The EMBO Journal (2010) 29, 1215–1224 | © 2010 European Molecular Biology Organization | All Rights Reserved 0261-4189/10
www.embojournal.org

THE EMBO JOURNAL

Chromatin association and regulation of rDNA transcription by the Ras-family protein RasL11a

Mariaelena Pistoni[1], Alessandro Verrecchia, Mirko Doni, Ernesto Guccione[2] and Bruno Amati*

Department of Experimental Oncology, European Institute of Oncology (IEO), IFOM-IEO Campus, Milan, Italy

RasL11a and RasL11b are Ras super-family proteins of unknown function. Here, we show that RasL11a is a chromatin-associated modulator of pre-ribosomal RNA (pre-rRNA) synthesis. RasL11a was found in the nucleolus of interphase mouse fibroblasts, where it co-localized with the RNA polymerase I-specific transcription factor UBF. Similar to UBF, RasL11a also marked the active subset of rDNA repeats (also called nucleolar organizers, or NORs) on mitotic chromosomes. In cells, RasL11a existed in stable complexes with UBF and, as shown by chromatin immunoprecipitation, distributed along the rDNA transcription unit. Upon treatment of cells with actinomycin D, RasL11a and UBF persisted on the transcription unit beyond the release of RNA polymerase I, and remained co-localized in peri-nucleolar cap structures. Ectopic expression of RasL11a enhanced pre-rRNA levels in cells, whereas RasL11a knockdown had the opposite effect. In transient transfection experiments, RasL11a enhanced the transcriptional activity of an RNA polymerase I-specific reporter controlled by the rDNA enhancer/promoter region. We speculate that RasL11a acts in concert with UBF to facilitate initiation and/or elongation by RNA polymerase I in response to specific upstream stimuli.

The EMBO Journal (2010) **29,** 1215–1224. doi:10.1038/emboj.2010.16; Published online 18 February 2010
Subject Categories: chromatin & transcription
Keywords: nucleolus; Ras; ribosomal DNA; RNA polymerase I; UBF

Introduction

Proteins of the Ras super-family constitute a basic signalling module that has been adapted to a variety of pathways and processes. These proteins are small GTPases, and alternate between GTP- and GDP-bound states under control of specific guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). These two states regulate differential interactions with a variety of other proteins: the GTP-bound form is generally recognized by specific effectors, and is thus considered the active state in signalling. Hence, Ras super-family proteins act as molecular switches, regulating a multitude of events in diverse intracellular compartments and in response to numerous upstream signals (for reviews, see Takai *et al*, 2001; Colicelli, 2004; Bos *et al*, 2007). For several Ras-like proteins, however, information on cellular distribution and function is missing altogether. Here, we present the initial characterization one of these 'orphan' Ras super-family members, RasL11a.

Mammalian genomes contain two genes of the *RasL11* subfamily: *RasL11a* and *RasL11b* (Colicelli, 2004; Louro *et al*, 2004; Piek *et al*, 2004). A third gene, *RasL11c*, is present in marsupials but has been lost in placental mammals (Duret *et al*, 2006; Stolle *et al*, 2007). RasL11 proteins share the basic modular structure of the Ras super-family, although specific deviations in amino-acid sequence and negative biochemical results (for RasL11b, also called RLP) have cast doubt on whether RasL11 proteins are able to bind and hydrolyse GTP (Louro *et al*, 2004; Piek *et al*, 2004; Stolle *et al*, 2007). RasL11 proteins show another feature that is atypical within the Ras super-family, namely, the lack of a prenylation motif (CAAX) at their C-terminus, which serves to target Ras proteins to membrane compartments. Instead, RasL11 proteins have a distinct, positively charged C-terminal domain. This feature is shared with a minority of Ras-like proteins, in particular Ran, which is involved in nucleo-cytoplasmic shuttling (Weis, 2003; Colicelli, 2004). We thus investigated the subcellular distribution of RasL11a, leading to the unexpected finding that it is a nucleolar protein associated with ribosomal DNA (rDNA) and with the RNA polymerase I (RPI)-specific transcription factor UBF (Grummt, 2003; Moss *et al*, 2007; McStay and Grummt, 2008). Our data altogether identify RasL11a as a positive regulator of RPI transcription.

Results

Nucleolar localization of RasL11a

We raised two rabbit antisera directed against a C-terminal peptide of human RasL11a. The two antibodies (named Ab48 and Ab49) were affinity purified against the immunogenic peptide. As shown by immunoblot analysis after transient transfection in 293T cells, Ab48 and Ab49 specifically recognized either human or mouse RasL11a, but not the related protein RasL11b (Supplementary Figure S1A). In mouse NIH-3T3 fibroblasts, these antibodies recognized a protein of ca. 27 kDa: this band was downregulated on retroviral expression of a small hairpin RNA targeting the mouse *RasL11a* mRNA (shL11a), showing its identity as RasL11a (Supplementary Figure S1B). Most importantly, in the context of this work, neither of these antibodies cross-reacted with the nucleolar protein UBF (Supplementary Figure S1C). By indirect immunofluorescence analysis on NIH-3T3 fibroblasts, Ab49 stained multiple foci that were clustered within

*Corresponding author. Department of Experimental Oncology, European Institute of Oncology (IEO), IFOM-IEO Campus, Via Adamello 16, Milan 20139, Italy. Tel.: +39 02 57489824; Fax: +39 02 94375990; E-mail: bruno.amati@ifom-ieo-campus.it
[1]Present address: Division of Regenerative Medicine, San Raffaele Scientific Institute, Milan 20132, Italy
[2]Present address: Institute of Molecular and Cell Biology (IMCB), 61 Biopolis Drive, 138673, Singapore

Received: 7 September 2009; accepted: 26 January 2010; published online: 18 February 2010

nuclear sub-compartments reminiscent of nucleoli; this staining was specific, as it was eliminated either by infection with the shL11a retrovirus, or by pre-blocking of the antibody with the immunogenic peptide (Supplementary Figure S1D and E). Although with a higher cytoplasmic background, Ab48 yielded the same specific pattern in nuclei (Supplementary Figure S1F). A similar nuclear staining was observed with Ab49 in primary mouse embryo fibroblasts (MEFs) (Supplementary Figure S1G). We used confocal microscopy to address the co-localization of RasL11a with known nucleolar proteins (Boisvert *et al*, 2007) in NIH-3T3 cells: RasL11a foci were all included within the areas delimited by nucleophosmin (NPM), nucleolin, fibrillarin or UBF, showing nucleolar localization of RasL11a (Figure 1A–D). Merging of the two signals indicated precise co-localization of RasL11a with UBF, a factor that has a central role in transcription of rDNA repeats by RPI (Grummt, 2003; Moss *et al*, 2007; McStay and Grummt, 2008).

The haploid human and mouse genomes contain 200–300 rDNA genes, organized into five major clusters of direct repeats (the nucleolar organizers, or NORs) (Rowe *et al*, 1996; Moss *et al*, 2007). Only a fraction of the 10 NORs in a diploid cell are active, the others being in a repressed, heterochromatic state (Grummt, 2003; Moss *et al*, 2007; McStay and Grummt, 2008). On entry into mitosis the nucleolus disassembles and rDNA transcription is silenced, but active NORs retain their open configuration and remain associated with the RPI transcription machinery, including

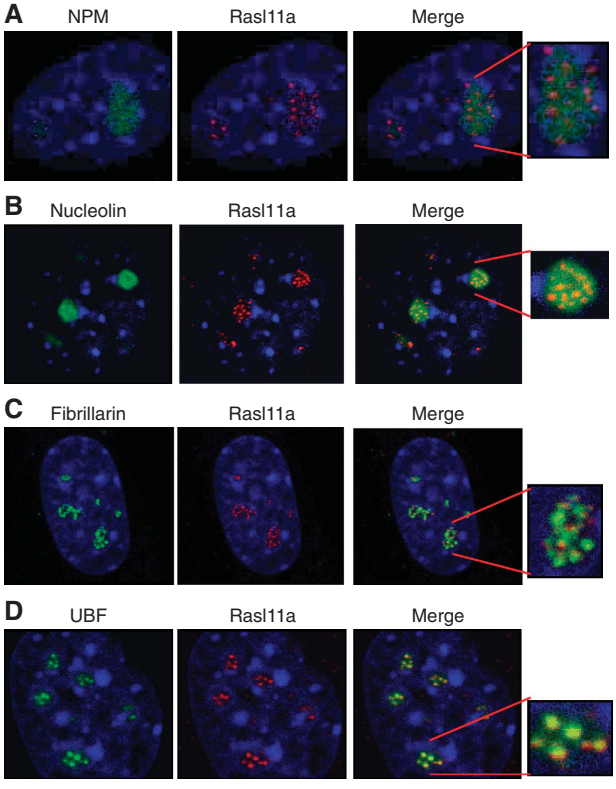

Figure 1 Subcellular localization of RasL11a. (**A**–**C**) Asynchronously proliferating NIH-3T3 cells were analysed by indirect immunofluorescence and examined under the confocal microscope. Nuclear DNA was counter-stained with DAPI. Representative confocal sections of a single nucleus are shown for interphase cells co-stained for (**A**) RasL11a (Ab49) and NPM, (**B**) RasL11a and nucleolin, (**C**) RasL11a and fibrillarin and (**D**) RasL11a and UBF.

UBF (Roussel *et al*, 1993, 1996; Jordan *et al*, 1996; Gebrane-Younes *et al*, 1997; Grummt, 2003; Leung *et al*, 2004; Mais *et al*, 2005; Moss *et al*, 2007; McStay and Grummt, 2008). In our immunofluorescence experiments, examination of cells at different stages of mitosis showed that RasL11a and UBF formed prevalent foci on chromosome arms. At virtually all of these foci, the two proteins where co-localized (Figure 2A). Serial optical sections confirmed that all chromosomal foci stained by one protein also contained the other (Supplementary Figure S2A). Co-staining with γ-tubulin and the centromeric marker CREST showed that RasL11a foci were not at centromeres (Supplementary Figure S2B). For either protein, additional staining was observed without co-localization: this was most abundant at the early stages of mitosis, and was invariably situated outside of—albeit adjacent to—chromosome arms (Figure 2A). Although the specificity of this extra-chromosomal staining remains to be established, it may conceivably correspond to a fraction of either protein that was not rDNA associated (see below) and was released on disassembly of the nucleolus. We then used immuno-FISH, combining immune labelling of RasL11a and fluorescent *in situ* hybridization with an rDNA probe, and analysed serial confocal sections on mitotic cells (i–viii, Figure 2B): this allowed us to visualize six pairs of dots (as expected from their occurrence on sister chromatids) in which the rDNA and RasL11a signals merged (yellow), whereas other dots stained for rDNA only (red). We found no chromosomal dots containing RasL11a without rDNA (green). The green cytosolic staining was only apparent on over-exposure, needed to visualize rDNA staining, and is thus considered background. Additional immuno-FISH serial sections on cells in metaphase, telophase or interphase confirmed the localization of RasL11a with rDNA at all stages (Supplementary Figures S3 and S4). In summary, RasL11a is a nucleolar protein that co-localizes with rDNA and UBF throughout the cell cycle. It follows that, similar to UBF, RasL11a remains associated with active NORs on mitotic chromosomes.

RasL11a binds the rDNA transcription unit in vivo

UBF binds the rDNA promoter to activate RPI transcription and similar to the RP1 complex (Stefanovsky *et al*, 2006) associates with the whole rDNA transcription unit *in vivo* (O'Sullivan *et al*, 2002; Grandori *et al*, 2005; Mais *et al*, 2005). We, thus, sought to address whether RasL11a also associates with rDNA *in vivo*, and whether its distribution along the rDNA repeat would parallel that of UBF or RPI. Asynchronously proliferating NIH-3T3 cultures were analysed by quantitative chromatin immunoprecipitation (qChIP) with antibodies against RasL11a, UBF or the RP1 subunit RPA194. DNA recovered with the immunoprecipitated chromatin was used as template for real-time PCR amplification of short amplicons distributed along the mouse rDNA repeat, including the promoter (amplicons #1–5), the whole transcription unit (#6–20) and the intergenic spacer (IGS) (#21–27) (Figure 3A; Supplementary Table S1). Recovery of the amplified sequences was quantified as per cent of input chromatin (Frank *et al*, 2001). To compare binding profiles, the data were normalized to the maximum peak value along the rDNA (Figure 3B–D). Importantly, control ChIPs with no antibody yielded no significant enrichment (Supplementary Figure S5A; data not shown).

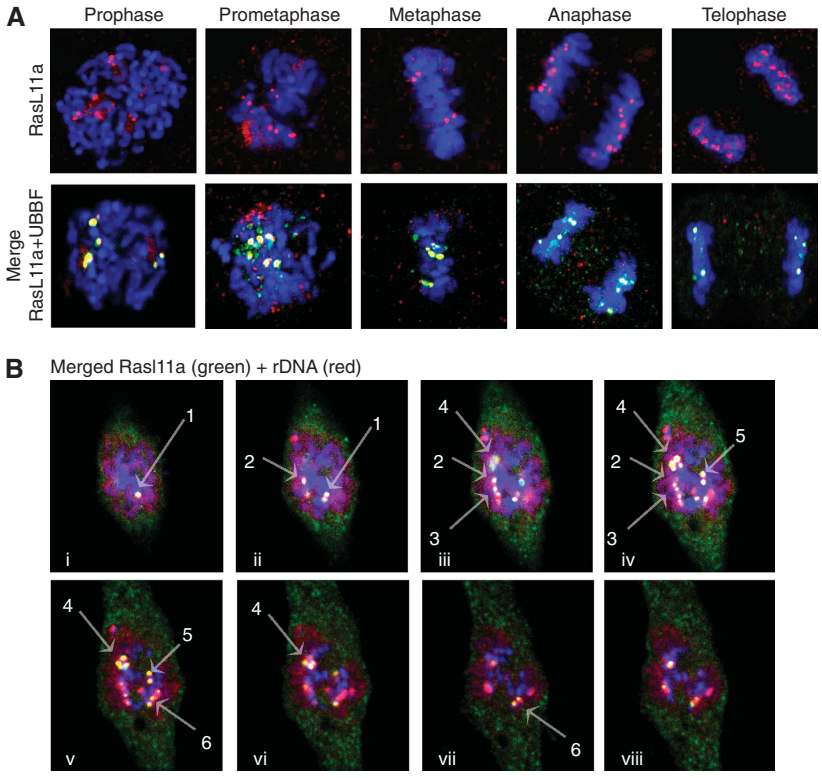

Figure 2 Similar to UBF, RasL11a stains active NORs and is associated with rDNA. (**A**) Cells at the indicated stages of mitosis are shown from the same RasL11a/UBF co-localization experiment as in Figure 1D. (**B**) Immuno-FISH analysis was used to co-stain RasL11a (Ab49) and rDNA. The rDNA probe for FISH was located in the IGS (accession number AF441733). The successive panels (i–viii) show serial confocal sections of a representative mitotic cell. The six pairs of dots showing merged signals (yellow) are numbered 1–6. Other chromosomal dots are red, indicating the presence of rDNA without RasL11a, whereas the opposite (green chromosomal dots) is not observed. Note that the green cytosolic staining is due to the over-exposure required for FISH.

As expected, RPA194 (Figure 3B) and UBF (Figure 3C) distributed along the promoter and the whole transcription unit, with a sharp drop following the termination site (compare amplicons #20 and #21) and no interaction throughout the IGS (see Discussion). Albeit in one series, UBF apparently cross-linked at the 3′ end of the IGS (amplicon #27, Figure 3C), this was not reproduced in other experiments (Figure 4B): we attribute this to poorer resolution in the qChIPs of Figure 3C, which is generally because of poorer shearing of chromatin during sonication. Importantly, RPA194 did not bind #27, but readily associated with nearby promoter sequences (#1, 2: Figures 3B and 4A; Supplementary Figure S6A; note that Figure 3B and C/D originate from different ChIP series). No interaction was observed between RPA194 or UBF and several control sequences, including the repetitive element *Sine B2* and single-copy RPII promoters (*CAD, TMP, TUB*). Interestingly, differences were observed between the RPA194 and UBF cross-linking patterns, in particular within the promoter: both proteins also cross-linked at different levels with adjacent sequences (e.g. #3, 5, 6), probably owing to conformational constraint within pre-initiation complexes.

The two anti-RasL11a antibodies (Ab48 and Ab49) yielded essentially identical qChIP profiles, showing that RasL11a also interacted with the rDNA promoter and the transcription unit (Figures 3D and 4C). Albeit the levels of RasL11a, cross-linking to the promoter region was variable between different experiments, we consistently observed maximal and sustained binding throughout the transcription unit, and absence of enrichment on the IGS or control sequences (compare Figures 3D and 4C; Supplementary Figures S5B and S6B).

RasL11a/UBF co-localization and rDNA association persist after inhibition of RNA PolI

To address the dependency of the RasL11a–rDNA interaction on transcription, we treated NIH-3T3 cells with the intercalating agent actinomycin D (actD), which inhibits transcription and causes release of RPI from chromatin (Stefanovsky *et al*, 2006). Treatment of cells for 1 h with 0.05 or 0.5 μg/ml actD caused a complete release of RPA194 from the transcription unit (Figure 4A: note that the two RPA194 antibodies showed the same effect). Interestingly, the polymerase was also released at the 5′ end of the promoter (amplicons #1, 2, preceding the enhancer repeats), whereas it remained bound close to the transcription start site and was more resistant to release in the very initial part of the transcription unit (#6, 7), an effect that was fully reproducible (compare Figure 4A; Supplementary Figure S6A). Althoutgh actD caused a decrease in UBF and RasL11a binding throughout the rDNA (Figure 4B and C), both proteins were significantly more resistant than RPA194. An independent experiment with doses up to 5 μg/ml actD showed stronger effects on RasL11a binding, although still less pronounced than the complete release of

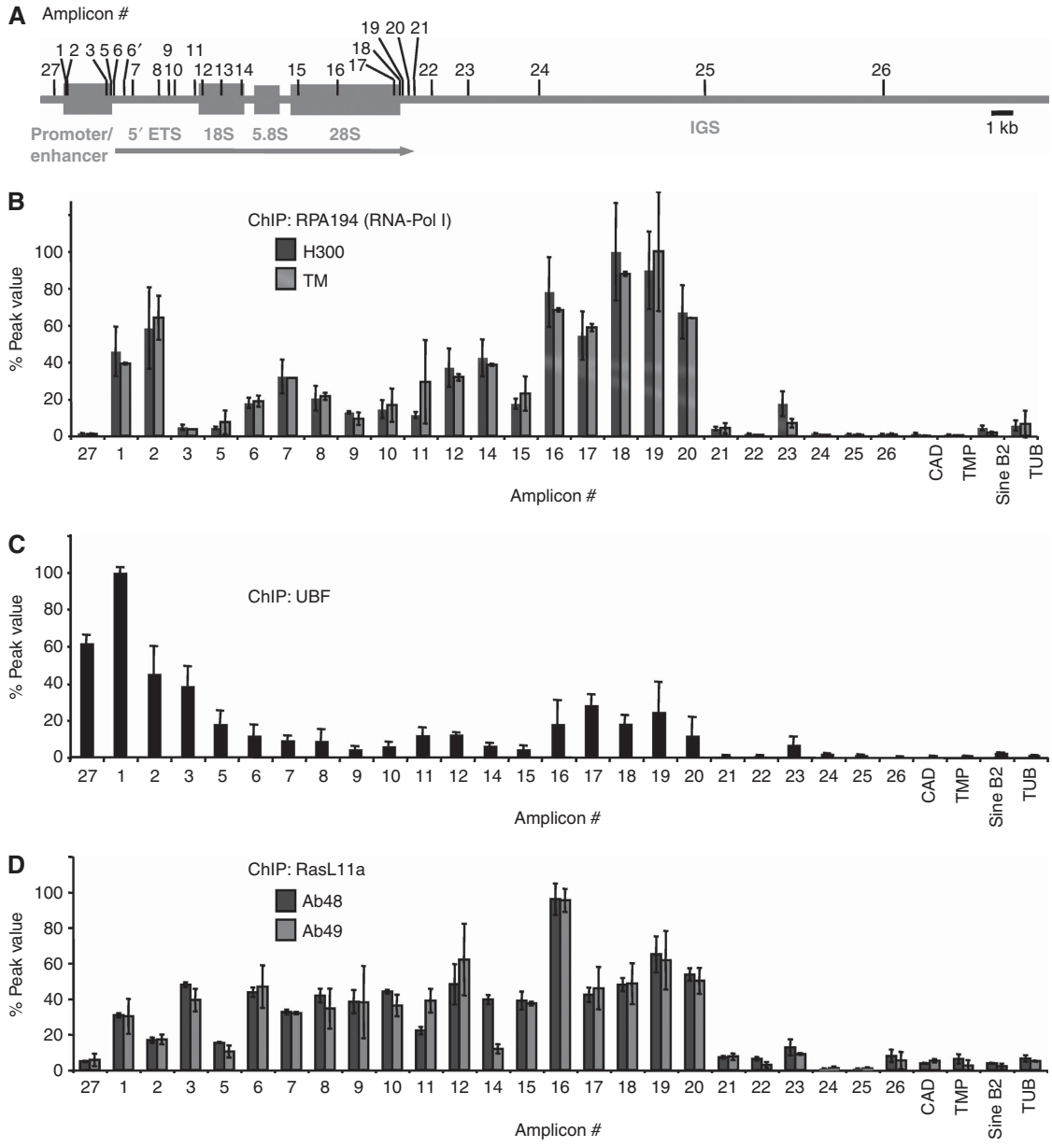

Figure 3 RasL11a is associated with rDNA *in vivo*. (**A**) Schematic representation of the mouse rDNA repeat. The sequence coordinates and annotation were taken from Genbank BK000964 (note that the promoter/enhancer region was moved from the 3′ to the 5′ end, for display). The position and numbering of all PCR amplicons used for qChIP analysis is indicated (amplicon #), with the corresponding PCR primers listed in Supplementary Table S1. All amplicons are 100–150 bp in length. qChIP analysis was performed with (**B**) the RPA antibodies H300 and TM, (**C**) anti-UBF or (**D**) the RasL11a antibodies Ab48 and Ab49. Data represent the average and standard deviation from two (**B**) or four (**C**, **D**) independent qChIP experiments with each antibody. All were quantified as percent of input DNA (Frank *et al*, 2001) and normalized to the maximum peak value in the rDNA profile. The maximum peak value for RPA194 was 1.27% of input with antibody H300 (on amplicon #18) and 3.24% with TM (on amplicon #19). For UBF, it was 1% of input (amplicon #1). For RasL11a it was 0,96% of input with Ab48 and 0.95% with Ab49 (both on amplicon #16).

RPA194 from the transcription unit (Supplementary Figure S6A and B). Interestingly, prolonged treatment (3 h) with the lowest dose of actD (0.05 μg/ml) led to reaccumulation of RPA194 in the promoter/TSS region but not in the trancription unit, while it had no further effect on RasL11a binding (data not shown). Altogether, our data indicate that the binding of RasL11a and UBF to rDNA persists after cessation of RPI transcription.

As assayed by immunofluorescence, actD treatment led to the co-segregation of RasL11a and UBF into a cap structure adjacent to the nucleolar body, marked by nucleolin and NPM. At all actD concentrations, co-localization of RasL11a and UBF was retained (Figure 5; data not shown). Such actD-induced caps have been described earlier: these structures contain rDNA, UBF, RPI and SL1, and coincide with transcriptional shutdown (Jordan *et al*, 1996; Dousset *et al*, 2000; Valdez *et al*, 2004). In summary, RasL11a retained its co-localization with UBF on inhibition of transcription by actD, and both proteins were preferentially retained on the rDNA relative to RP1.

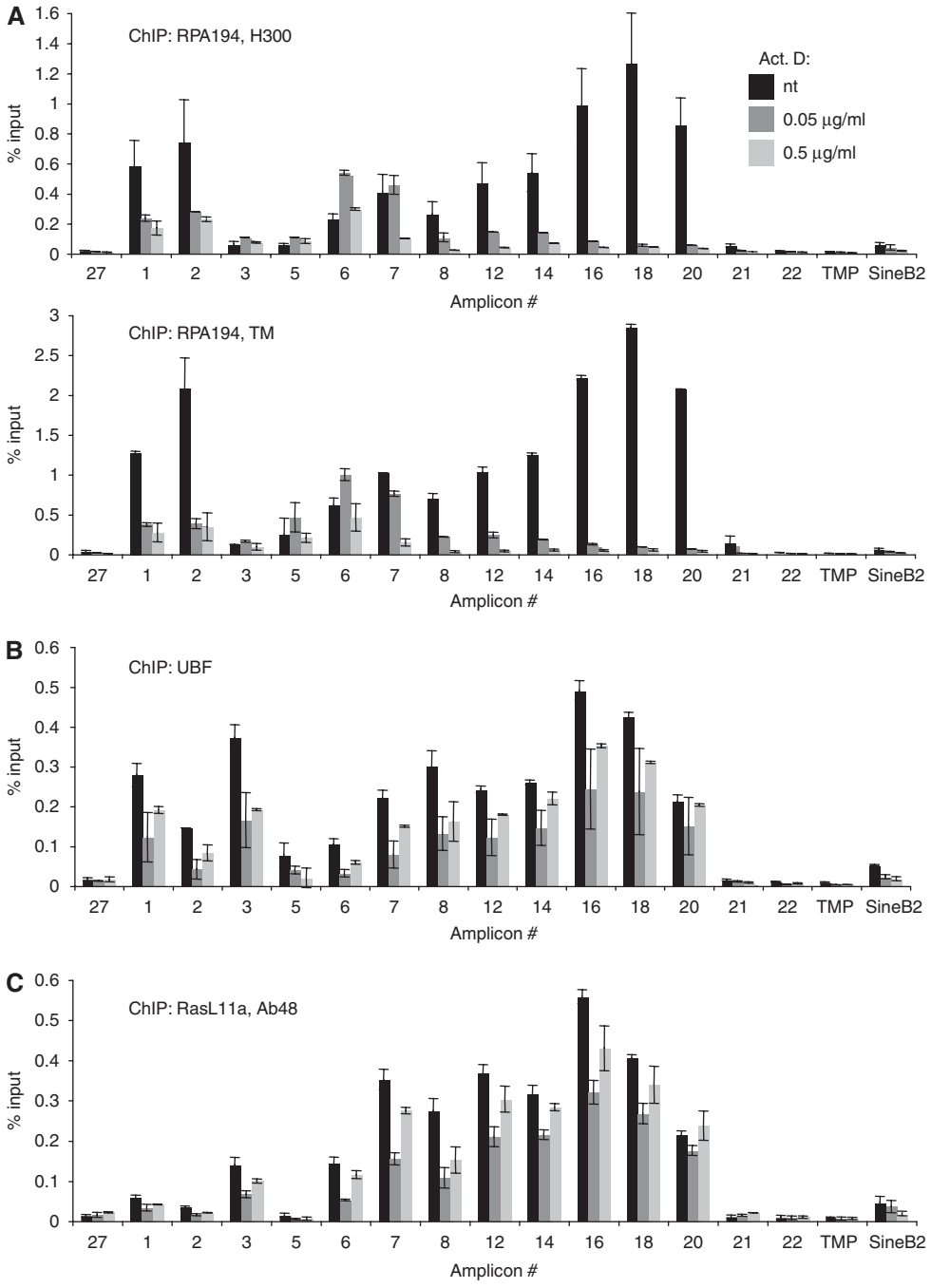

Figure 4 RasL11a and UBF persist on the rDNA transcription unit after removal of RPI. Proliferating NIH-3T3 cells were treated for 1 h with the indicated concentrations of actinomycin D. qChIP analysis was performed with (**A**) the RPA194 antibodies H300 and TM, (**B**) anti-UBF or (**C**) the RasL11a antibody Ab48. nt, not treated.

RasL11a binds UBF in cells

Altogether, the above data suggested that RasL11a might associate with UBF *in vivo*. To address this question, we prepared nuclear lysates from exponentially growing NIH-3T3 cells for co-immunoprecipitation analysis. Immunoprecipitation was performed with a mouse monoclonal antibody against RasL11a (BP6), or a control antibody, followed by immunoblotting with rabbit antibodies against UBF, or RasL11a (Ab48). BP6 quantitatively immunoprecipitated the RasL11a protein present in the lysate (Figure 6A) and reproducibly co-precipitated a minor fraction of UBF (Figure 6A and B). Importantly, BP6 did not directly recognize UBF (Supplementary Figure S1C). We conclude that RasL11a and UBF exist in a complex in cells. That the majority of UBF was not recovered with RasL11a indicates either that most of UBF is free of RasL11a, or that most of the complex dissociated during the lysis and co-immunoprecipitation procedure. This notwithstanding, these interaction data are fully consistent with the co-localization of RasL11 and UBF, as well as their co-existence on rDNA in all of the above experiments.

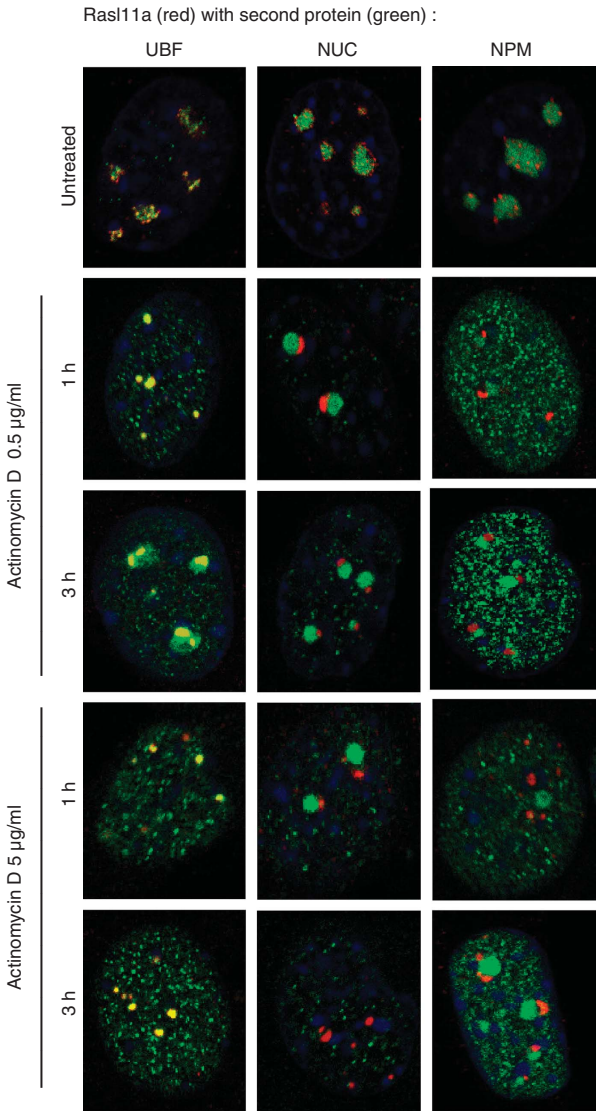

Figure 5 RasL11a co-segregates with UBF after actinomycin D treatment. Proliferating NIH-3T3 cells were treated with actinomycin D as indicated to the left, stained by indirect immunofluorescence as indicated on top and examined under a confocal microscope.

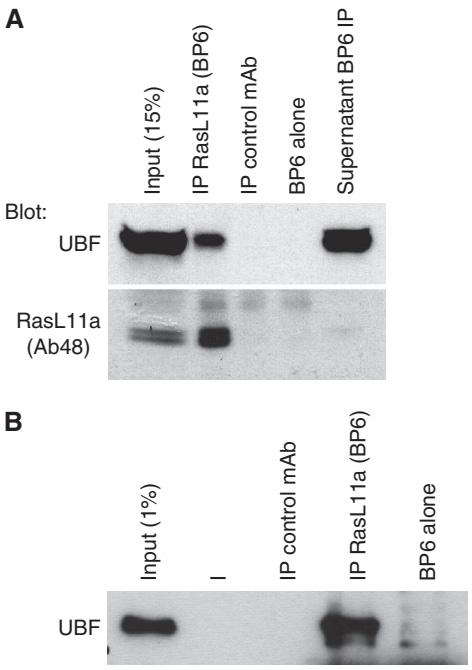

Figure 6 RasL11a and UBF interact in cells. (**A**, **B**) NIH-3T3 nuclear lysates were immunoprecipitated (IP) with the antibodies indicated on top, and the precipitates analysed on immunoblots with the antibodies indicated to the left. BP6: anti-RasL11a monoclonal (see Materials and methods). Control mAb: non-specific monoclonal antibody of the same isotype. BP6 alone: mock IP with BP6 and no lysate. Supernatant BP6 IP: supernatant from the RasL11a immunoprecipitation.

RasL11a positively regulates RPI transcription

To address whether RasL11a influences RPI transcription, we performed a transient transfection assay with an RPI-specific reporter gene driven by the mouse rDNA promoter (Budde and Grummt, 1999). The activity of this reporter was normalized against that of a co-transfected RNA PolII-specific reporter, the levels of both RNA products being measured by quantitative RT–PCR (see Materials and methods). Co-transfection of these reporters with increasing amounts of a RasL11a-expression vector in NIH-3T3 cells led to progressive increases in reporter activity, paralleled by enhanced accumulation of the RasL11a protein (Figure 7A). Thus, under these conditions, elevating RasL11a levels in cells had a positive effect on RPI transcription. To address whether the same was true on the endogenous rDNA repeats, we infected NIH-3T3 cells with a retrovirus encoding human RasL11a: this led to a six-fold increase in *RasL11a* mRNA (data not shown) but only to a very modest increase in total RasL11a protein levels (Figure 7B, top). Retroviral expression of Flag-tagged RasL11a, allowing distinction from endogenous RasL11a by size, showed that the exogenous protein accumulated at lower than physiological levels, and caused a reduction of endogenous RasL11a. This result suggests the existence of a feedback mechanism controlling total RasL11a levels, explaining its moderate accumulation in retrovirally infected cells. This notwithstanding, the stable expression of RasL11a caused a two-fold increase in cellular pre-rRNA levels, as assayed by RT–PCR with primers amplifying the 5′ ETS (Figure 7B; amplicon #6′: see map in Figure 3A). Unlike native RasL11a, the Flag-tagged protein had no effect on pre-rRNA levels (data not shown): this suggests that Flag-RasL11a levels were too low to deregulate rDNA transcription or, alternatively, that its capacity to do so was altered by the epitope tag. Altogether, our data show that increasing RasL11a levels in proliferating NIH-3T3 cells causes a moderate, albeit significant increase in pre-rRNA synthesis.

We then used RNA interference to address whether endogenous RasL11a was limiting for RP1 transcription in NIH-3T3 cells. We first infected cells with the retrovirus encoding the shL11a hairpin. As shown above, RasL11a protein levels were substantially reduced in shL11a-infected cells (Supplementary Figure S1B), paralleling the reduction in mRNA levels (Figure 8A, grey bars). In the same cells, pre-rRNA levels were reduced by ca. 25% (grey bars). To obtain an independent confirmation of this effect, we transfected NIH-3T3 cells with two different small interfering RNA

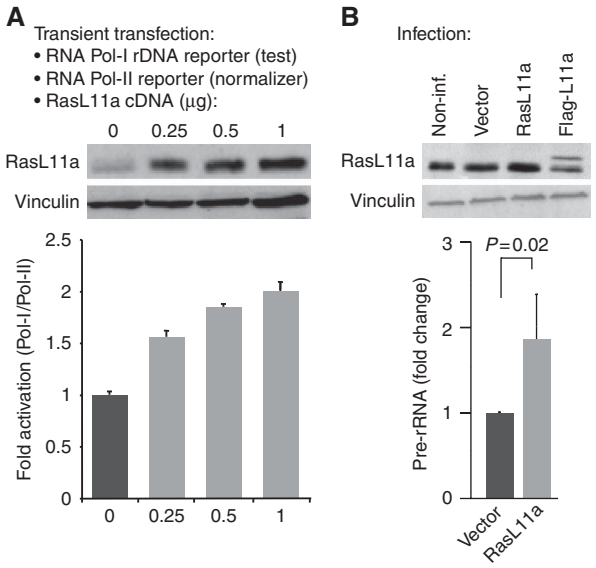

Figure 7 RasL11a positively regulates RPI transcription. (**A**) RasL11a transactivates an RPI reporter. NIH-3T3 cell were co-transfected with the RPI reporter plasmid pMR5783-BH (Budde and Grummt, 1999) and increasing amounts of pCMV5-hRasL11a. The RNA produced from this reporter was quantified by RT–PCR, and normalized to the RNA from a co-transfected RPII-specific *Renilla* luciferase reporter, which was not influenced by RasL11a. All values are expressed relative to the control sample without RasL11a (black bar). The panels above the graph show RasL11a and vinculin levels from the same transfected cells, as analysed by immunoblotting. (**B**) NIH-3T3 cells were infected with retroviruses expressing human RasL11a, with or without a Flag epitope tag, as indicated. Top panels: immunoblot analysis of RasL11a and vinculin in the infected cells. Bar graph: pre-RNA levels in infected cells, measured by quantitative RT–PCR. The data are the average of four independent experiments. RasL11a mRNA levels were enhanced ca. six-fold in the pBabe-hRasL11a-infected cells, as assayed with primers complementary to both the mouse and human cDNAs (data not shown).

(siRNA) oligonucleotides targeting the *RasL11a* mRNA (indicated A13, A14) or with a control siRNA. Quantitative RT–PCR measurements confirmed that each specific siRNA knocked down RasL11a expression and, in parallel, reduced pre-rDNA levels in cells (Figure 8B). The same was observed in primary MEFs (Figure 8C). The same experiment was repeated in NIH-3T3 cells with the A13 siRNA, alongside a pool of siRNAs targeting UBF (Dharmacon), which led to a reduction of ca. 40% in *UBF* mRNA levels. Remarkably, targeting *RasL11a* and *UBF* caused similar decreases in pre-rRNA accumulation (Figure 8D), a pattern that was confirmed by RT–PCR with a second pair of PCR primers in the 5′ ETS (amplicon #7 see map in Figure 3A). The opposite pattern, that is an accumulation of the same RNA sequences on either *RasL11a* or *UBF* knockdown, was observed when analysing their abundance in nascent (chromatin-associated) RNA (Figure 8E). In line with the known role of UBF in transcriptional elongation (Stefanovsky *et al*, 2006; Moss *et al*, 2007), these data suggest that knockdown of either UBF or RasL11a may decrease the processivity of RNA PolI, leading to an accumulation of elongating molecules on chromatin, but to their depletion in total RNA.

It is important to outline here that the above data do not imply that RasL11a is essential *per se* for rDNA transcription. In addition, RasL11a protein levels were highly variable among different cell lines, without clear correlation to their proliferative or transformed state (Supplementary Figure S7). It also remains to be understood whether RasL11a and RasL11b are redundant in rDNA regulation. Our siRNA experiments in NIH-3T3 cells gave no indications in this regard (data not shown), although it remains unclear how much RasL11b protein will be expressed in those cells, relative to the high levels of RasL11a (Supplementary Figure S7).

Discussion

We have shown that RasL11a is a nucleolar protein that co-localizes and associates with UBF, the master regulator of rDNA transcription by RPI (Grummt, 2003; Moss *et al*, 2007; McStay and Grummt, 2008). RasL11a co-localizes with UBF not only at nucleoli in interphase, but also on chromosomes in mitotic cells. UBF and the RPI machinery mark a subset of rDNA repeats on chromosome arms (NORs), which correspond to the active repeats (Roussel *et al*, 1993, 1996; Jordan *et al*, 1996; Gebrane-Younes *et al*, 1997; Leung *et al*, 2004; Mais *et al*, 2005). The fact that RasL11a co-localizes on chromosome arms with either UBF (by immunofluorescence) or rDNA (by immuno-FISH) shows that RasL11a also marks active NORs in mitosis.

Consistent with the co-localization data, qChIP analysis showed that RasL11a associates with rDNA *in vivo*. Binding was detected starting at the promoter and throughout the transcription unit, with the exclusion of the IGS. In agreement with previous reports, the RPI subunit RPA194 (Stefanovsky *et al*, 2006) and UBF (O'Sullivan *et al*, 2002; Grandori *et al*, 2005; Mais *et al*, 2005) were also cross-linked to the rDNA repeat. The distribution of UBF deserves a note here: consistent with another report (Grandori *et al*, 2005), we detected no significant enrichment of UBF on the IGS. This conclusion appears at odds with two other reports, in which UBF was mapped to the whole rDNA repeat, including the IGS (O'Sullivan *et al*, 2002; Mais *et al*, 2005). On the basis of the fine mapping and resolution seen in our data, and on the consistency on the UBF, RPI and RasL11a maps across different experiments, in particular their sharp drop after the termination site (compare amplicons #20 and #21 in all our qChIP profiles), we favour the conclusion that UBF binding is restricted to the promoter and transcription unit. Although RasL11a, UBF and RPI were cross-linked to the same areas of the rDNA repeat, careful examination of several experiments indicated important differences among their profiles. In particular, RasL11a appeared significantly more enriched along the transcribed unit relative to the promoter region. Finally, treatment of cells with increasing doses of actD, which interferes with RPI transcription and chromatin association (Stefanovsky *et al*, 2006), showed that RasL11a and UBF could persist throughout the transcribed region even after release of RPI. UBF is critical both for the initiation and elongation phases (Stefanovsky *et al*, 2006; Moss *et al*, 2007). On the basis of its prevalent enrichment along the rDNA transcribed region, RasL11a seems ideally positioned to modulate either transcriptional elongation (Stefanovsky *et al*, 2006) or co-transcriptional events such as assembly of the ribosome or the pre-rRNA processome (Gallagher *et al*, 2004).

Our functional assays for RasL11a included (i) co-transfection of a RasL11a-expression vector with an rDNA-reporter

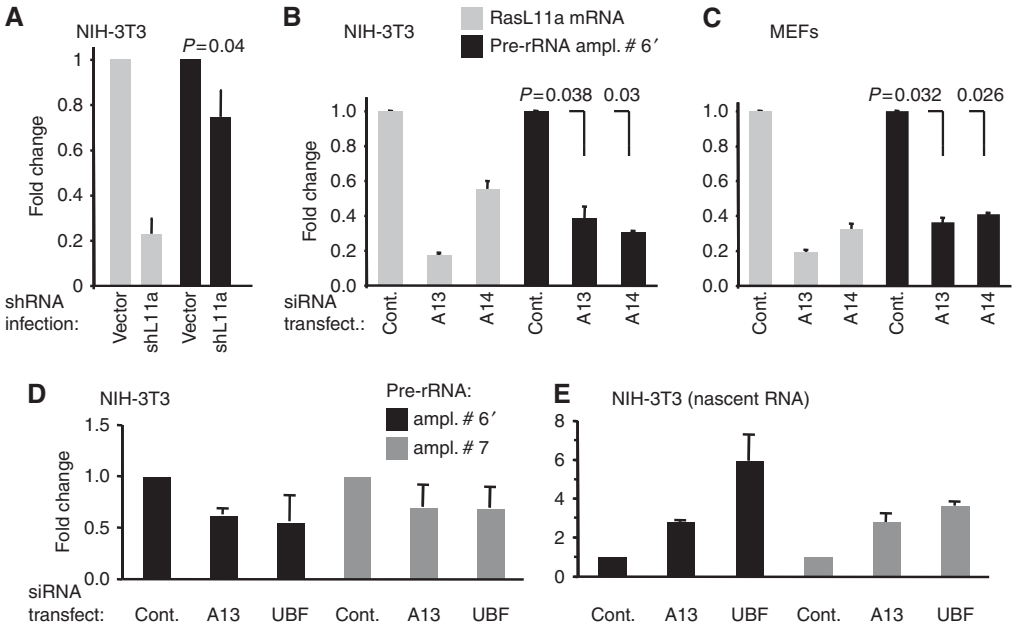

Figure 8 RasL11a is limiting for pre-rRNA synthesis in NIH-3T3 cells. (**A**) Cells were infected with a retrovirus expressing an RasL11a-specific shRNA hairpin (shL11a) or with a control vector, as indicated. *RasL11a* mRNA and pre-rRNA levels were measured by RT–PCR amplification (for the pre-rRNA 5′ ETS, amplicon #6′, Figure 3A) with total RNA as template, normalized to the *TBP* mRNA and expressed relative to the control (Vector). Note that knockdown of the RasL11a protein by shL11a is shown in Supplementary Figure S1B and D. (**B**) NIH-3T3 cells or (**C**) primary MEFs were transfected with two different siRNA oligonucleotides against the *RasL11a* mRNA (A13, A14), or with a control oligonucleotide (all from Dharmacon), as indicated. *RasL11a* mRNA and pre-rRNA levels were quantified as in (**A**). (**D**, **E**) NIH-3T3 cells were transfected with a control siRNA, the A13 siRNA against *RasL11a*, or a siRNA pool targeting the *UBF* mRNA. Pre-rRNA levels were analysed by RT–PCR with amplicons #6′ or #7 (see map, Figure 3A), with either total RNA (**D**), or the nascent (chromatin-associated) RNA fraction (**E**) as template.

construct and (ii) measurements of endogenous pre-rRNA levels after either stable expression or knockdown of RasL11a. Altogether, the data indicated that RasL11a positively regulates RPI transcription and, similar to UBF (Stefanovsky *et al*, 2006; Moss *et al*, 2007), may act at the level of initiation as well as elongation, although additional effects on pre-rRNA processing and/or stability cannot be formally ruled out. It is important to stress, however, that our data should not be interpreted to mean that RasL11a has an essential role in RPI transcription and, by inference, in cell proliferation. For example, we have not observed negative effects on cell cycle progression after infection with the shL11A retrovirus. Others have observed similar reductions of pre-rRNA level in cells without concomitant growth defects (Murayama *et al*, 2008). Furthermore, expression of the RasL11a protein was detected only in a fraction of the mouse or human cell lines examined, with a number of rapidly growing lines showing no detectable RasL11a protein. One possible reason for this would be redundancy of RasL11a with the related protein RasL11b. So far, however, we did not obtain appropriate reagents to conclusively address expression, localization and function of the RasL11b protein, and whether this variant also regulates RPI transcription remains to be addressed. Another plausible explanation is that RasL11a (and possibly also RasL11b) has an accessory, but non-essential role in RPI transcription, functioning to relay-specific regulatory signals to UBF and the RPI machinery.

Ras-family proteins function as switches in a diversity of signalling pathways, and do so by binding and hydrolysing GTP. As explained in 'Introduction', it remains presently unclear whether RasL11 proteins are able to bind and hydrolyse GTP. In our own experiments, we detected no nucleotide-binding activity of RasL11a when over-expressed in transfected cells (data not shown). Such negative results cannot be considered as definitive, however, and may be due to the lack of appropriate co-factors and/or biochemical conditions in those assays. Thus, the existence of a GDP/GTP cycle for RasL11 proteins remains a key open question, the resolution of which will also require identification of specific co-factors, including GEFs, GAPs and effector proteins.

Assuming that RasL11a is actually subject to a GDP/GTP cycle, this cycle may conceivably respond to signals that regulate RPI transcription. Alternatively, RasL11a may not cycle and, whether bound or not to a nucleotide, may thus be locked in a unique state: even in such state, however, RasL11a may transduce upstream inputs through alternative post-translational modifications. Regardless of which of the above scenarios proves correct, a variety of stimuli exist, which may use RasL11a to control RPI. For example, phosphorylation of UBF by the ERK kinase enhances RPI elongation, thereby linking pre-rRNA synthesis to mitogenic stimulation (Stefanovsky *et al*, 2006; Moss *et al*, 2007). Other stimuli that directly regulate rDNA function include energy metabolism (Murayama *et al*, 2008) and genotoxic stress (Yuan *et al*, 2007). It will be relevant to determine whether any of these pathways also targets RasL11a and/or modulates its interaction with UBF.

Somewhat paradoxically for a positive regulator of rDNA transcription, *RasL11a* has been identified as a candidate tumour-suppressor gene, its promoter being a target for CpG methylation (Weber *et al*, 2005) and its mRNA being

downregulated in some tumours (Louro *et al*, 2004). Further work will be required to assess whether RasL11a actually qualifies as a tumour suppressor, whether it functionally influences tumour progression, whether the latter would relate to its function in rDNA transcription, and whether any of these activities are shared with RasL11b. This notwithstanding, the association of RasL11a with transcribed chromatin establishes a novel paradigm for Ras-family proteins: it will be most interesting to ask whether RPII- or RPIII-transcribed genes are targeted in a similar manner, if not by RasL11a perhaps by other family members.

Materials and methods

Cells, plasmids, transfection and infection

The NIH-3T3 and Phoenix-eco cell lines (ATCC, Manassas, VA) were cultured in DMEM supplemented with 4.5 g/l glucose, 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. actD (Calbiochem; 200 μg/ml stock in DMSO) was added to the culture medium at the indicated concentrations. Infectious retroviruses were produced by transfection of Phoenix-eco cells and used to infect NIH-3T3 cells by standard methods. For expression of hRasL11a, the cDNA was cloned in pBabe-Puro and pBabe-Hygro retroviruses. For RNA interference, we constructed the recombinant retrovirus TMP_{OFF}-shL11a. TMP_{OFF} is a derivative of the tetracycline-regulated retroviral vector TMP (provided by M Hemann, MIT) (Dickins *et al*, 2005), in which we inserted a DNA fragment expressing the tTA regulator: the insert was excised from pRetro-Off (Clontech) by sequential treatment with *Hind*III, T4 DNA polymerase and *Pac*I and ligated into TMP (linearized with BstXI, T4 DNA polymerase and *Pac*I). shL11a is a microRNA-based short hairpin RNA (Dickins *et al*, 2005) targeting the sequence ATGGTGGATTCTCTGACCAAA in mouse *RasL11a* exon 2 (Genbank NM_026864).

Antibodies

Antisera Ab48 and Ab49 were raised against a peptide corresponding to the C-terminal 19 residues of human RasL11a (KRRFKQALSPKVKAPSALG) coupled to KLH through an N-terminal cysteine. The final bleed was affinity purified on the same peptide. Mouse RasL11a has four different residues in the same region (KRRFRQALSSKAKASSALG) but was recognized by Ab48 and Ab49 as well as human RasL11a (Supplementary Figure S1A). These antibodies showed no direct cross-reaction with UBF (Supplementary Figure S1C). The mouse monoclonal antibody BP6 was raised against a GST–hRasL11a fusion protein. The hybridoma supernatant recognized human as well as mouse RasL11a by immunoblotting, and immunoprecipitated RasL11a from NIH-3T3 lysates (Figure 6A; data not shown). The anti-NPM antibody was provided by Manuela Colombo, anti-γ-tubulin and anti-CREST by Andrea Musacchio and anti-RPA194 (TM) by Tom Moss. Other antibodies were from Santa Cruz: RPA194 (H300), UBF (F-9); TAFI p48 (M-19); fibrillarin (N-15); nucleolin (H-6). From Sigma: vinculin (V-19131) and FLAG M2. Nuclear extracts for immunoprecipitation were prepared as described in Sambrook and Russel: Molecular Cloning, A laboratory manual, Vol. 3, p.17.9 (3rd ed., CSHL Press).

Immunofluorescence and immuno-FISH analysis

Immunofluorescence and immuno-FISH images were acquired under a LEICA confocal microscope. For immunofluorescence, NIH-3T3 cells were grown on slides and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were permeabilized with PBS, 0.1% triton X100, 0.2% BSA for 10 min at room temperature. Primary antibodies (listed above) were as indicated in the Figures. Secondary antibodies were donkey anti-mouse, -rabbit or -goat IgG, conjugated with either Cy3 or FITC (Jackson Lab). DAPI (4′-6-diamidino-2-phenylindole) was used for DNA staining. For immuno-FISH, mRasL11a staining with Ab48 was combined with fluorescent *in situ* hybridization with a mouse rDNA probe (Genbank nr. AF441733). The probe was labelled with Cy3-dUTP (Amersham) using a nick-translation kit (Roche), resuspended in hybridization solution (2 × SSC, 20% dextran sulphate, 50% formamide) denatured for 7 min at 75°C and stored at −20°C until use. NIH-3T3 cells fixed as above were sequentially permeabilized (1 h in PBS, 0.5% Saponin, 0.5% Triton X-100), immunolabelled with Ab48 followed by an FITC-conjugated goat-anti-rabbit antibody, fixed again, subjected to acid denaturation (0.1 M HCl, 20 min), RNase treatment, hybridization with the Cy3-labelled rDNA probe and DAPI staining.

Quantitative chromatin immunoprecipitation

NIH-3T3 cells were grown and processed for qChIP following our original protocol (Frank *et al*, 2001). Immunoprecipitated DNA from 10^7 cell equivalents was resuspended in 200 μl of 10 mM Tris, pH 8. Real-time PCR was performed with 6 μl of DNA per reaction and 200 nM primers, diluted in a final volume of 20 μl in SYBR Green Reaction Mix (Perkin Elmer, Boston, MA) with the primers listed in Supplementary Table S1.

Reporter assay

For the RPI reporter assay of Figure 7A, NIH-3T3 cells were transfected using lipofectamine (Invitrogen). The mouse rDNA promoter-reporter plasmid pMR5783-BH (Budde and Grummt, 1999) was co-transfected with either pCMV5-hRasl11a or the empty pCMV5 vector, and with phRL-TK (Promega). phRL-TK is an RPII-transcribed a *Renilla* luciferas reporter, and was used for normalization. The RNAs transcribed by both reporters were quantified by RT–PCR with the primers listed in Supplementary Table S2.

mRNA analysis

Total cellular RNA was prepared with the RNeasy kit (Qiagen). Nascent, chromatin-associated transcripts were isolated as described (Masternak *et al*, 2003). cDNA was produced using the reverse-transcriptase SuperScriptII (Invitrogen). Real-time PCR was performed with 10 ng of cDNA per reaction and 800 nM primers, diluted in a final volume of 20 μl in SYBR Green Reaction Mix (Perkin Elmer, Boston, MA) with the primers listed in Supplementary Table S3. *TBP* was used for normalization.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Gioacchino Natoli for critical reading of the manuscript, Giorgio Scita and members of the Amati lab for discussion and feedback throughout this work, Manuela Colombo, Andrea Musacchio, Larry Rothblum, Tom Moss and Michael Hemann for materials and advice, Marianne Schroeder for the initial identification of RasL11a, Giuseppe Ossolengo, Daniele Piccini, Massimiliano Garrè, Pietro Transidico and other members of the facilities at the IFOM-IEO Campus for technical support and Pier Giuseppe Pelicci for his continuous support and enthusiasm. This work was supported by grants from the Italian Association for Cancer Research (AIRC) and from the Italian health ministry.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Boisvert FM, van Koningsbruggen S, Navascues J, Lamond AI (2007) The multifunctional nucleolus. *Nat Rev Mol Cell Biol* **8:** 574–585

Bos JL, Rehmann H, Wittinghofer A (2007) GEFs and GAPs: critical elements in the control of small G proteins. *Cell* **129:** 865–877

Budde A, Grummt I (1999) p53 represses ribosomal gene transcription. *Oncogene* **18:** 1119–1124

Colicelli J (2004) Human RAS superfamily proteins and related GTPases. *Sci STKE* **2004:** RE13

Dickins RA, Hemann MT, Zilfou JT, Simpson DR, Ibarra I, Hannon GJ, Lowe SW (2005) Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. *Nat Genet* **37:** 1289–1295

Dousset T, Wang C, Verheggen C, Chen D, Hernandez-Verdun D, Huang S (2000) Initiation of nucleolar assembly is independent of RNA polymerase I transcription. *Mol Biol Cell* **11:** 2705–2717

Duret L, Chureau C, Samain S, Weissenbach J, Avner P (2006) The Xist RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. *Science* **312:** 1653–1655

Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B (2001) Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev* **15:** 2069–2082

Gallagher JE, Dunbar DA, Granneman S, Mitchell BM, Osheim Y, Beyer AL, Baserga SJ (2004) RNA polymerase I transcription and pre-rRNA processing are linked by specific SSU processome components. *Genes Dev* **18:** 2506–2517

Gebrane-Younes J, Fomproix N, Hernandez-Verdun D (1997) When rDNA transcription is arrested during mitosis, UBF is still associated with non-condensed rDNA. *J Cell Sci* **110:** 2429–2440

Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, White RJ (2005) c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat Cell Biol* **7:** 311–318

Grummt I (2003) Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes Dev* **17:** 1691–1702

Jordan P, Mannervik M, Tora L, Carmo-Fonseca M (1996) *In vivo* evidence that TATA-binding protein/SL1 colocalizes with UBF and RNA polymerase I when rRNA synthesis is either active or inactive. *J Cell Biol* **133:** 225–234

Leung AK, Gerlich D, Miller G, Lyon C, Lam YW, Lleres D, Daigle N, Zomerdijk J, Ellenberg J, Lamond AI (2004) Quantitative kinetic analysis of nucleolar breakdown and reassembly during mitosis in live human cells. *J Cell Biol* **166:** 787–800

Louro R, Nakaya HI, Paquola AC, Martins EA, da Silva AM, Verjovski-Almeida S, Reis EM (2004) RASL11A, member of a novel small monomeric GTPase gene family, is down-regulated in prostate tumors. *Biochem Biophys Res Commun* **316:** 618–627

Mais C, Wright JE, Prieto JL, Raggett SL, McStay B (2005) UBF-binding site arrays form pseudo-NORs and sequester the RNA polymerase I transcription machinery. *Genes Dev* **19:** 50–64

Masternak K, Peyraud N, Krawczyk M, Barras E, Reith W (2003) Chromatin remodeling and extragenic transcription at the MHC class II locus control region. *Nat Immunol* **4:** 132–137

McStay B, Grummt I (2008) The epigenetics of rRNA genes: from molecular to chromosome biology. *Annu Rev Cell Dev Biol* **24:** 131–157

Moss T, Langlois F, Gagnon-Kugler T, Stefanovsky V (2007) A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. *Cell Mol Life Sci* **64:** 29–49

Murayama A, Ohmori K, Fujimura A, Minami H, Yasuzawa-Tanaka K, Kuroda T, Oie S, Daitoku H, Okuwaki M, Nagata K, Fukamizu A, Kimura K, Shimizu T, Yanagisawa J (2008) Epigenetic control of rDNA loci in response to intracellular energy status. *Cell* **133:** 627–639

O'Sullivan AC, Sullivan GJ, McStay B (2002) UBF binding *in vivo* is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat. *Mol Cell Biol* **22:** 657–668

Piek E, Van Dinther M, Parks WT, Sallee JM, Bottinger EP, Roberts AB, Ten Dijke P (2004) RLP, a novel Ras-like protein, is an immediate-early transforming growth factor-beta (TGF-beta) target gene that negatively regulates transcriptional activity induced by TGF-beta. *Biochem J* **383:** 187–199

Roussel P, Andre C, Comai L, Hernandez-Verdun D (1996) The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. *J Cell Biol* **133:** 235–246

Roussel P, Andre C, Masson C, Geraud G, Hernandez-Verdun D (1993) Localization of the RNA polymerase I transcription factor hUBF during the cell cycle. *J Cell Sci* **104:** 327–337

Rowe LB, Janaswami PM, Barter ME, Birkenmeier EH (1996) Genetic mapping of 18S ribosomal RNA-related loci to mouse chromosomes 5, 6, 9, 12, 17, 18, 19, and X. *Mamm Genome* **7:** 886–889

Stefanovsky V, Langlois F, Gagnon-Kugler T, Rothblum LI, Moss T (2006) Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. *Mol Cell* **21:** 629–639

Stolle K, Schnoor M, Fuellen G, Spitzer M, Cullen P, Lorkowski S (2007) Cloning, genomic organization, and tissue-specific expression of the RASL11B gene. *Biochim Biophys Acta* **1769:** 514–524

Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. *Physiol Rev* **81:** 153–208

Valdez BC, Henning D, So RB, Dixon J, Dixon MJ (2004) The Treacher Collins syndrome (TCOF1) gene product is involved in ribosomal DNA gene transcription by interacting with upstream binding factor. *Proc Natl Acad Sci USA* **101:** 10709–10714

Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* **37:** 853–862

Weis K (2003) Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* **112:** 441–451

Yuan X, Feng W, Imhof A, Grummt I, Zhou Y (2007) Activation of RNA polymerase I transcription by cockayne syndrome group B protein and histone methyltransferase G9a. *Mol Cell* **27:** 585–595

