175/-1933-BH; Fig 1B). Significantly, the steady-state level of cellular IGS rRNA was almost 1,000-fold lower than pre-rRNA (Fig 1C), suggesting that spacer transcripts are either synthesized from a minor fraction of rRNA genes or are rapidly degraded. To distinguish between both possibilities, we compared the association of the Pol I transcription machinery with the spacer and the main gene promoter by chromatin immunoprecipitation (ChIP) assays. Consistent with the *in vitro* transcription experiments and the Pol I reporter assays in Fig 1A,B, binding of the promoter selectivity factor SL1 (also known as

transcription initiation factor TIF-IB) and the Pol I-associated factor TIF-IA was slightly decreased at the spacer promoter (Fig 1D). By contrast, Pol I occupancy was elevated at the spacer promoter. As Pol I loading does not correlate with the transcriptional output, Pol I is either stalled at the spacer promoter or IGS rRNA is synthesized from a minor fraction of rRNA genes, or is rapidly degraded.

Previous studies have revealed that growth factors stimulate Pol I transcription by extracellular-signal-regulated kinase-dependent and ribosomal-S6-kinase-dependent phosphorylation

of TIF-IA and the upstream binding factor (UBF; Zhao *et al*, 2003; Stefanovsky *et al*, 2006). To examine whether the synthesis of IGS rRNA is responsive to the growth-dependent TIF-IA, we monitored transcripts from the spacer and the main gene promoter after overexpression of TIF-IA. Transcription from both promoters was stimulated by ectopic TIF-IA (supplementary Fig S1 online), supporting the idea that the basal Pol I transcription machinery directs the synthesis of IGS rRNA and pre-rRNA. Nevertheless, the level of cellular IGS transcripts seems to be regulated by different mechanisms than pre-rRNA. As shown in Fig 1E, mitogenic stimulation of serum-starved NIH3T3 cells by serum or phorbol 12-myristate 13-acetate increased pre-rRNA synthesis; however, it did not affect the level of IGS rRNA. This indicates that the spacer promoter and the main rDNA promoter are differentially sensitive to mitogenic signals.

### IGS rRNA levels transiently increase in mid S phase

The synthesis of pre-rRNA fluctuates during cell-cycle progression, steadily increasing from G1 to G2 phase and being shut off during mitosis (Klein & Grummt, 1999). By contrast, the steady-state level of IGS rRNA was almost identical in G0, G1/S and G2/M cells, demonstrating that the synthesis and turnover of spacer transcripts does not change during the cell cycle (supplementary Fig S2 online). To uncover a potential link between IGS rRNA synthesis and rDNA replication, we synchronized NIH3T3 cells by treatment with aphidicolin, and monitored pre-rRNA and IGS rRNA levels after release into the S phase. The aim of this experiment was to link transcription of IGS rRNA to replication timing. Previous studies have established that hypomethylated, active rRNA genes replicate early (2–3 h after release), whereas CpG-methylated, silent rRNA genes replicate later, at about 5–6 h after entry into S phase (Li *et al*, 2005; supplementary Fig S3 online). Consistent with active rDNA copies replicating early, the level of pre-rRNA doubled at 2–3 h after release from the aphidicolin block (Fig 2A, left panel). Notably, the level of unprocessed spacer transcripts (IGS rRNA, middle panel) and processed spacer transcripts (pRNA, right panel) increased at 4 h, a first indication that IGS rRNA is synthesized from other rDNA repeats than pre-rRNA. Consistent with previous results showing that IGS rRNA needs to be processed into pRNA that is stabilized by binding to NoRC (Mayer *et al*, 2006), IGS rRNA decreased in the second half of S phase, whereas pRNA remained unchanged. Different levels of IGS rRNA and pRNA were also observed in fluorescence *in situ* hybridization (FISH) experiments (Fig 2B). Although there is an obvious difference in the amount of IGS rRNA determined by reverse transcriptase PCR (RT-PCR) and by *in situ* hybridization, IGS rRNA is clearly enriched in nucleoli 4 h after entry into S phase, indicating that elevated levels of IGS rRNA precede replication of silent rDNA copies. The pRNA, however, which is an integral component of NoRC and is required for silencing a fraction of rDNA repeats, was permanently present in nucleoli.

The decline in IGS rRNA, but not pRNA, in late S phase indicates that cells transcribe more IGS RNA than they accumulate, implying the existence of an active RNA degradation mechanism. In support of this, knockdown of a subunit of the exosome, ExoSC3, led to a 3.8-fold increase in the level of IGS rRNA and a 2.2-fold increase in that of pRNA without affecting the level of pre-rRNA. This indicates that cells carefully regulate

the level of spacer transcripts to produce the amount of pRNA required for propagation of the heterochromatic state of silent rRNA genes (supplementary Fig S4 online).

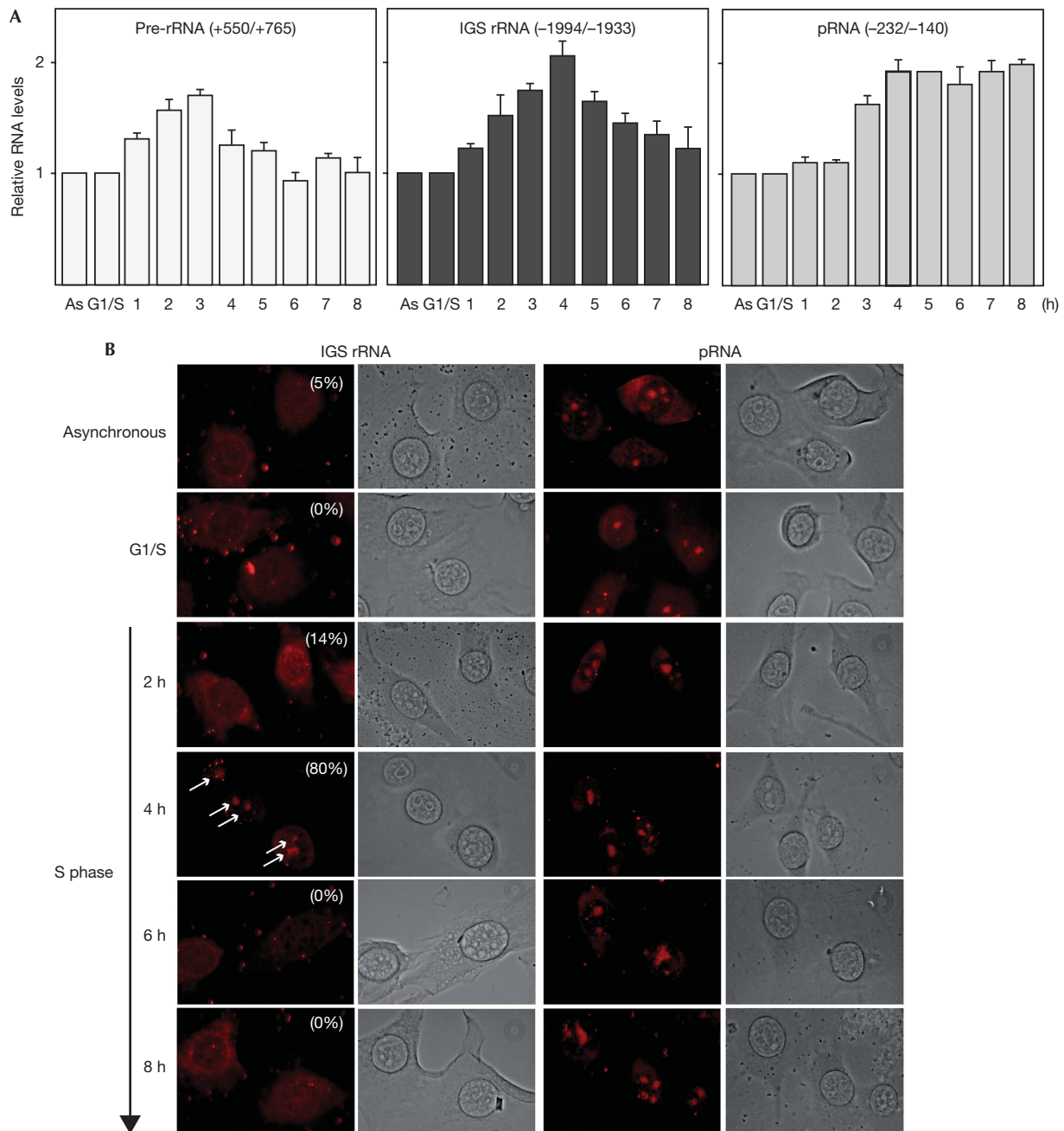
### IGS rRNA is transcribed from a subclass of rRNA genes

To determine whether IGS rRNA derives from hypomethylated or from CpG-methylated rDNA repeats, we performed ChIP-chop assays; that is, we precipitated chromatin-associated Pol I and monitored CpG methylation of co-precipitated rDNA by methylation-sensitive restriction analysis. Consistent with enhanced pre-rRNA synthesis occurring in early S phase, the association of Pol I with the main rDNA promoter increased 2 h after release from the aphidicolin block (Fig 3A, right panels). Pol I occupancy in the IGS was enhanced after 4 h (Fig 3A, left panels), a further indication that both promoters are differentially regulated and are not active at the same time. To analyse whether IGS transcripts originate from hypomethylated or hypermethylated rDNA repeats, the immunoprecipitated DNA was digested with *HpaII*, and resistance to *HpaII* digestion was measured by quantitative PCR (qPCR). As expected, Pol I was associated with hypomethylated rDNA promoters that are sensitive to *HpaII* digestion. Similarly, Pol I occupied hypomethylated IGS sequences, supporting the view that IGS rRNA is transcribed from a subfraction of unmethylated rRNA genes to propagate the heterochromatic state of silent rDNA repeats through cell division.

So far, one mouse and one human rDNA repeat has been sequenced (Third Party Annotation accession numbers BK000964 and U13369), suggesting that all rDNA repeats are identical. However, restriction fragment length polymorphism was noted in the 5'-end of mouse rRNA genes, which was attributed to a variable number of enhancer repeats located between the spacer promoter and the promoter-proximal terminator  $T_0$  (Arnheim & Kuehn, 1979; Tseng *et al*, 2008). The existence of rDNA variants with a different number of enhancer repeats enabled us to examine whether IGS transcripts derive from a specific subset of rRNA genes. Southern blot analysis of genomic DNA from NIH3T3 cells revealed distinct lengths of IGSs (Fig 3B). Cloning and sequencing indicated that these rDNA variants have identical spacer and promoter sequences but contain a variable number (9, 10, 11, 12 and 22) of enhancer repeats. A similar restriction fragment length polymorphism in the IGS could be detected after amplification of sequences between the spacer and gene promoter both in NIH3T3 cells and in mouse tissues (Fig 3C).

To examine whether IGS rRNA is transcribed from a specific subclass of rRNA genes, we analysed the length of IGS rRNA by RT-PCR using RNA from NIH3T3 cells and mouse liver (Fig 3D). Amplification of IGS rRNA predominantly yielded DNA fragments containing nine enhancer repeats. An additional band comprising six enhancer elements was observed in mouse liver, supporting the view that IGS rRNA is transcribed from a specific subset of rRNA genes to facilitate heterochromatin formation and silencing of a fraction of rDNA repeats *in trans*.

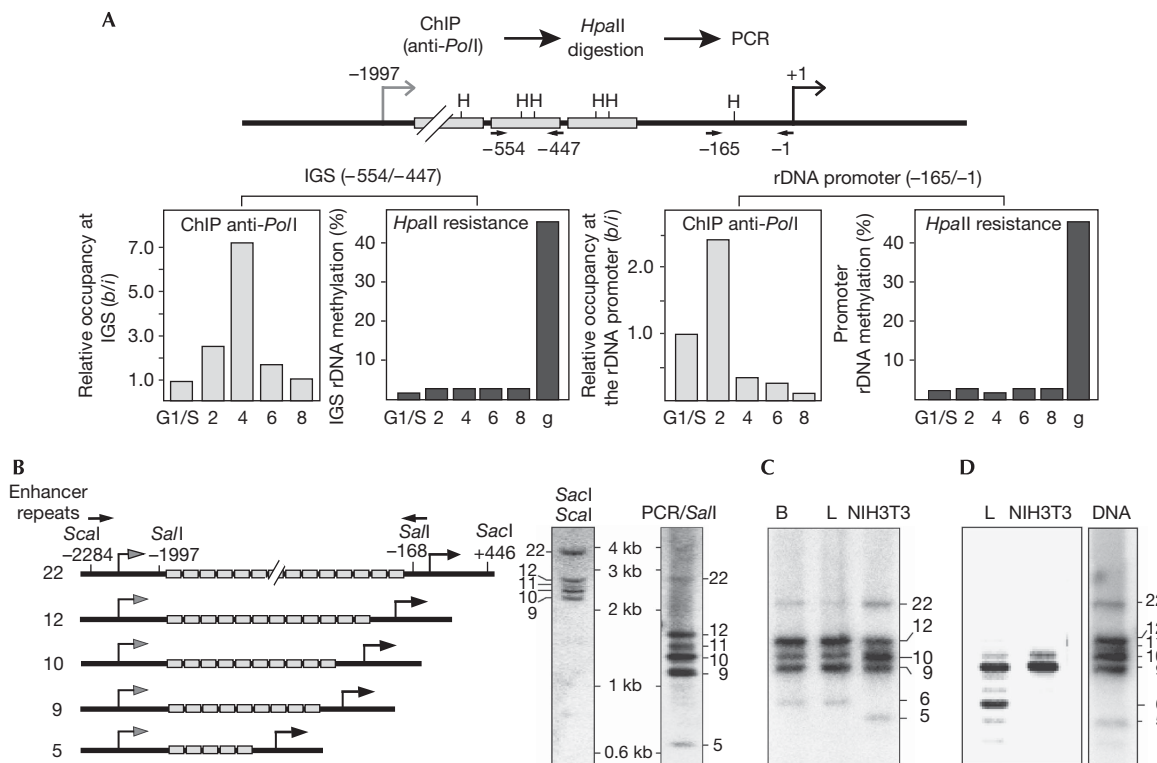
To verify that IGS rRNA functions in *trans*, we artificially raised the level of spacer transcripts by transfecting NIH3T3 cells with pE3SP-mrDNA-232/-43, a Pol I-driven vector that comprises rDNA sequences from -232 to -43 (Fig 4A). On transfection, a 10–15-fold increase in spacer transcripts was observed (Fig 4B). Consistent with previous results showing that pRNA is required for NoRC-dependent heterochromatin formation and transcriptional



**Fig 2** | The level of IGS rRNA transiently increases during mid S phase. (A) Levels of pre-rRNA, IGS rRNA and pRNA during S-phase progression. NIH3T3 cells arrested at G1/S were released into S phase and transcription was measured in 1 h intervals by RT-qPCR. Values were normalized to 28S rRNA. Error bars indicate  $\pm$  s.d. values of three independent experiments. (B) RNA FISH showing IGS rRNA and pRNA in asynchronous cells or in NIH3T3 cells that were synchronized by aphidicolin treatment and released into S phase. IGS rRNA was visualized by hybridization with a probe containing rDNA sequences from -554 to -447, pRNA with a probe comprising rDNA sequences from -232 to -140. Signals were visualized with extravidin-Cy3 conjugate. A total of 100 cells were counted; the percentage of IGS rRNA-positive cells is indicated in the images. Arrows mark nascent IGS rRNA in nucleoli. As, asynchronous cells; FISH, fluorescence *in situ* hybridization; IGS, intergenic spacer; Pol I, RNA polymerase I; pre-rRNA, rRNA precursor; pRNA, promoter RNA; rRNA/DNA, ribosomal RNA/DNA; RT-qPCR, reverse transcriptase quantitative PCR.

silencing, elevated levels of spacer RNA triggered hypermethylation of rRNA genes. In addition, heterochromatic histone marks (H3K9me3 and H4K20me3) increased, whereas rDNA promoter

occupancy of the euchromatic histone mark H3K4me3 decreased (Fig 4C). Similarly, elevation of cellular pRNA levels by transfection with *in vitro*-synthesized pRNA-205/-1 led to hypermethylation of



**Fig 3** IGS rRNA is transcribed from a subclass of rRNA genes. (A) IGS rRNA is transcribed from hypomethylated rRNA genes. Light bars show ChIP data monitoring Pol I occupancy at the main gene promoter and the IGS promoter during S-phase progression. Data show Pol I-associated rDNA compared with input DNA (*b/i*), normalized to Pol I occupancy in G1/S cells. Dark bars show the percentage of *HpaII*-resistant immunoprecipitated rDNA. As a control, methylation of genomic DNA (g) from asynchronous cells is shown. Methylation of rDNA is shown as the ratio of *HpaII*-resistant immunoprecipitated rDNA and undigested total rDNA. The positions of *HpaII* sites (H) and primers used to amplify the IGS and the rDNA promoter are indicated in the scheme (top). (B) Southern blot analysis. DNA from NIH3T3 cells was digested with *SacI* and *Scal* and hybridized with an IGS-specific probe. As a control, IGS rDNA (-2175/-1) was amplified by PCR and digested with *Sall* (right lane). The scheme on the left depicts part of the 5'-terminal region of rDNA variants containing a different number of enhancer repeats (light boxes). Arrows represent the PCR primers used to amplify spacer sequences. Nucleotide positions refer to mouse rDNA sequence BK000964. (C) PCR of IGS (from -2175 to -1) using 50–100 ng of genomic DNA from mouse brain (B), mouse liver and NIH3T3 cells. (D) IGS rRNA originates from a subclass of rDNA repeats. RNA from mouse liver and NIH3T3 cells was subjected to RT-PCR using the Expand Long Template PCR Kit (Roche) and primers that amplify rDNA sequences from -1994 to -20. Amplification of the respective region of genomic DNA from NIH3T3 cells is shown on the right. ChIP, chromatin immunoprecipitation; IGS, intergenic spacer; Pol I, RNA polymerase I; rRNA, ribosomal RNA; RT-PCR, reverse transcriptase PCR.

the rDNA promoter and decreased pre-rRNA synthesis, indicating that spacer transcripts mediate heterochromatin formation and silencing of a fraction of rDNA repeats in *trans* (Fig 4D).

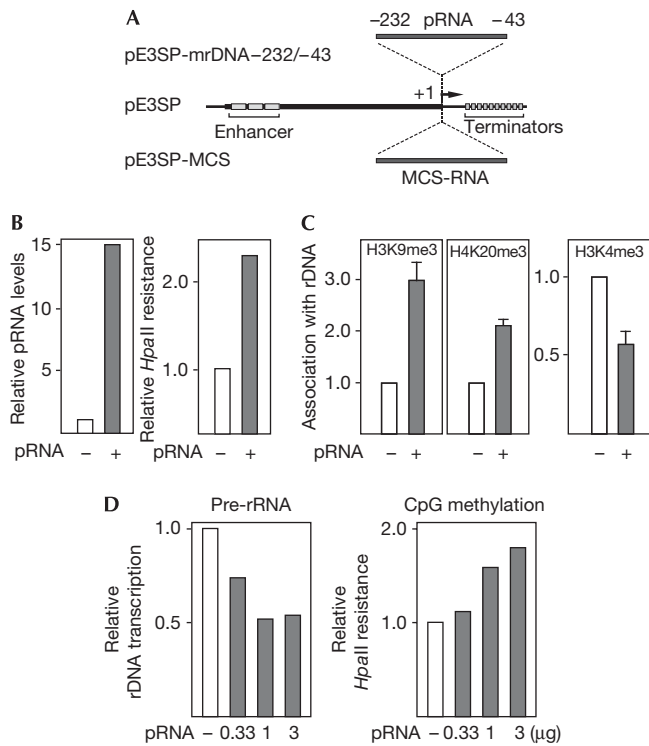
## DISCUSSION

Recently, Tseng *et al* (2008) identified several mouse rDNA variants that are genetically distinct and show a tissue-specific expression pattern, challenging the idea that rDNA is an array of identical, co-regulated repetitive units. Our study supports the idea of the existence of rDNA variants with distinct functions. We show that the spacer promoter of a specific set of hypomethylated rRNA genes directs the synthesis of IGS rRNA in early S phase. These spacer transcripts are processed during mid to late S phase to yield pRNA that is indispensable for NoRC-dependent rDNA silencing (Mayer *et al*, 2006). Although the spacer promoter and the main gene promoter have some sequence homology and Pol I is enriched at the spacer promoter, IGS transcripts are rare and

cannot be detected on northern blots, indicating that IGS rRNA is rapidly degraded unless stabilized by binding to NoRC. In turn, NoRC requires the association with pRNA for binding to chromatin and heterochromatin formation.

Notably, timing of IGS rRNA transcription and processing into pRNA correlates with NoRC binding to silent rRNA genes during mid to late S phase; that is, the time when NoRC associates with newly replicated silent genes (Li *et al*, 2005). Thus, the cell carefully tunes the timing of IGS rRNA transcription/processing to inherit rDNA silencing during cell division, suggesting that replication timing represents a 'window of opportunity' to propagate the epigenetic state and transcriptional activity of individual rDNA clusters, allowing activators or repressors to bind selectively to newly replicated chromatin.

The finding that transcripts from the spacer promoter have an indispensable function in epigenetic silencing of rDNA is in apparent disagreement with previous studies showing that,



**Fig 4** | pRNA functions in *trans*. (A) Scheme of pE3SP-mrDNA-232/-43 used to express ectopic pRNA. The vector pE3SP contains 5'-terminal rDNA sequences (-639/-1) including three enhancer elements (grey boxes) fused to 3'-terminal Pol I transcription terminators (Brenz Verca *et al*, 2007). The rDNA sequences or unrelated sequences from the MCS of pBluescript SK+ were inserted downstream from the Pol I transcription start site (+1) to yield pE3SP-mrDNA-232/-43 or pE3SP-MCS. (B) Ectopic pRNA induces hypermethylation of rDNA in *trans*. NIH3T3 cells were transfected with pE3SP-MCS (white bars) or pE3SP-mrDNA-232/-43 (grey bars). The graph on the left illustrates the level of ectopic pRNA relative to cellular pRNA. The graph on the right shows the ratio of *HpaII*-resistant rDNA to total rDNA in cells overexpressing pRNA or MCS RNA. (C) Ectopic pRNA triggers heterochromatin formation in *trans*. ChIP assays monitoring the indicated histone modifications (H3K9me3, H4K20me3 and H3K4me3) at the rDNA promoter after transfection of NIH3T3 cells with pE3SP-MCS (white bars) or pE3SP-mrDNA-232/-43 (grey bars). (D) Synthetic pRNA induces hypermethylation and rDNA silencing. NIH3T3 cells were transfected with the indicated amounts of *in vitro* transcribed MCS RNA (white bars) or pRNA-205/-1 (grey bars). Left panel: pre-rRNA synthesis was monitored by RT-qPCR and normalized to *GAPDH* mRNA. Right panel: data represent the ratio of *HpaII*-resistant rDNA to total rDNA in pRNA-transfected compared with MCS RNA-transfected cells. ChIP, chromatin immunoprecipitation; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; MCS, multiple cloning site; Pol I, RNA polymerase 1; pre-rRNA, rRNA precursor; pRNA, promoter RNA; rDNA, ribosomal DNA; RT-qPCR, reverse transcriptase quantitative PCR.

in *Xenopus* and mice, spacer promoters enhance pre-rRNA synthesis in *cis*, suggesting that spacer promoters form a functional unit in conjunction with adjacent enhancers to activate transcription (de Winter & Moss, 1986; Tower *et al*, 1989; Paalman *et al*, 1995; Caudy & Pikaard, 2002). This implies that the spacer

promoter has a dual function in rDNA transcription, regulating the readout of rDNA both in *cis* and in *trans*. It is probable that loop formation between the spacer promoter and the gene promoter activates Pol I transcription at specific rRNA genes (R.S., unpublished data), whereas the production of IGS rRNA from other repeats is required for NoRC-dependent rDNA silencing. These results reveal a further level of complexity in the regulation of rDNA transcription, suggesting that different gene clusters are independently regulated and have distinct functions.

## METHODS

**Plasmids.** The plasmid pMr-168 contains rDNA sequences from -168 to +155 with respect to the transcription start site. The plasmid pMr-2175 contains spacer sequences from -2175 to -1905. The rDNA reporter plasmids are fusions of 5'-terminal rDNA fragments containing the pre-rRNA gene promoter (pMr-1922/+130-BH) or the spacer promoter (pMr-2175/-1933-BH) with a 3'-terminal fragment including two terminator elements. Reporter transcripts were monitored by RT-qPCR using primers that amplify pUC sequences present between the promoter and the terminators. pE3SP-mrDNA-232/-43, rDNA sequences from -232 to -43 were inserted into pE3SP (Brenz Verca *et al*, 2007). To synthesize pRNA *in vitro*, pT7mr-205/-1 was used, a pTOPO<sup>®</sup> vector containing mouse rDNA sequences from -205 to -1 fused to the T7 bacteriophage promoter.

**Transfection and RNA analysis.** NIH3T3 cells were transfected with plasmid DNA by using the calcium phosphate co-precipitation method or with synthetic pRNA (1  $\mu$ g) by using the Mirus *TransIT* TKO reagent (Mobitec, Göttingen, Germany). Cellular IGS rRNA and pre-rRNA were measured by RT-PCR with random hexameric primers or a primer encompassing rDNA sequences from -20 to -1. For RNA-FISH, NIH3T3 cells were fixed for 15 min with 4% formaldehyde and permeabilized with 0.5% Triton X-100 for 5 min. Cells were incubated at 37 °C overnight with a biotin-labelled RNA probe (5 ng/ $\mu$ l) in 50% formamide, 2  $\times$  saline sodium citrate, transfer RNA (1 mg/ml), 1% bovine serum albumin and 10% dextran sulphate. Signals were visualized with extravidin-Cy3 conjugate (Sigma, Deisenhofen, Germany). The IGS rRNA was visualized by hybridization with a biotin-labelled RNA probe encompassing sequences from -554 to -447; pRNA was detected using a probe comprising sequences from -232 to -140 with respect to the transcription start site.

***In vitro* transcription assays.** The assays (25  $\mu$ l) contained 50 ng of template DNA (pMr-168 or pMr-2175 linearized with *NdeI*), 30  $\mu$ g of nuclear extract proteins from exponentially growing FM3A cells, 12 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-KOH (pH 8.0), 0.1 mM EDTA, 5 mM MgCl<sub>2</sub>, 80 mM KCl, 10 mM creatine phosphate, 12% (v/v) glycerol, 0.66 mM each of ATP, GTP and CTP, 0.01 mM UTP and 0.5 mCi [ $\alpha$ -<sup>32</sup>P]UTP (5,000 Ci/mmol). After incubation for 60 min at 30 °C, RNA was extracted and analysed on 4.5% polyacrylamide gels. To synthesize pRNA, a DNA fragment containing the T7 promoter fused to mouse rDNA sequences from -205 to -1 was used as a template in T7 RNA polymerase-driven transcription assays. Control RNA (MCS-RNA) was synthesized to generate T7 RNA polymerase from pBluescript II SK<sup>+</sup>/*EcoRI*.

**Cell synchronization.** The NIH3T3 cells were arrested at the G1/S boundary by treatment with aphidicolin (2  $\mu$ g/ml for 20 h), released and labelled in 1 h intervals for 30 min with 30  $\mu$ M BrdU.

Newly replicated DNA was immunoprecipitated with BrdU antibody in the presence of BrdU-labelled *Escherichia coli* DNA, and measured by semi-quantitative PCR (Li *et al*, 2005).

**ChIP and DNA methylation assays.** Sheared cross-linked chromatin (40 µg) was incubated with antibodies at 4 °C overnight in 16.7 mM Tris–HCl (pH 8.0), 167 mM NaCl, 0.01% SDS and 1.1% Triton X-100. Immunoprecipitates were captured on protein G/A–Sepharose saturated with salmon sperm DNA (0.25 mg/ml) for 2 h. Protein–DNA complexes were washed twice in a low salt buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 5 mM MgCl<sub>2</sub> and 1% Triton), twice in the same buffer but containing 500 mM NaCl, once in a buffer containing 250 mM LiCl, 10 mM Tris–HCl (pH 8.0), 5 mM EDTA, 0.5% Na-deoxycholate and 0.5% Triton, and twice in Tris–EDTA (TE) buffer. After reversal of the cross-links (6 h at 65 °C) and digestion with proteinase K, DNA was extracted and amplified by qPCR. DNA in the immunoprecipitates and input chromatin was normalized to control reactions from mock-transfected cells. To monitor CpG methylation, 1 µg genomic DNA was digested with 5 U *Hpa*II or *Msp*I before PCR amplification with promoter-specific primers (Mayer *et al*, 2006). Primer sequences are listed in the supplementary information online.

**Supplementary information** is available at *EMBO reports* online (<http://www.emboreports.org>).

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#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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