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The structure of NoRC-associated RNA is crucial for targeting the chromatin remodelling complex NoRC to the nucleolus

Christine Mayer[+], Melanie Neubert & Ingrid Grummt

Division of Molecular Biology of the Cell II, German Cancer Research Center, Heidelberg, Germany

Silencing of ribosomal RNA genes (rDNA) requires binding of the chromatin remodelling complex NoRC to RNA that is complementary to the rDNA promoter. NoRC-associated RNA (pRNA) folds into a conserved stem–loop structure that is required for nucleolar localization and rDNA silencing. Mutations that disrupt the stem–loop structure impair binding of TIP5, the large subunit of NoRC, to pRNA and abolish targeting of NoRC to nucleoli. Binding to pRNA results in a conformational change of TIP5, as shown by enhanced sensitivity of TIP5 towards trypsin digestion. Our results indicate an RNA-dependent mechanism that targets NoRC to chromatin and facilitates the interaction with co-repressors that promote heterochromatin formation and rDNA silencing.

INTRODUCTION

In eukaryotes, most DNA does not have any protein coding ability; many of these sequences, however, are transcribed, generating an extensive noncoding (nc) RNA transcriptome that correlates with the increasing complexity of regulatory networks in higher eukaryotes. Thus, ncRNAs are important as biological regulators, rather than simply being the by-products of background transcription. Recently, transcripts originating from a spacer promoter located about 2 kb upstream from the pre-ribosomal RNA transcription start site have been shown to be important in ribosomal RNA gene (rDNA) silencing (Mayer *et al*, 2006). The data suggested that short-lived intergenic transcripts are processed into 150–250 nucleotide (nt) RNAs that overlap the rDNA promoter. This 'promoter RNA' (pRNA) is stabilized by binding to TIP5 (TIF interacting protein 5), the large subunit of the chromatin remodelling complex NoRC. NoRC is associated

Division of Molecular Biology of the Cell II, German Cancer Research Center, DKFZ-ZMBH-Alliance, INF 581, D-69120 Heidelberg, Germany
[+]Corresponding author. Tel: + 49 6221 42 1424; Fax: + 49 6221 42 3467; E-mail: christine.mayer@dkfz.de

with silent rDNA and mediates heterochromatin formation and transcriptional silencing of a fraction of rDNA repeats (Santoro *et al*, 2002; Santoro & Grummt, 2005; Zhou & Grummt, 2005). Antisense-mediated depletion of pRNA leads to displacement of NoRC from nucleoli, decreased rDNA methylation and activation of RNA polymerase I (Pol I) transcription, indicating the requirement of pRNA in rDNA silencing (Mayer *et al*, 2006).

Here, we have studied the molecular principles that control NoRC–pRNA recognition. We show that TIP5 recognizes a phylogenetically conserved hairpin structure that is contained within the minimal RNA binding domain. Mutations that disrupt the hairpin alleviate the interaction with TIP5, whereas compensatory mutations that maintain the hairpin structure restore binding to TIP5. Furthermore, RNase footprinting and protease sensitivity experiments show that TIP5 binds to pRNA in an induced fit mechanism, resulting in structural changes that might facilitate NoRC function. Depletion of pRNA causes translocation of NoRC from nucleoli to the nucleoplasm, whereas 'refeeding' with ectopic pRNA restores nucleolar localization. This targeting process requires both the hairpin structure of pRNA and sequences upstream from the hairpin. The results show an important role of RNA in targeting a chromatin remodelling complex to a gene promoter to trigger heterochromatin formation and transcriptional silencing.

RESULTS

### NoRC recognizes a conserved hairpin within pRNA

Mammalian rDNA promoters share little sequence homology (Fig 1A), which accounts for the species specificity of rDNA transcription that requires factors from the same or closely related organisms. In cell-free transcription assays, transcription of the murine template requires factors present in nuclear extracts from mouse cells, whereas transcription of human rDNA templates requires human factors (Fig 1B). Similarly, in transient transfection assays, the murine Pol I reporter was efficiently transcribed in mouse but not in human cells, and vice versa (Fig 1C). By contrast, NoRC-dependent repression of Pol I transcription was not species specific. Overexpression of murine TIP5 repressed Pol I transcription in both human and mouse cells (Fig 1D), which is an unexpected result, given that NoRC function requires binding of TIP5 to pRNA—that is, RNA complementary to the rDNA promoter (Mayer *et al*, 2006).
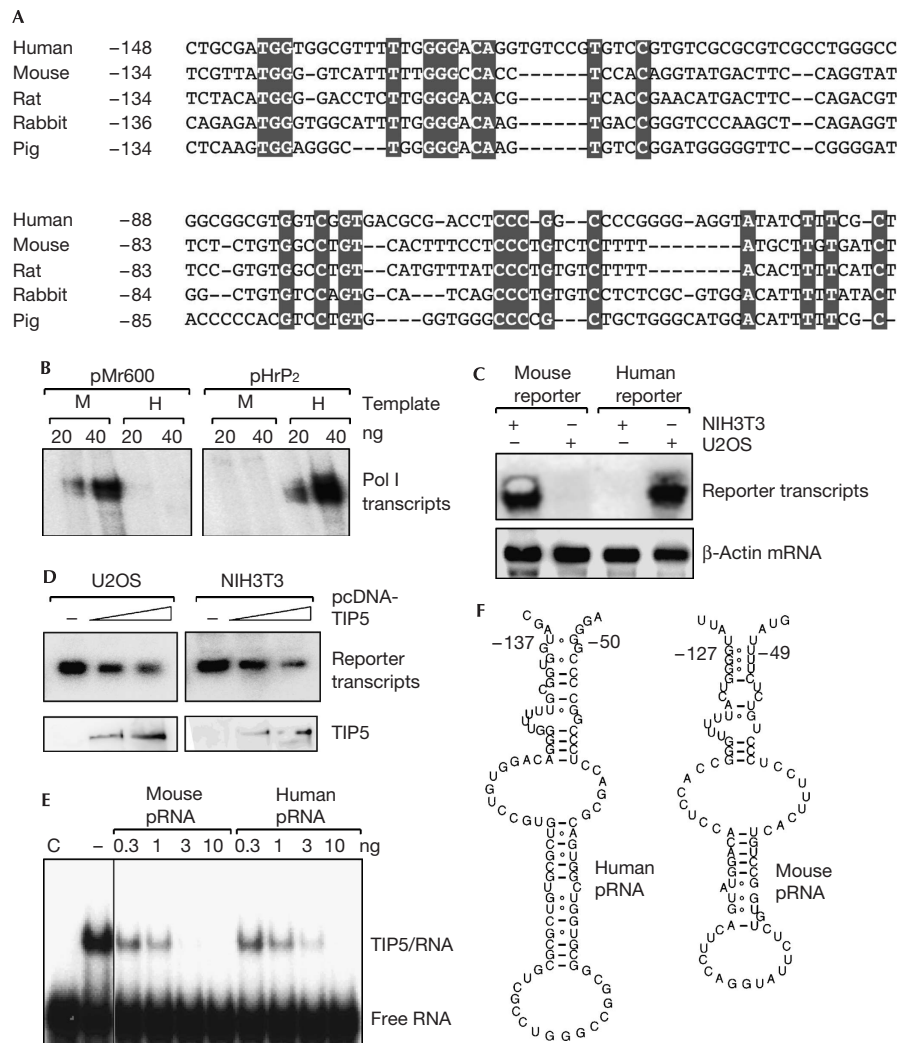
Fig 1 | Transcription of ribosomal RNA genes but not NoRC-dependent silencing is species specific. (A) Alignment of mammalian rDNA promoter sequences. Conserved bases are highlighted, and numbers refer to the position of nucleotides with respect to the polymerase I (Pol I) transcription start site. Sequence alignment was performed using the CLUSTALW 1.74 software. **(B)** Pol I transcription is species specific. *In vitro* transcription assays contained either a linearized mouse (pMr600) or human (pHrP$_2$) rDNA template and 20 or 40 µg nuclear extract (NE) from human (HeLa; H) or mouse (FM3A; M) cells. **(C)** *In vivo* reporter assays. NIH3T3 cells (left) and U2OS cells (right) were transfected with 2.5 µg of a human (pHrP$_2$-BH) or murine (pMr1930-BH) Pol I reporter plasmid. Transcripts from the reporter plasmids and β-actin mRNA were analysed on Northern blots. **(D)** NoRC represses transcription in both mouse and human cells. U2OS (left panel) and NIH3T3 cells (right panel) were co-transfected with pcDNA-HA/Flag-TIP5 (4 and 8 µg) and either the human reporter pHrP$_2$-BH or the mouse reporter pMr1930-BH, and RNA was analysed on Northern blots. The Western blot (bottom) shows the expression of HA/Flag-tagged TIP5 in U2OS and NIH3T3 cells. **(E)** Murine TIP5$_{1-598}$ binds to human and mouse pRNA with similar affinity. *In vitro* transcripts corresponding to selected regions of murine rDNA were used in electrophoretic mobility shift assays to compete for binding of TIP5$_{1-598}$ to radiolabelled run-off transcripts from pBluescript (MCS-RNA). **(F)** Computer-predicted secondary structure of human (left) and mouse (right) pRNA. The numbers refer to the position of nucleotides with regard to the Pol I transcription start site. Secondary structure prediction of RNA folds was performed using the 'RNA Alifold' program of the Vienna RNA structure prediction package. HA, haemagglutinin; pRNA, promoter RNA; rDNA, ribosomal RNA genes.

To examine whether TIP5 interacts with both human and mouse pRNA, we performed electrophoretic mobility shift assays (EMSAs) using unspecific radiolabelled T7 run-off transcripts (MCS-RNA). The competition assays in Fig 1E show that pRNA binds with high affinity to TIP5, displacing unspecific MCS-RNA from TIP5, regardless of whether human or mouse pRNA was used as a competitor. Thus, TIP5 binds to pRNA from both species with similar affinity, which is consistent with NoRC silencing rDNA in both species.

In search for structural motifs that are shared by pRNA from various species, a computational approach showed that RNAs corresponding to rDNA promoters of several mammals, including
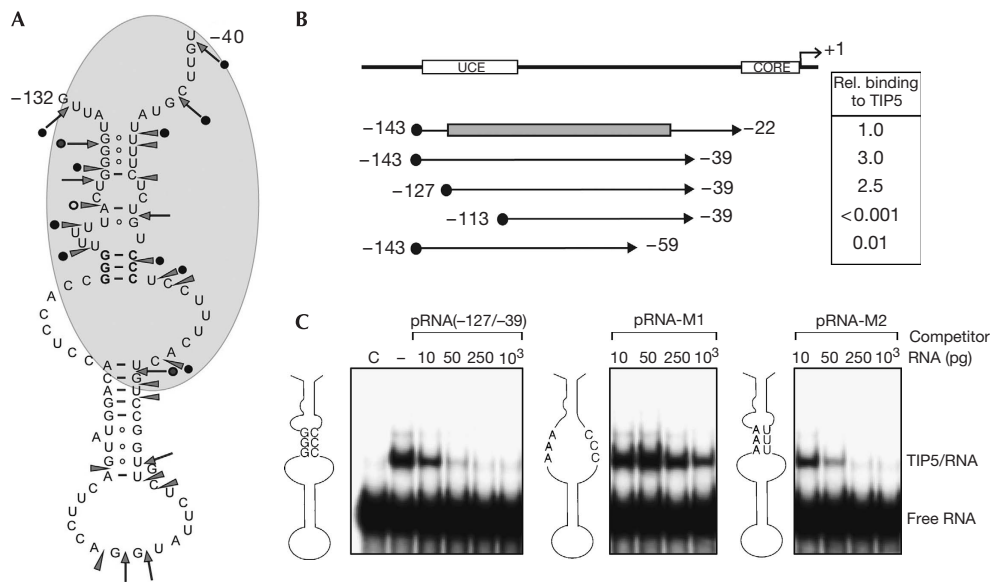
Fig 2 | TIP5 recognizes the secondary structure of promoter RNA. (**A**) Schematic depicting the secondary structure of mouse pRNA as shown by RNase footprinting. Cleavages by RNase T1 are indicated by arrows, and RNase V1 cleavages by arrowheads. Cleavages that are decreased as a consequence of human TIP5 binding are indicated by black circles. The area of pRNA that is shielded from RNase cleavage is shaded in grey. The three G-C base pairs mutated in pRNA-M1 are indicated in bold. (**B**) Mapping of the minimal TIP5 interacting RNA domain. Black circles indicate T7 promoter-derived sequences common to all run-off transcripts used. The grey box marks the part of pRNA that is involved in hairpin formation. The numbers on the right are taken from Table 1, representing the relative binding affinities of murine $TIP5_{1-598}$. (**C**) Mutations that disrupt the secondary structure abrogate TIP5 binding to pRNA. Mouse pRNA and mutants pRNA-M1 and pRNA-M2 comprising the minimal sequence required for TIP5 binding (from −127 to −39) were used in EMSA competition assays containing murine $TIP5_{1-598}$ and labelled MCS-RNA. The schemes indicate nucleotide exchanges in pRNA-M1 and -M2 and the predicted structure of the individual pRNAs. pRNA, promoter RNA.

human, mouse, rat and pig, can fold into a common secondary structure. The most striking feature of this hypothetical structure is a hairpin that contains rDNA sequences from nucleotides −127 to −49 in mouse and from −137 to −50 in human pRNA (Fig 1F).

TIP5 recognizes the stem–loop structure of pRNA

RNase structure mapping and footprinting experiments showed that TIP5 binding protects sequences near the base of the hairpin (supplementary Fig S1 online). As summarized in Fig 2A, the results support the algorithm-predicted hairpin in pRNA, showing that TIP5 specifically recognizes the basal part of the hairpin, thus stabilizing the RNA structure.

To define the minimal RNA sequence that interacts with TIP5, we synthesized pRNAs comprising different lengths of promoter sequences and assayed the ability of these transcripts to compete for TIP5 binding to unspecific MCS-RNA. This analysis showed that TIP5 bound with high affinity to pRNAs containing rDNA sequences −127 to −39 (Fig 2B). Truncation at the 5′ or 3′ end lowered the binding affinity at least 100-fold, indicating that sequences from −127 to −39 are crucial for binding to TIP5 (Table 1). Strikingly, this sequence contains the predicted hairpin structure (from −127 to −49), suggesting that NoRC recognizes this specific RNA structure.

To examine whether the hairpin fold is required for NoRC binding to pRNA, we disrupted the basal part of the predicted stem

Table 1 | Binding of murine $TIP5_{1-598}$ to promoter-proximal murine and human pRNA transcripts

pRNA transcripts	Relative binding
Murine	
rDNA −168/+7	50
rDNA −205/−1	350
rDNA −143/−22	500
rDNA −143/−39	500
rDNA −127/−39	400
rDNA −143/−59	5
rDNA −113/−39	<0.1
rDNA −127/−39 (M1)	0.4
rDNA −127/−39 (M2)	330
Human	
rDNA −143/−22	125

Radiolabelled MCS-RNA was incubated with purified $TIP5_{1-598}$ and increasing amounts of the indicated competitor RNAs, and RNP complexes were analysed by electrophoretic mobility shift assay. Numbers of pRNA transcripts denote positions relative to the pre-rRNA transcription start site. The amount of total RNA required to reduce binding of TIP5 to 50% was set to 1.0. rDNA, ribosomal RNA genes.
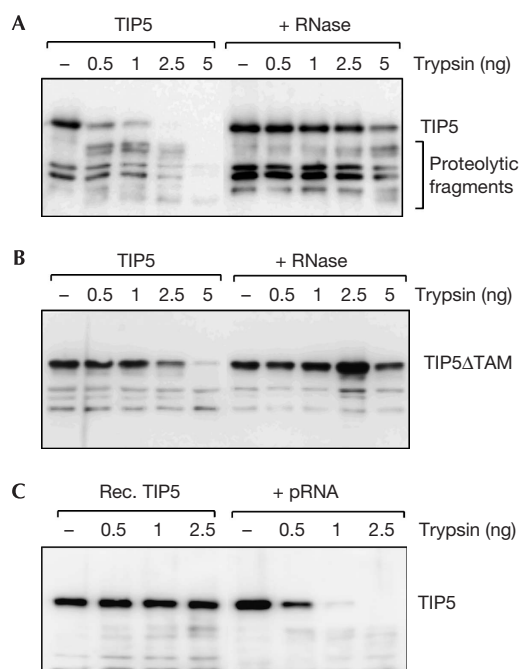
Fig 3 | Interaction with promoter RNA changes the conformation of TIP5.
(**A**) RNase treatment confers a more compact structure to TIP5.
Immunopurified HA/Flag-murine $TIP5_{1-731}$ (50 ng) was incubated with
increasing amounts of trypsin in the absence or presence of RNase A,
and cleavage products were analysed on immunoblots. (**B**) Same as in
(**A**) except that murine $TIP5_{1-731}$ lacking the RNA-binding domain
(TIP5ΔTAM) was used. (**C**) Interaction with pRNA renders TIP5
hypersensitive towards proteolysis. Recombinant (Rec.) murine $TIP5_{1-598}$
was preincubated with 200 ng yeast transfer RNA or with mouse pRNA
(−127/−39), and subjected to partial proteolysis and immunoblotting.
HA, haemagglutinin; pRNA, promoter RNA.

structure by replacing three guanosine residues (nucleotides
−113, −114 and −115) with adenosine residues, and measured
the relative binding affinity of TIP5 to wild-type and mutant pRNA
in EMSA competition assays. Mutations that prevent base pairing
between nucleotides −113 to −115 and −60 to −58, and
therefore destroy the stem–loop (pRNA-M1), abrogate binding to
TIP5, whereas introduction of compensatory C to U mutations
(pRNA-M2), allowing hairpin formation, restored high-affinity
binding (Fig 2C). These results are consistent with TIP5 recogniz-
ing an evolutionarily conserved RNA conformation, suggesting
that the secondary structure rather than the primary pRNA
sequence is selected.

Binding to pRNA induces a structural change of TIP5
To examine whether binding to RNA induces a conformational
change in TIP5, we preincubated immunopurified TIP5 in the
absence or presence of RNase A before digestion with limited
amounts of trypsin. The immunoblot in Fig 3A shows that
$TIP5_{1-731}$ was efficiently cleaved by low concentrations of trypsin.
However, after treatment with RNase A, $TIP5_{1-731}$ was about
20-fold more resistant towards proteolysis, indicating that in

the absence of RNA the protein has adopted a more compact
structure. In support of this, TIP5ΔTAM, a TIP5 mutant that lacks
the TAM domain and is deficient in RNA binding (Mayer *et al*,
2006), was also more resistant towards trypsin digestion (Fig 3B).

Finally, we compared the protease sensitivity of TIP5 in the
absence or presence of exogenous pRNA. TIP5 was first treated
with RNase A, incubated with a control RNA (yeast transfer RNA,
lanes 1–4) or mouse pRNA (lanes 5–8), and then subjected to partial
proteolysis (Fig 3C). Binding to pRNA, but not tRNA, induced the
trypsin-hypersensitive 'open' protein structure that is characteristic
of cellular TIP5. These results indicate that on pRNA binding, TIP5
undergoes a conformational change in the region encompassing the
TAM domain, and this structural change might facilitate the
interaction with other proteins required for NoRC function.

pRNA regulates the nucleolar localization of NoRC
Depletion of pRNA by antisense locked nucleic acid (LNA)-DNA
gapmers leads to translocation of NoRC from nucleoli to the
nucleoplasm (Mayer *et al*, 2006). To show the direct role of pRNA
in targeting NoRC to nucleoli, we sought to restore the nucleolar
localization of NoRC in pRNA-depleted NIH3T3 cells by adding
an excess of exogenous pRNAs to permeabilized cells. As
shown in Fig 4A, knockdown of pRNA (supplementary Fig S2
online) resulted in translocation of NoRC into the nucleoplasm
without affecting the nucleolar localization of upstream binding
factor (UBF). Notably, 'feeding' of pRNA-depleted cells with
size-fractionated nucleolar RNA (<500 nt), but not with tRNA,
restored the nucleolar localization of NoRC (Fig 4B). Similarly, *in
vitro* transcripts comprising mouse or human pRNA sequences
from −205 to −1 rescued NoRC localization (Fig 4B,C). However,
mutant pRNA in which the predicted stem structure has been
disrupted (pRNA-M1) did not restore NoRC localization. Thus,
exogenous pRNA is able to trigger relocalization of NoRC into
nucleoli, provided that its secondary structure is kept intact.

Next, we examined whether the pRNA hairpin loop—that is,
the minimal sequence required for NoRC binding to pRNA—is
sufficient for targeting NoRC to nucleoli or whether other
sequences are crucial for NoRC localization. For this, we used
the 'refeeding' assay to compare the ability of 'long' pRNA (from
−205 to −1) and 'short' pRNA (from −127 to −39) to mediate
nucleolar relocation of NoRC after knockdown of endogenous
pRNA. Although 'short' pRNA bound to NoRC with higher affinity
than the 'long' pRNA (Table 1), it was not able to restore the
nucleolar localization of NoRC (Fig 4C, upper panel), indicating
that sequences adjacent to the stem–loop are necessary for pRNA-
mediated nucleolar localization of NoRC. To examine whether
these adjacent regions are species specific, we generated
chimaeric 'human–mouse' or 'mouse–human' pRNAs in which
rDNA sequences from −205 to −126 from one species were fused
to the hairpin fold (−127 to −39) from the other species, and
assayed these RNAs for their ability to restore NoRC localization.
In accordance with the cross-species relevance of pRNA in NoRC
function, both pRNA chimaera facilitated NoRC translocation to
nucleoli (Fig 4C). However, if the adjacent region was replaced by
an unrelated RNA sequence, the hybrid RNA was much less able
to relocate NoRC (Fig 4C). This shows that TIP5 binding to the
hairpin structure of pRNA is necessary but not sufficient for
targeting NoRC to nucleoli, and NoRC recruitment to nucleolar
chromatin requires 5′ adjacent sequences of pRNA that cover
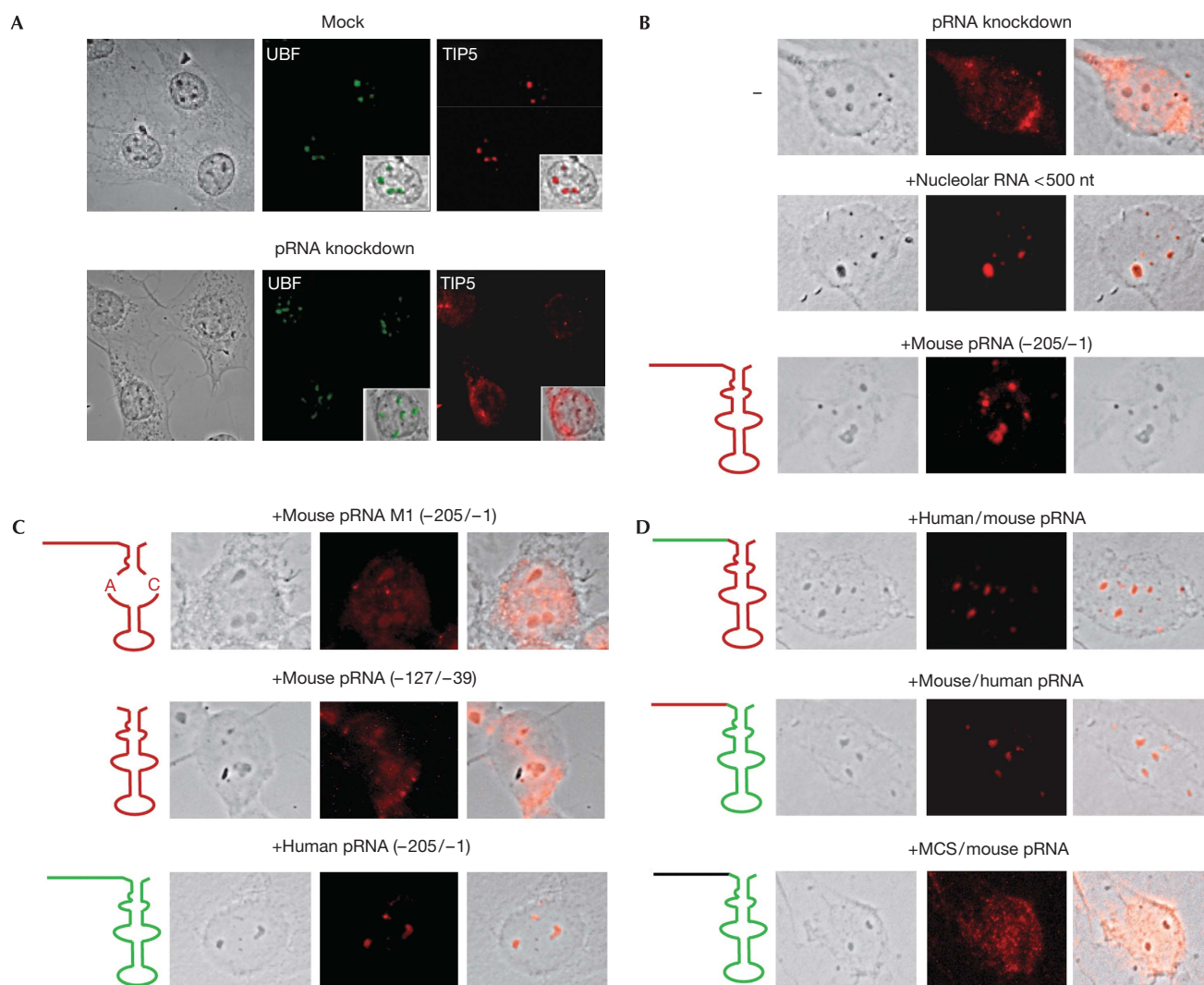
Fig 4 | Promoter RNA is required for nucleolar localization of NoRC. (**A**) Exogenous pRNA restores the nucleolar localization of NoRC in pRNA-depleted cells. NIH3T3 cells were transfected with 50 nM of control (mock) or antisense LNA-DNA (pRNA knockdown), and TIP5 and UBF were visualized by immunostaining. The insets show an overlay of phase-contrast images and immunostaining. (**B**) Permeabilized NIH3T3 cells were incubated with transfer RNA, nucleolar RNA (<500 nt) or in vitro-synthesized mouse pRNA ($-205/-1$), and NoRC localization was visualized with TIP5 antibodies (red). Phase-contrast images, an immunofluorescence of TIP5 and an overlay with the respective phase-contrast image are shown (left to right). (**C**) Same as in (**B**) but permeabilized cells were incubated with mouse pRNA-M1 ($-205/-1$), mouse pRNA ($-127/-39$) and human pRNA ($-205/-1$) as indicated. The schematics show characteristics of the pRNA variants used for refeeding. Mouse rDNA sequences are drawn in red, and human rDNA sequences in green. (**D**) Same as in (**B**) but permeabilized cells were incubated with chimaeric human/mouse or mouse/human pRNAs ($-205/-1$) as indicated. In the bottom panels, pRNA sequences from -205 to -128 were replaced by unrelated sequences from the multiple cloning site (MCS/mouse pRNA). Mouse rDNA sequences are drawn in red, human rDNA sequences in green and unspecific MCS sequences in black. pRNA, promoter RNA; rDNA, rRNA genes.

important regulatory elements such as the upstream terminator T_0 and the upstream control element.

DISCUSSION

Here, we have used RNA binding assays in conjunction with partial proteolysis and RNA replenishment experiments to decipher the structure–function relationship of NoRC binding to pRNA. We have shown that mammalian rDNA-specific pRNAs fold into a conserved secondary structure, and that this structure is

required for high-affinity binding of TIP5. Mutations that affect the maintenance of the hairpin stem impair TIP5 binding, whereas introduction of compensatory base changes restores the interaction with TIP5. The results indicate that TIP5 recognizes a specific stem–loop structure within pRNA that is conserved across mammalian species. The interaction of TIP5 with this specific RNA fold might provide the selection pressure necessary for the conservation of RNA conformation, and might allow NoRC to tolerate mutational changes without loss of function. Such

plasticity of the interacting partners would allow for evolution of a broad spectrum of RNA binding specificities despite the selective pressure that conserves the primary sequence of the RNA-binding site. Of note, the fact that unrelated sequences can fold into similar secondary structures raises the exciting possibility that other RNA binding partners might interact with TIP5 and guide NoRC to non-ribosomal target genes outside of the nucleolus to induce heterochromatin formation and transcriptional silencing.

The structure and function of proteins are affected and regulated by interacting partners, including RNAs (Williamson, 2000). In accordance with this, we found that association with RNA induces a conformational change of TIP5. If bound to RNA, TIP5 adapts an 'open', trypsin-hypersensitive structure, whereas both RNase A treatment and deletion of the RNA-binding domain confer a more compact structure that is less sensitive towards proteolysis. This implies an induced fit mechanism—a common feature of RNA–protein complexes (Puglisi *et al*, 1995)—using the binding energy to drive conformational changes to ensure that the RNA–protein complex exerts its function only after the appropriate conformational adaptation has occurred. Such a structural change might facilitate or modulate the interaction of TIP5 with other proteins required for NoRC function such as histone deacetylases (HDACs) and DNA methyltransferases (DNMTs; Santoro & Grummt, 2005; Yuan *et al*, 2007). As TIP5 is apparently more extended if bound to RNA, this 'open' configuration would allow for additional interaction surfaces to become available.

Regarding the role of pRNA in targeting NoRC to rDNA, we have previously shown that heterochromatin formation and nucleolar localization of NoRC require the interaction of TIP5 with pRNA (Mayer *et al*, 2006). After depletion of pRNA, NoRC was no longer nucleolar, being distributed throughout the nucleoplasm. Here, we have used a 'refeeding' or 'RNA replenishment' assay, involving a brief incubation of permeabilized, pRNA-depleted cells with size-fractionated nucleolar RNA or with *in vitro* transcripts. These assays showed that exogenous pRNA was able to re-establish the nucleolar localization of NoRC in pRNA-depleted cells. Notably, rescue of NoRC localization required pRNA containing sequences from −205 to −1—that is, the part of rDNA that comprises essential regulatory elements, the core promoter, the upstream control element and the upstream terminator T_0. Consistent with the prevalent use of RNA to target chromatin-associated factors to subcellular structures, our results show that pRNA is necessary and sufficient to target NoRC to nucleoli. As complex rearrangements in the chromatin structure are unlikely to occur within the short time needed for NoRC relocalization, pRNA seems to have a role in targeting NoRC to nucleolar chromatin. This targeting process requires the stem–loop structure that is required for TIP5 binding (nucleotides −127/−39), as well as further upstream sequences (up to nucleotide −205). Notably, both mouse and human pRNA sequences are able to target NoRC to nucleoli, whereas replacement of the upstream part of pRNA by unrelated sequences impairs nucleolar localization of NoRC, underscoring the role of the hairpin in TIP5 binding and upstream pRNA sequences in targeting NoRC to nucleoli. Given that silencing of a fraction of rDNA repeats has an important role in genome stability (Kobayashi *et al*, 2004; Peng & Karpen, 2007), NoRC-associated pRNA might control the number of silent rDNA repeats, thereby contributing to rDNA stability, nucleolar structure and longevity.

METHODS

Plasmids. pMr1930-BH and pHrP$_2$-BH, pMr600 and pHrP$_2$ have been described previously (Budde & Grummt, 1999; Mayer *et al*, 2005). pcDNA-haemagglutinin (HA)/Flag-TIP5 encodes full-length murine TIP5 and pcDNA-HA/Flag-TIP5$_{1−731}$ encodes amino acids 1–731 of murine TIP5 fused to an amino-terminal HA/Flag tag.

Transfections and RNA analysis. A total of 3×10^5 NIH3T3 cells or 8×10^5 HEK293T cells were transfected with 1 µg of Pol I reporter plasmids and various amounts of pcDNA-HA/Flag-TIP5. Transcripts from reporter plasmids were monitored on northern blots. To normalize for RNA loading, blots were probed for β-actin messenger RNA. Alternatively, transcripts were analysed by real-time quantitative PCR with primers specific for the rDNA reporter and glyceraldehyde-3-phosphate dehydrogenase mRNA.

Expression and purification of recombinant proteins. His-tagged murine TIP5$_{1−598}$ and human TIP5$_{510−611}$ were expressed in *Escherichia coli* BL21(DE3) codon$^+$ and purified on Ni-NTA agarose. For expression in mammalian cells, HEK293T cells were transfected with pcDNA-HA/Flag-TIP5, and TIP5 was purified with HA or Flag antibodies.

In vitro **transcription assays.** A 25 µl assay sample contained 50 ng of template DNA (pMr600/*Eco*RI or pHrP$_2$/*Eco*RI), 30 µg of nuclear extract proteins from exponentially growing FM3A or HeLa cells, 12 mM Hepes–KOH (pH 8.0), 0.1 mM EDTA, 5 mM MgCl$_2$, 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 [α-^{32}P]UTP (5,000 Ci/mmol). After incubation for 60 min at 30 °C, RNA was extracted and analysed on 4.5% polyacrylamide gels.

Electrophoretic mobility shift assays. Radiolabelled MCS-RNA was synthesized by T7 RNA polymerase using pBluescript-KS/*Eco*RI as a template. A 1–2 fmol portion of RNA was incubated for 5 min on ice with 1–3 pmol of recombinant TIP5 in 20 µl binding buffer (20 mM Tris–HCl (pH 8.0), 5 mM MgCl$_2$, 100 mM KCl and 0.2 mM EDTA). After the addition of competitor RNA, incubation was continued for 15 min. RNA–protein complexes were analysed on 6% native polyacrylamide gels and visualized by autoradiography.

Refeeding assay and immunofluorescence. To examine the ability of pRNA to restore NoRC localization after knockdown of endogenous pRNA, NIH3T3 cells were transfected with antisense LNA-DNA gapmers (5′-CAGGtatgacttccaGGTA-3′; LNA bases, uppercase; DNA bases, lowercase) using *Trans*IT-TKO Transfection Reagent. After 48 h, cells were permeabilized for 3 min with 0.04% Triton X-100 in 20 mM Tris–HCl (pH 7.4), 5 mM MgCl$_2$, 0.5 mM EDTA and 25% glycerol and incubated for 30 min with 100 ng/ml of RNA in the presence of 25 U/ml RNasin (Promega, Madison, WI, USA). Cells were fixed with methanol (7 min) and acetone (1 min) at −20 °C, blocked with 1% BSA and immuno-stained with TIP5 and UBF antibodies.

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

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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