CRM1 controls the composition of nucleoplasmic pre-snoRNA complexes to licence them for nucleolar transport

Bérengère Pradet-Balade¹,
Cyrille Girard¹, Séverine Boulon,
Conception Paul, Karim Azzag,
Rémy Bordonné, Edouard Bertrand*
and Céline Verheggen*

Institut de Génétique Moléculaire de Montpellier, UMR 5535 CNRS,
Université Montpellier I and II, Montpellier Cedex 5, France

Transport of C/D snoRNPs to nucleoli involves nuclear export factors. In particular, CRM1 binds nascent snoRNPs, but its precise role remains unknown. We show here that both CRM1 and nucleocytoplasmic trafficking are required to transport snoRNPs to nucleoli, but the snoRNPs do not transit through the cytoplasm. Instead, CRM1 controls the composition of nucleoplasmic pre-snoRNP complexes. We observed that Tgs1 long form (Tgs1 LF), the long isoform of the cap hypermethylase, contains a leucine-rich nuclear export signal, shuttles in a CRM1-dependent manner, and binds to the nucleolar localization signal (NoLS) of the core snoRNP protein Nop58. *In vitro* data indicate that CRM1 binds Tgs1 LF and promotes its dissociation from Nop58 NoLS, and immunoprecipitation experiments from cells indicate that the association of Tgs1 LF with snoRNPs increases upon CRM1 inhibition. Thus, CRM1 appears to promote nucleolar transport of snoRNPs by removing Tgs1 LF from the Nop58 NoLS. Microarray/IP data show that this occurs on most snoRNPs, from both C/D and H/ACA families, and on the telomerase RNA. Hence, CRM1 provides a general molecular link between nuclear events and nucleocytoplasmic trafficking.

The EMBO Journal (2011) 30, 2205–2218. doi:10.1038/emboj.2011.128; Published online 26 April 2011

Subject Categories: RNA

Keywords: CRM1; nucleolus; nucleocytoplasmic transport; snoRNP

Introduction

Non-coding RNPs perform essential functions in the cell: (i) they are at the heart of the translation apparatus and catalyse the peptidyl-transferase reaction (Simonovic and

Steitz, 2009); (ii) they have key roles in the processing of other RNAs (Wahl *et al*, 2009); and (iii) they have various regulatory functions, best exemplified by miRNAs, which regulate mRNA translation and stability (Chekulaeva and Filipowicz, 2009). To perform their function, non-coding RNAs must be processed and assembled with specific proteins to form stable RNP particles (Staley and Woolford, 2009). This often occurs in specific compartments where the RNAs have to localize transiently before reaching their site of function (Matera and Shpargel, 2006). How these RNAs are transported during their biogenesis is not well understood, in particular in the case of transit between sub-nuclear compartments.

The m^{2,2,7}G-capped U1, U2, U4 and U5 snRNAs are key components of the pre-mRNA splicing machinery and are one of the best understood paradigm to study RNP assembly and transport (for review, see Wahl *et al* (2009)). In vertebrate cells, the snRNA precursors are transcribed by RNA polymerase II. They receive an m⁷G-cap, and are exported to the cytoplasm by a complex containing the cap binding complex (CBC; Izaurralde *et al*, 1995), the phosphorylated export adaptor PHAX (Ohno *et al*, 2000), Ran and the exportin CRM1 (Fornerod *et al*, 1997). In the cytoplasm, snRNAs are assembled with the core Sm proteins by the survival of motor neuron (SMN) complex (for review, see Chari *et al* (2009)). SMN and the Sm core proteins then recruit the cap hypermethylase Tgs1 and this allows the conversion of the m⁷G-cap to a 2,2,7-trimethylguanosine (TMG; Mouaikel *et al*, 2002; Lemm *et al*, 2006; Girard *et al*, 2008). Consequently, snRNPs are reimported to nuclei and transported to Cajal bodies (CB), to finalize their maturation before being targeted to the nucleoplasm or to speckles (for review, see Chari *et al* (2009)).

SnoRNAs are small, evolutionarily conserved RNAs that localize to nucleoli and function in rRNA biogenesis (for review, see Matera *et al* (2007)). On the basis of short consensus motifs, snoRNAs can be grouped into two families, the C/D and the H/ACA RNAs. SnoRNAs from each family fold into a similar secondary structure and associate with a small set of common proteins (reviewed in Matera *et al* (2007) and Reichow *et al* (2007)): 15.5K, Nop56, Nop58 and fibrillarin for C/D snoRNAs; Nhp2, Nop10, Gar1 and dyskerin for H/ACA snoRNAs. The snoRNAs are transcribed in the nucleoplasm either from their own gene or as part of introns of pre-mRNAs. The intronic snoRNAs are released from the pre-mRNA via a splicing-dependent pathway (Leverette *et al*, 1992; Hirose *et al*, 2003), and are uncapped. In contrast, snoRNAs from independent gene, such as U3, U8 and U13, are synthesized from snRNA-type RNA polymerase II promoters (Tyc and Steitz, 1989), and their precursors contain a short 3' extension and an m⁷G-cap structure that is hypermethylated into a TMG by Tgs1. SnoRNA precursors are present in large multiprotein complexes (Boulon *et al*,

*Corresponding authors. E Bertrand or C Verheggen, UMR 5535 CNRS, Institut de Génétique Moléculaire de Montpellier, 1919 route de Mende, 34293 Montpellier Cedex 5, France. Tel.: +33 434359646; Fax: +33 467040231; E-mail: edouard.bertrand@igmm.cnrs.fr or celine.verheggen@igmm.cnrs.fr

¹These authors contributed equally to this work

Received: 27 February 2011; accepted: 28 March 2011; published online: 26 April 2011

2004, 2008; Watkins *et al*, 2004). In addition to core snoRNP proteins, these complexes contain factors involved in transport (CBC, PHAX and CRM1; Boulon *et al*, 2004; Watkins *et al*, 2004), RNP assembly (Nufip, the R2TP complex and Hsp90) (Boulon *et al*, 2008; Zhao *et al*, 2008) and RNA processing (Tgs1, La, Lsm, the exosome) (for review, see Matera *et al* (2007)). Transport of C/D snoRNAs to nucleoli is only partially characterized. It was shown that C/D snoRNAs transit through CB where cap hypermethylation, 3'-end trimming and final assembly steps are thought to occur (Narayanan *et al*, 1999; Verheggen *et al*, 2002). More recently, we showed that PHAX and CRM1 are involved in the transport of capped C/D snoRNAs to nucleoli (Boulon *et al*, 2004). PHAX was shown to direct these complexes to CB, while CRM1 was required for their transport from CB to nucleoli. PHAX and CRM1 are not recruited concomitantly but sequentially: PHAX binds m⁷G-capped pre-snoRNA, while CRM1 associates predominantly with TMG-capped mature snoRNAs (Boulon *et al*, 2004). Given that C/D snoRNA transport does not seem to involve a cytoplasmic step (Terns *et al*, 1995), it is surprising that PHAX and CRM1 are required for targeting snoRNPs to nucleoli. It was, therefore, important to further investigate the role of these transport factors in snoRNP biogenesis and trafficking. In this study, we have elucidated the role of CRM1. We found that it controls the intranuclear trafficking of snoRNPs by promoting the dissociation of Tgs1 from their nucleolar localization signal (NoLS).

Results

Both functional nuclear pores and CRM1 are required to transport capped and uncapped C/D snoRNAs to the nucleolus

We previously showed that CRM1 binds U3 precursor RNAs, and by microinjection experiments, that it was required to transport U3 from CB to nucleoli (Boulon *et al*, 2004). Indeed, in HeLa cells, inhibition of CRM1 with Leptomycin B (LMB) led to the accumulation of microinjected U3 in CB and prevented its localization to nucleoli. Since CRM1 is an exportin, we wondered whether its role in the trafficking of U3 was dependent on functional nuclear pores or not. To test this, we microinjected HeLa cells with m⁷G-capped fluorescent U3 in the presence of wheat germ agglutinin (WGA), which binds to the glycosylated residues of nucleoporins and inhibits their function (Yoneda *et al*, 1987). Upon WGA microinjection, U3 RNAs were not localized to nucleoli but accumulated in CB as seen by colocalization with coilin (Figure 1A). Microinjected RNAs had the same localization as when cells were pre-treated with LMB (Boulon *et al*, 2004), indicating that CRM1 inhibition and blocking nuclear pores had the same effect on snoRNA transport. To verify that the effect of WGA was specific, we repeated the experiment by coinjecting WGA with N-acetyl glucosamin, which prevents binding of WGA to nucleoporins. In this case, the ability of U3 snoRNA to localize to nucleoli was not affected (Supplementary Figure S1).

Since U3 is transcribed from an snRNA-type RNA polymerase II gene and its precursor possess an m⁷G-cap (Peculis and Steitz, 1993), we wondered whether the effect of LMB would extend to intronic C/D snoRNAs that do not have a cap. To this end, fluorescent uncapped U14 snoRNAs were synthesized *in vitro* and microinjected into the nuclei of HeLa

cells. As shown in Figure 1B, after 2 h, most of the RNAs localized to CB and nucleoli. Interestingly, the nucleolar localization of U14 was lost when cells were treated with LMB or when WGA was coinjected. However, in contrast to capped C/D snoRNAs, U14 did not accumulate in large amount in CB but mostly remained in the nucleoplasm. This could be due to a difference in kinetic properties between U14 and U3 trafficking. Nevertheless, the loss of nucleolar targeting for U14 showed that CRM1 and functional nuclear pores were also required for proper transport of uncapped C/D snoRNAs.

Capped and uncapped snoRNAs do not transit through the cytoplasm during their biogenesis

One explanation of the above results would be that snoRNAs are recognized by CRM1 and exported to the cytoplasm. Given that trimethylation of capped C/D snoRNAs occurs in the nucleus and that CRM1 is associated with TMG-capped mature snoRNAs (Boulon *et al*, 2004), such a cytoplasmic step would involve almost completely mature species. To test whether snoRNAs were exported during their biogenesis, we performed heterocaryon assays (Fok *et al*, 2006). HeLa cells were first transfected with a plasmid coding for a tagged RNA and then fused to mouse Balb C cells, whose nuclei can be easily recognized due to their spotted DAPI staining. The tagged RNAs were then detected by fluorescent *in situ* hybridization (FISH) to see if they had migrated from human to mouse nuclei, which would provide evidence for a cytoplasmic phase during their biogenesis. In order to verify the fusion of the cytoplasms, HeLa cells were also co-transfected with a plasmid coding for GFP-fibrillarin. Cells were further maintained in nocodazole to prevent entry into mitosis and fusion of nuclei (Figure 2A). To first verify that we could visualize shuttling of neo-synthesized RNAs, human cells were transfected with a plasmid coding for a tagged U4 snRNA and then fused with mouse cells. Export of U4tag could be detected by the heterocaryon assay (Figure 2B). Moreover, we found that shuttling increased with time and that LMB abolished it, as expected for an snRNA (Figure 2B; Supplementary Figure S2). We then did similar assays with plasmids coding for tagged U3 or an artificial intronic C/D snoRNA. In both cases, snoRNAs were detected in the nucleoli of human cells but were absent from mouse nuclei despite high expression levels and incubation times of 16 h (Figure 2C; Supplementary Figure S2B). To rule out the possibility that the tag interfered with RNA export, we also performed the experiment with a non-tagged U3 snoRNA. We transfected mouse cells with a plasmid encoding rat U3B.7 and took advantage of a previously developed probe that specifically detects this U3 variant (Verheggen *et al*, 2002). Whereas U3B.7 was overexpressed when transfected in mouse nuclei, it was not detectable in the recipient nuclei of heterocaryons (Figure 2D). These experiments demonstrate that capped and uncapped C/D snoRNAs do not have a cytoplasmic phase during their biogenesis. This excludes the possibility that CRM1 acts as an export receptor for these RNAs and suggests that it has an alternative role in their biogenesis.

We then wondered if the absence of a cytoplasmic step during C/D snoRNA biogenesis would also extend to H/ACA snoRNAs and hTR, the human telomerase RNA. hTR resembles a TMG-capped H/ACA scaRNA that localizes to CB and it

Figure 1 CRM1 and nuclear pores are required for nucleolar targeting of microinjected C/D snoRNAs. (A) Cy3-labelled (red) fluorescent snoRNA U3 was microinjected in the nucleus of HeLa cells and cells were fixed 2 h later. CB were labelled with anti-coilin antibodies (green). On the merged image, DNA staining with DAPI (blue) is shown. In the control, U3 was localized to CB and nucleoli but after coinjection with WGA, it was only to CB. (B) Microinjection as for (A) but with uncapped U14 snoRNA. In the control, U14 was localized to CB and nucleoli but remained in the nucleoplasm upon LMB pre-treatment or WGA coinjection. The bar represents 5 μ m.

associates with PHAX (Kiss, 2002; Terns and Terns, 2002; Boulon *et al*, 2004), while U64 is a canonical intronic H/ACA snoRNA. We transfected HeLa cells with constructs coding for hTR or U64 and GFP-fibrillarin, generated heterocaryons and looked at their localization by FISH, 16 h after fusion. We detected hTR and U64 in human nuclei that had been transfected but not in the recipient nuclei of the heterocaryon (Figure 2E), indicating that similar to C/D snoRNAs, H/ACA snoRNA and hTR were not exported during their biogenesis.

Binding of core proteins to C/D snoRNA is not affected by CRM1 inhibition

It was previously shown that nucleolar localization of C/D snoRNAs requires their assembly with all four core proteins (Samarsky *et al*, 1998; Verheggen *et al*, 2001). A simple explanation for the effect of CRM1 would thus be that it affects the assembly of snoRNAs with core proteins. To test this, we immunoprecipitated (IP'd) nascent snoRNP complexes with antibodies against Nop56, Nop58 and fibrillarin from LMB- or mock-treated cells which had been metaboli-

cally labelled with 32 P for snoRNA visualization. As shown in Figure 3A, nascent U3 snoRNAs were efficiently IP'd with the three antibodies, whether cells were treated with LMB or not. These results showed that inhibition of CRM1 neither prevent synthesis of U3 snoRNA nor its assembly with C/D core proteins.

CRM1 controls the subcellular localization of Tgs1, the sn/snoRNA cap hypermethylase

As CRM1 is neither involved in export of snoRNAs nor in their assembly with core proteins, it might control the localization or binding of a key biogenesis factor. Indeed, pre-snoRNPs occur as complex assembly intermediates, and a number of factors involved in C/D snoRNA biogenesis have been characterized (for review, see Matera *et al* (2007)). Some are nuclear (Nufip, BCD1, Nopp140, La and Lsm) while others are both cytoplasmic and nuclear (hSpagh, hPih1, hRvb1, hRvb2 and Tgs1; data not shown). We tested a possible association of these factors with CRM1 in two ways: (i) for the nuclear factors, we determined in

Figure 2 C/D and H/ACA snoRNAs do not transit in the cytoplasm during their biogenesis. **(A)** Description of the heterocaryon assay. Heterocaryons were cultured for 16 h before fixation. **(B)** A plasmid encoding a tagged snRNA U4 was used as a positive control. By FISH, we stained U4tag in red. GFP-fibrillarin in green labels nucleoli in all nuclei of the heterocaryon. DNA staining in blue allows human and mouse nuclei to be distinguished. U4tag was localized to CB of both types of nuclei. After LMB treatment, U4tag was only detected in the human nuclei that were initially transfected. Mouse recipient nuclei are rimmed. **(C)** Legend as in **(B)** but with cells transfected with plasmids coding for U3tag and C/Dtag snoRNAs. RNAs were detected neither in mouse nuclei nor in non-transfected human nuclei of the heterocaryon. **(D)** Legend as in **(B)** except a plasmid coding for rU3B.7, a natural U3 variant, was used, and that mouse nuclei were transfected instead of the human nuclei. No rU3B.7 snoRNAs were seen in the untransfected, recipient nuclei of the heterocaryon (rimmed). **(E)** Legend as in **(B)** except that the RNA component of telomerase (hTR) and U64, two H/ACA snoRNAs, were transfected and analysed. Similarly to C/D snoRNAs, H/ACA RNAs did not localize to untransfected nuclei of the heterocaryon. The bar represents 5 μ m.

heterocaryon assays if they were shuttling in a LMB-dependent manner; (ii) for those that had a cytoplasmic localization, we treated cells with LMB to see if this led to a nuclear relocalization. This was done for all the factors listed above, but only the localization of Tgs1, the enzyme required for m⁷G-cap hypermethylation of snRNA and snoRNA (Mouaikel *et al*, 2002; Verheggen *et al*, 2002), was affected by CRM1 inhibition (Figure 3). Tgs1 exists as two isoforms in HeLa cells: either as a full-length protein called Tgs1 long form (Tgs1 LF) or as shorter form (Tgs1 SF) produced by proteolytic cleavage of its N-terminal domain (Girard *et al*, 2008). Tgs1 SF contains the catalytic methyltransferase domain (Mtase, Figure 3B) and interacts with snoRNAs through Nop56 and Nop58 core proteins (Mouaikel *et al*, 2002; Girard *et al*, 2008). It is a nuclear protein concentrated in CB and it does not shuttle (Supplementary Figure S3). In contrast, Tgs1 LF is mainly cytoplasmic and its N-terminal domain binds SmB (Girard *et al*, 2008). GFP-Tgs1 LF is however not only observed in the cytoplasm but also in the nucleus where it accumulates in CB (Verheggen *et al*, 2002). When cells were treated with LMB, the cytoplasmic localization of GFP-Tgs1 LF was lost and the protein relocalized to the nucleoplasm (Figure 3C). Heterocaryon experiments confirmed that GFP-Tgs1 LF shuttles in a CRM1-dependent manner (Supplementary Figure S3). This suggested that the N-terminal domain of Tgs1 LF, which is removed when the protein is processed into Tgs1 SF, might bind CRM1 via a nuclear export signal (NES).

An NES in Tgs1 LF is responsible for its shuttling

In order to identify potential NES sequences in Tgs1 LF, we used the online NetNES server (CBS, Technical University of Denmark). To confirm the functionality of the predicted NES sequences, we first constructed truncation mutants of Tgs1 LF fused to GFP and studied their localization and shuttling properties (Supplementary Figure S4). These results pointed to a potential involvement of two NES: (i) one from aa 288 to 295 (NES1) and (ii) another from aa 317 to 326 (NES2; Supplementary Table SI). We tested their role in the export of Tgs1 LF by creating point mutants by site-directed mutagenesis. NES1 was mutated by conversion of Val 290 and Leu 292 to two Ala (NES1mut) or by deletion of Leu 292 to F295 (NES1Δ4). GFP-Tgs1 NES1mut and GFP-Tgs1 NES1Δ4 were both cytoplasmic and nuclear and relocalized to nuclei upon LMB treatment (Figure 3D, upper and middle panels), indicating that NES1 is not essential for shuttling. Conversion of Ile 324 and Leu 326 to two Ala in NES2 (GFP-Tgs1 NES2mut) induced a strong accumulation of the protein in the nucleus, but again LMB treatment led to more complete nuclear accumulation of GFP-Tgs1 NES2mut (Figure 3D, upper panels; and data not shown). However, a deletion of NES2 (GFP-Tgs1 NES2Δ5) led to a complete nuclear relocalization of the mutant protein (Figure 3D, lower panels), and in a heterocaryon experiments, GFP-Tgs1 NES2Δ5 remained in the transfected nuclei (Supplementary Figure S3). Thus, NES2 is the key sequence recognized by CRM1 as it is absolutely required for the nuclear export of Tgs1 LF.

Tgs1 directly interacts with CRM1 in a Ran-GTP-dependent manner

To test whether Tgs1 LF directly interacts with CRM1, we performed *in vitro* binding assay using recombinant Tgs1 LF

and CRM1 in the absence and presence of the Ran Q69L mutant, which remains in the GTP-bound form and increases the affinity of exportins for their substrates (Fornerod *et al*, 1997). Specific binding of GST-Tgs1 LF to CRM1 was observed in the presence of Ran-GTP but not in its absence (Figure 4A). Moreover, CRM1 did not associate with GST alone or with the NES mutant GST-Tgs1 NES2Δ5. These results showed that CRM1 binds directly to Tgs1 LF, and that this binding depends on NES2.

Association of Tgs1 LF with snoRNP is regulated by CRM1

To decipher the links between snoRNPs and CRM1, we tested whether the association of Tgs1 with snoRNPs was affected by CRM1 inhibition. To this end, we IP'd C/D snoRNP complexes from HeLa whole cell extracts in both LMB-treated and non-treated cells using a monoclonal antibody against fibrillarin. As shown in Figure 4B, we found that fibrillarin-containing complexes were strongly associated with Tgs1 SF, and only weakly with Tgs1 LF. Remarkably, however, the association of Tgs1 LF was increased in cells treated with LMB. To confirm these data, we generated a stable cell line expressing GFP-Tgs1 LF. We then IP'd GFP-Tgs1 LF-containing complexes from nuclear extracts and analysed the presence of C/D snoRNP core proteins. We showed that Nop58 associated more strongly with GFP-Tgs1 LF in LMB-treated cells, as compared with non-treated cells (Figure 4C). To confirm that such changes were related to the association of Tgs1 LF with C/D snoRNPs and not unassembled core proteins, we directly measured its association with snoRNAs using RNase protection assays. To this end, cells were transfected with a rat U3B.7 gene and analysed with a probe covering its 3'-end to discriminate the precursor and mature U3 RNAs (Verheggen *et al*, 2002). We found that LMB slightly decreased association of Tgs1 with rat U3B.7 RNAs, when both Tgs1 LF and Tgs1 SF were IP'd using anti-Tgs1 antibodies that recognize both isoforms (Figure 5A, left panel). To discriminate Tgs1 LF from Tgs1 SF, we next IP'd GFP-containing complexes from stable cell lines expressing GFP-Tgs1 LF or GFP-Tgs1 SF. The amount of rU3B.7 RNA co-precipitated with GFP-Tgs1 SF in LMB-treated cells was decreased as compared with non-treated cells (Figure 5A, middle panel). In contrast, with GFP-Tgs1 LF, a higher amount of rU3B.7 was found in the pellet from LMB-treated cells, as compared with non-treated cells (Figure 5A, right panel). Thus, CRM1 inhibition led to an increased association of U3 snoRNA with Tgs1 LF but not with Tgs1 SF.

Next, we wanted to verify that Tgs1 LF association also occurs on endogenous snoRNAs and that CRM1 inhibition increases this association. Levels of endogenous U3 in pellets of GFP-Tgs1 LF IP were measured by quantitative RT-PCR (qRT-PCR). For each IP, RNA levels were normalized to GAPDH and expressed as fold enrichment over a control IP without antibody. As shown in Figure 5B, U3 was specifically enriched in the GFP-Tgs1 LF IP, and LMB treatment increased this association by more than two-fold. As a control, U2 snRNA showed a decreased association with Tgs1 LF after LMB treatment, while U6 snRNA did not show any specific association, as expected. This demonstrated the specificity of the effect of CRM1 inhibition on C/D snoRNAs, and suggested that CRM1 could be involved in the removal of Tgs1 LF from C/D snoRNP.

Since LMB affects transport of intronic, uncapped snoRNAs (Figure 1B), we next wondered if Tgs1 LF was also associated with this class of snoRNAs. To test this, cells were transiently

transfected with a construct expressing an artificial C/D snoRNA placed in the intron of β -globin gene (pCMV-C/DdBB), and RNP complexes were IP'd with anti-Tgs1

antibodies that recognize both Tgs1 LF and SF. The pellets were analysed by RNase protection assays. As shown in Figure 5C, this artificial intronic snoRNA was specifically pelleted by anti-Tgs1 antibodies. Furthermore, using the GFP-Tgs1 LF cell line and anti-GFP IP, we also measured by qRT-PCR the association of GFP-Tgs1 LF with the endogenous intronic snoRNA U14. This association was increased two-fold after LMB treatment (Figure 5B), demonstrating that Tgs1 LF also associates with intronic snoRNAs, and that this interaction is again regulated by CRM1.

To test whether CRM1 would influence the binding of Tgs1 LF to other C/D snoRNAs and to other RNA species, we analysed the Tgs1 IP with microarrays. To this end, we used the Flp-In system to generate isogenic HeLa cell lines that stably express the short and long isoforms of Tgs1 with a 3xFlag tag. IPs were then performed with anti-Flag beads, using these cells or the parental cell line as control. Enrichment of the various IPs over control was then calculated, and RNAs were ranked according to this score. The first 500 RNA were significantly enriched in the IP (their enrichment was higher than mean + 2s.d., P -value > 0.95). We first compared the IPs between the two Tgs1 isoforms. It was previously shown using anti-Fibrillarin IPs that this protein is

preferentially associated with Tgs1 SF (Girard *et al*, 2008; Figure 4B). In agreement, we found a strong enrichment of C/D snoRNAs in the Tgs1 SF IPs, several fold higher than that of snRNAs (Table I). Likewise, scaRNAs and H/ACA snoRNAs were also strongly bound to Tgs1 SF, and snoRNAs and scaRNAs were in fact the most frequent families found in the first 500 RNAs of the Tgs1 SF IP (Table I; Figure 5D, 46 RNAs species in the first 500). Conversely, Tgs1 LF was more strongly associated with snRNAs than scaRNAs and snoRNAs, as might be expected from previous studies (Table I). Then, we analysed the effect of LMB on the association of Tgs1 LF with cellular RNAs. In this case, we IP'd Tgs1 LF in presence and absence of LMB and calculated the enrichment of each RNA in the LMB-treated versus non-treated cells. RNAs were then ranked according to this score, and mean enrichment values for specific families were also calculated (Table I; Figure 5D). Remarkably, binding of Tgs1 LF to C/D snoRNAs was the most affected by LMB treatment, and this occurred for both capped and uncapped intronic species. Indeed, binding of C/D snoRNAs to Tgs1 LF increased in average two-fold after LMB treatment (with a highest value of 20-fold), and 30% of all C/D snoRNAs were in the first 500 RNA species that respond most strongly to LMB (Figure 5D).

Figure 4 Tgs1 LF binds CRM1 and snoRNP proteins. (A) His-CRM1, His-RanQ69L and GST-Tgs1 LF were produced in bacteria, purified and used for *in vitro* binding assay. Western blot using anti-CRM1 revealed direct Ran-dependent binding of CRM1 on immobilized GST-Tgs1 LF. CRM1 binding requires the NES2 in Tgs1 LF, as no binding could be detected with or without RanQ69L using GST-Tgs1 NES2Δ5. Membrane stained with Ponceau shows the material loaded. (B) In HeLa whole cell extract (WCE), both forms of Tgs1 are revealed by western blot using a polyclonal antibody against Tgs1. After IP of fibrillarin-containing complexes, a larger amount of Tgs1 SF was co-precipitated compared with Tgs1 LF. LMB treatment increased the amount of Tgs1 LF that was co-precipitated. Inputs represent 10% of extracts. (C) Immunoblotting of Nop58 after IP of GFP-Tgs1 LF from nuclear extract (NE). More Nop58 was associated with GFP-Tgs1 LF after LMB treatment. Immunoblotting of Tgs1 showed that GFP-Tgs1 LF was present at similar level as endogenous Tgs1 LF (Input), and the same amount of GFP-Tgs1 LF was IP'd in cells treated or not with LMB (Pellet, Ip).

Figure 3 CRM1 inhibition does not affect C/D snoRNP assembly but affects the localization of the Tgs1 hypermethylase. (A) RNA metabolic labelling with 32 P was performed in HeLa cells in the presence or absence of LMB and specific IPs with antibodies against core proteins Nop56, Nop58 and fibrillarin were carried out. U3 snoRNA was resolved on sequencing gels. For each antibody, the amount of pelleted U3 was the same whether cells were treated or not with LMB. (B) Scheme of Tgs1 LF and Tgs1 SF (Mtase, methyltransferase domain). Positions of NES1 and NES2 in Tgs1 LF sequence are indicated. (C) Constructs coding for GFP-Tgs1 LF were transiently transfected into HeLa cells (green). CB were labelled using anti-coilin antibodies (red). In the merged image, DNA is stained with DAPI (blue). Most of GFP-Tgs1 LF is cytoplasmic but a fraction also colocalized with CB in the nucleus (upper panel). After LMB treatment, the cytoplasmic localization is completely lost. (D) Legend as in (C) except that GFP-Tgs1 NES1mut, NES2mut, NES1Δ4 and NES2Δ5 were transfected. GFP-Tgs1 NES1mut and NES1Δ4 showed nuclear and cytoplasmic localization (upper and middle panels) and the faint cytoplasmic signal was lost after LMB treatment (middle panels). GFP-Tgs1 NES2mut showed pronounced nuclear accumulation (second upper panel). GFP-Tgs1 NES2Δ5 exclusively localized to the nucleus in the absence of LMB treatment (lower panel). The bar represents 5 μ m.

Figure 5 Association of Tgs1 LF with capped and uncapped snoRNAs is increased upon CRM1 inhibition. (A) RNAse protection assays were performed to study the association of both forms of Tgs1 with U3 snoRNA. Vertical lines indicate the band corresponding to pre-snoRNAs (pre-U3) and mature snoRNA (U3-m). LMB treatment increases the association of U3 snoRNA with GFP-Tgs1 LF (right panel) but not with GFP-Tgs1 SF (middle panel). This increased association upon LMB is not detectable when both Tgs1 LF and Tgs1 SF were IP'd (left panel). Inputs represent 10% of the extracts. (B) By qRT-PCR, four different RNA species were measured after IP of GFP-Tgs1 LF from LMB treated and untreated. Each value represents the fold enrichment as compared with control beads after normalization to GAPDH, and represents the average of three different IP experiments. Error bars represent s.d. (C) RNAse protection assay using a probe specific to an exogenously expressed intronic snoRNA pCMV-C/dBB. The snoRNA was IP'd with an antibody recognizing both Tgs1 LF and SF (Ip). Input is 10% of the extract. (D) Isogenic HeLa cells stably expressing tagged 3xFlag-Tgs1 SF and LF were generated with the Flp-In system. The tagged proteins were IP'd from total cellular extract, in presence of LMB when indicated, and the RNA content of each pellet was analysed on microarrays. The control was the parental cell line that did not express any tagged protein. The graph represents the frequency with which the indicated families are found in the 500 RNAs most enriched in the IP (*P*-value that the enrichment is significant >0.95). The number of genes for each family is indicated.

Interestingly, LMB also increased the binding of Tgs1 LF to H/ACA snoRNA and scaRNAs (Table I), as well as to hTR RNA (3.5-fold, data not shown). In addition, a slight but significant increase in the binding of Tgs1 LF to snoRNA host genes also occurred after LMB treatment, suggesting that the association of Tgs1 LF with snoRNA occurred at an early step of their biogenesis. Altogether, these data indicating that CRM1 controlled the binding of Tgs1 LF to a large number of RNA species. Because most of these species are uncapped, these results suggest a new role for Tgs1 LF in snoRNP biogenesis,

which is unrelated to cap hypermethylation, and this role appears to be regulated by CRM1.

CRM1 inhibition and overexpression of Tgs1 NES2Δ5 lead to reduced nucleolar localization of a C/D snoRNA reporter

Next, we established an experimental system to analyse the role of CRM1 and Tgs1 LF on snoRNA biogenesis at the cellular level. To this end, a canonical C/D snoRNA was placed in the efficiently spliced MINX intron, and the

Table 1 Microarray analysis of RNAs IP'd with Tgs1 isoforms

Log2 ratios	Tgs1 SF—Cont ^a	Tgs1 LF—Cont ^a	Tgs1 LF: LMB-NT ^b
SnRNA (9 genes)	0.25 (3.1×10^{-3})	0.82 (3.1×10^{-3})	0.25 (3.1×10^{-3})
ScaRNA (25 genes)	1.85 (0.1×10^{-3})	0.22 (3.8×10^{-3})	0.53 (0.7×10^{-3})
C/D (112 genes)	1.2 (1.9×10^{-3})	-0.34 (3.3×10^{-3})	0.75 (0.1×10^{-3})
H/ACA (108 genes)	0.61 (0.1×10^{-3})	-0.11 (3.7×10^{-3})	0.53 (0.1×10^{-3})
Host genes (36 genes)	0.06 (0.22)	-0.17 (0.1×10^{-3})	0.27 (0.1×10^{-3})

^aAverage of fold enrichment of RNA species in IP versus control for each RNA families.

^bAverage of fold enrichment for RNA species in Tgs1 FL IP for LMB-treated cells compared with non-treated (NT) cells. *P*-values were obtained from Mann and Whitney test and are indicated in brackets.

Figure 6 Nucleoplasmic accumulation of pre-snoRNA following CRM1 inhibition or overexpression of GFP-Tgs1 NES2Δ5. **(A)** Scheme of the pHIV-MINX-C/D construct. Probes used for FISH are indicated by a star and recognized: exon 2 to localize mRNAs and pre-mRNAs (FISH exon 2, green); C/D snoRNA to localize precursor and mature snoRNAs (FISH snoRNA, red). **(B)** Graph showing the relative intensity of snoRNA signals in nucleoli compared with nuclear patches (P) in three different situations. In each image, the nuclear background was subtracted, and the nucleolar signal was then divided by the signal from patches. Mean values are calculated from 30 cells, and error bars represent s.d. **(C)** Cells stably expressing GFP-Tgs1 LF and transiently transfected with pHIV-MINX-C/D construct treated (2) or not (1) with LMB, were processed for FISH. As a control, the same construct devoid of C/D snoRNA sequence was transfected and cells were treated with LMB (lower panels). In the nucleus, GFP-Tgs1 LF mainly localized to CB. It relocalized to the nuclear patches after LMB treatment and this relocalization is dependent on the presence of C/D snoRNA in the construct. **(D)** Cells stably expressing GFP-Tgs1 NES2Δ5 and transiently transfected with the pHIV-MINX-C/D construct (3) were processed for FISH. More snoRNAs colocalized with pre-mRNA nuclear patches as compared with control cells expressing wild-type GFP-Tgs1 LF. The bar represents 5 μm.

localization of pre-snoRNA and mature snoRNA was studied by *in situ* hybridization (Zillmann *et al*, 1988; construct pHIV-MINX-C/D, Figure 6A). In transiently transfected cells, FISH

probes complementary to the second exon and to the intron of the reporter showed that the pre-mRNA accumulated in nuclear patches (Figure 6C; Supplementary Figure S5). These

patches were distinct from nucleoli where fibrillarin accumulates (Supplementary Figure S5, upper lane). A staining for the mature snoRNA also weakly labelled the patches, but most of it was localized to CB and nucleoli. This later result showed that the snoRNA was efficiently processed.

A stable cell line expressing GFP-Tgs1 LF was transfected with pHIV-MINX-C/D and Tgs1 LF localization was visualized together with the reporter snoRNA. In non-treated cells, GFP-Tgs1 LF was found in CB (Figure 6C, upper panels, arrow) and only a faint signal colocalized with the pre-mRNAs in the nuclear patches (designated by arrowhead). However, when cells were treated with LMB, GFP-Tgs1 LF accumulated more strongly in the patches with the pre-snoRNA (Figure 6C, middle panels). As a control, we transfected an MINX construct devoid of C/D snoRNA. In this case, spots of mRNAs were seen in the nucleoplasm with the exon 2 probe, but GFP-Tgs1 LF did not accumulate there, with or without LMB treatment (Figure 6C, lower panels). This indicated that Tgs1 LF specifically colocalized with nucleoplasmic pre-snoRNAs following CRM1 inhibition. This is in accordance with the observation that Tgs1 LF is more associated with C/D snoRNPs and their host genes in LMB-treated cells (Figures 4 and 5).

Following LMB treatment, we also observed a higher level of nucleoplasmic pre-snoRNAs and lower levels of snoRNA in nucleoli, while similar amounts of pre-mRNAs were expressed (Figure 6C, middle panels). To quantify such observation, we selected cells with similar levels of pre-mRNAs in the nucleoplasm using the exon 2 probe, and we compared the signal of snoRNA at nuclear patches and in nucleoli (Figure 6B). Upon LMB treatment, about twice more snoRNAs was present in the nuclear patches, while less reached nucleoli. This provided further evidence that nucleolar transport of intronic C/D snoRNA was disturbed by CRM1 inhibition.

We next analysed the effect of expressing the non-shuttling mutant of Tgs1, GFP-Tgs1 NES2Δ5. Expression of this mutant protein in the absence of LMB also gave rise to the accumulation of snoRNAs in the nucleoplasm, at the expense of nucleoli (Figure 6D and B for quantification). Taken together, these results support the idea that CRM1 acts on Tgs1 LF to release it from nascent snoRNP complexes, and that this release is required to transport C/D snoRNPs to the nucleolus. The fact that the NES2 mutant of Tgs1 LF recapitulates the effect of LMB further suggests the role of CRM1 is mainly mediated by Tgs1 LF.

CRM1 controls the interaction of Tgs1 LF with the NoLS of Nop58

Altogether, the data point towards an antagonism between the binding of Tgs1 LF to pre-snoRNAs and their localization in nucleoli. Interestingly, although no NoLS has been identified on snoRNP particles, the C-terminal domains of Nop56 and Nop58 have sequence similarities to NoLS, which often consists of long stretches of basic residues (Figure 7A, shown for Nop58). To test whether the C-terminal domain of Nop56 and Nop58 have NoLS properties, we fused them to GFP. After transfection into HeLa cells, we found that GFP-Nop56Ct and GFP-Nop58Ct were localized to CB and nucleoli, where they formed a punctate pattern that colocalized with U3 and thus likely corresponded to the dense fibrillar component, the site of snoRNP accumulation in nucleoli

(Figure 7B, upper panels, not shown for Nop56). Furthermore, C-terminal truncation mutants of Nop56 and Nop58 remained cytoplasmic, and when a nuclear localization signal (NLS) was added to the truncated proteins they localized to the nucleoplasm but remained excluded from CB and nucleoli (Figure 7B, middle and lower panels). This suggested that C-terminal domains of Nop56 and Nop58 function both as NLS and NoLS.

Since C-terminal domain of Nop56 and Nop58 have been reported to bind Tgs1 (Mouaikel *et al*, 2002; Girard *et al*, 2008), we next used *in vitro* experiments to address whether CRM1 affected the interaction of Tgs1 with the NoLS of these proteins. To this end, radiolabelled, *in vitro* translated Tgs1 LF was incubated with or without CRM1 and Ran-GTP and was pulled down with recombinant GST-Nop58Ct immobilized on glutathione-sepharose beads. Remarkably, while addition of CRM1 alone had no effect (data not shown), the binding of Tgs1 LF was reduced by more than two-fold when both CRM1 and Ran-GTP were present (Figure 7C and D). Interestingly, no such decrease was observed for the association of Nop58Ct with Tgs1 NES2Δ5, which lacks the NES that binds CRM1. These results indicate that the binding of CRM1 to the NES2 of Tgs1 LF antagonizes the binding of Tgs1 LF to the Nop58 NoLS. Altogether, this suggests a model where Tgs1 LF masks the NoLS of Nop58, and is released by the action of CRM1.

Discussion

CRM1 in interphase nuclei: more than an export receptor

CRM1 was known to be required for the nucleolar localization of C/D snoRNAs, but the underlying mechanism was not understood (Boulon *et al*, 2004). Early works in *Xenopus* oocytes gave conflicting results for C/D snoRNA transport pathways. It was shown that snoRNAs remained in the nucleus and could be hypermethylated without leaving this compartment (Terns and Dahlberg, 1994), whereas other studies concluded that snoRNA biogenesis required a cytoplasmic phase (Baserga *et al*, 1992). m⁷G-capped pre-U3, pre-U8 and pre-U13 snoRNAs were found associated with PHAX, which is an adaptor for CRM1 (Boulon *et al*, 2004), and in human cells, CRM1 and Ran both bind U3 and their inactivation affects the localization of nascent U3 particles (Cheng *et al*, 1995; Boulon *et al*, 2004; data not shown). However, cap hypermethylation of U3, U8 and U13 snoRNAs was found to be insensitive to CRM1 or RCC1 inhibition, indicating that the effect of CRM1 is posterior to this processing step (Cheng *et al*, 1995; Boulon *et al*, 2004). Recently, U8 was detected in a complex with the import factor snurportin in cytoplasmic extracts of HeLa cells (Watkins *et al*, 2007), suggesting that C/D snoRNAs could transit through the cytoplasm at a late stage of their biogenesis, after cap hypermethylation. For technical reasons, we could not analyse U8, so it remains possible that this snoRNA follows a particular transport pathway. However, our heterocaryon assays clearly demonstrate neither U3 nor a canonical intronic C/D snoRNA transit through the cytoplasm to reach nucleoli. Because CRM1 binds directly to U3 and is required for its transport (Boulon *et al*, 2004), these data show that CRM1 has a direct and strictly nuclear role during the trafficking of C/D snoRNAs to nucleoli.

Figure 7 Tgs1 LF interacts with the NoLS of Nop58 and CRM1 downregulates this interaction *in vitro*. (A) Scheme for human Nop58 protein and C-terminal domain sequence rich in charged amino acids (underlined, basic amino acids are in red and acidic in green). (B) HeLa cells transiently expressing GFP-Nop58Ct, GFP-Nop58ΔCt, GFP-Nop58ΔCt + NLS were processed for FISH with a probe against U3. GFP-Nop58Ct colocalized with U3 in CB and nucleoli (upper panel). In contrast, GFP-Nop58ΔCt is mainly cytoplasmic (lower panel). The bar represents 5 μm. (C) *In vitro* binding assays using GST-Nop58Ct, His-CRM1 and His-RanQ69L produced in bacteria and purified. *In vitro* translated, both ³⁵S-labelled Tgs1 LF and NES2Δ5 bind to GST-Nop58Ct but the binding was decreased for Tgs1 LF when both CRM1 and RanQ69L were added to the assay. Membrane coloured with Ponceau shows the material loaded. (D) Quantification of the radioactive signal seen for three experiments as in (C).

We have also analysed the transport of H/ACA snoRNAs and of hTR, the human telomerase RNA. hTR looks like a capped H/ACA scaRNA and it binds both snoRNP core

proteins and PHAX (Kiss, 2002; Terns and Terns, 2002; Boulon *et al*, 2004). Our results show that neither hTR nor intronic H/ACA snoRNA exit the nucleus during their

biogenesis, suggesting that they follow a strictly transport pathway, similar to the case of C/D snoRNAs. The situation is different in budding yeast where it was shown that telomerase RNA trafficking involves a cytoplasmic phase (Gallardo *et al*, 2008). In this regard, it is interesting to note that the yeast telomerase RNA bears an Sm site and associates with the Sm proteins rather than snoRNP proteins (Seto *et al*, 1999).

CRM1 dissociates Tgs1 LF from nucleoplasmic snoRNP complexes to unmask their NoLS

Our results indicate that at least some of the functions of CRM1 on snoRNP biogenesis are mediated by the long isoform of Tgs1 (Tgs1 LF). First, both CRM1 and Tgs1 LF are found preferentially associated with TMG-capped, nearly mature C/D snoRNAs (Verheggen *et al*, 2002; Boulon *et al*, 2004; Figure 5). This contrasts to the case of PHAX, which is associated with m⁷G-capped pre-snoRNAs and is thus unlikely to be responsible for the recruitment of CRM1 (Boulon *et al*, 2004). Second, *in vitro* binding experiments indicate that CRM1 directly binds the NES2 of Tgs1 LF and that its binding dissociates Tgs1 LF from the NoLS of Nop58. Third, immunoprecipitation experiments demonstrate that in cells, the binding of Tgs1 LF to snoRNPs is regulated by CRM1. Fourth, overexpression of Tgs1 NES2Δ5, which no longer binds CRM1, partially inhibits the transport of a snoRNA reporter to nucleoli. Altogether, these data suggest a model where CRM1 removes Tgs1 LF from Nop58, thereby activating the NoLS of Nop58 and promoting the nucleolar localization of snoRNPs. CRM1 appears to decrease the affinity of Tgs1 LF for Nop58. In addition, by exporting Tgs1 LF, CRM1 may decrease amount of Tgs1 LF in the nucleus and lower its association with nascent snoRNPs. This could also be the way CRM1 would be recycled, as dissociation of the Tgs1 LF-CRM1-RanGTP complex would be promoted by RanGAP in the cytoplasm.

Our microarray analysis indicates that Tgs1 LF binds to both capped and uncapped C/D snoRNA, and that CRM1 regulates its association with both types of RNA. In addition, Tgs1 LF binds hTR and H/ACA snoRNA in a CRM1-dependent manner and these snoRNA do not shuttle, suggesting that a similar mechanism may also operate for these RNAs. Thus, CRM1 and Tgs1 LF appear to be involved in the intranuclear trafficking of a large number of RNA species.

It has been previously shown that CRM1 regulates protein complexes composition, independently of its function in nuclear export. During mitosis, CRM1 was shown to form a complex with RanBP2 and RanGAP1 in a RanGTP-dependent manner, and this association is necessary for the deposition of the complex on kinetochores and for the formation of mitotic spindles (for review, see Arnaoutov *et al* (2005)). Our study extends these results and shows that CRM1 controls the composition of macromolecular complexes in interphase nuclei. As discussed below, such mechanisms may allow for a feedback of nucleocytoplasmic trafficking on other nuclear processes, allowing the integration of nuclear events with the physiological status of the cell.

Tgs1 has a conserved function in RNP trafficking

The C-terminal domains of the human Nop56 and Nop58 proteins are highly enriched in successive stretches of acidic and basic amino acids (K and E amino acids; Lyman *et al*,

1999). We show here that both are necessary and sufficient for the nucleolar localization of these proteins and function as bona fide NoLS. In budding yeast, Nop56 and Nop58 have also highly charged C-terminal domains that are enriched in KKD/E motifs and that bind Tgs1. Interestingly, Tgs1 is a nucleolar protein in yeast (Mouaikel *et al*, 2002), and its disruption or the removal of the C-terminal domain of Nop56 and Nop58 both affects the nucleolar structure and snoRNP localization in a very similar way (Colau *et al*, 2004). This indicates that in yeast, the interaction of the C-terminal domain of Nop56 and Nop58 with Tgs1 promotes the accumulation of snoRNPs in nucleoli and the coalescence of nucleolar components into well-defined structures (Colau *et al*, 2004). It thus seems that throughout evolution, the binding of Tgs1 to the C-terminal domain of Nop56 and Nop58 has conserved a dual role in driving cap hypermethylation and controlling snoRNP transport.

It is also possible that Tgs1 participates in snRNA trafficking. In humans, Tgs1 LF appears unable to interact with snRNA in the nucleus (data not shown), indicating that the Sm core that binds Tgs1 is probably masked by its association with other proteins, as in the case of U1 (Pomeranz Krummel *et al*, 2009). However, a partial disassembly of the snRNP would unmask the Sm core, and Tgs1 would then be able to bind the non-functional snRNPs and bring them back to CB or to the cytoplasm to prevent their participation in splicing.

CRM1 links nucleoplasmic events to nucleocytoplasmic trafficking

The fact that functional nucleoporins are required for the nucleolar localization of C/D snoRNA suggests that transit through nuclear pores is a necessary event. However, our experiments indicate that CRM1 functions in the nucleoplasm to control the composition of nascent snoRNP complexes. Several possibilities could resolve this paradox. One would be that blocking the pores would prevent export of CRM1 substrates, which would then rapidly accumulate in the nucleus and titrate CRM1, thereby inactivating it. In this respect, it is interesting to note that the most abundant CRM1 cargos are newly synthesized ribosomal subunits (for review, see Zemp and Kutay (2007)). Thus, the dependency of snoRNP intranuclear transport on CRM1 availability could be part of a feedback loop that coordinates snoRNP transport with ribosome biogenesis and nucleocytoplasmic trafficking. If ribosomal subunits were not properly exported, CRM1 activity would decrease, thereby lowering nucleolar transport of C/D snoRNAs, ultimately resulting in slowing down of ribosome biogenesis. Together with the study showing that CRM1 inhibition affects the retention of snRNP in CB (Sleeman, 2007), our results support the view that CRM1 could be an important player in coordinating the biogenesis of various RNAs to nucleocytoplasmic trafficking.

Materials and methods

Plasmids

Plasmids were generated by standard procedures or with the Gateway system (Invitrogen), and detailed maps are available upon request. pDon rU3B.7 and pT7-U3 have been described previously (Boulon *et al*, 2004). pT7-U14 and pU3tag were a gift from N Watkins. pDon-U4tag was obtained by PCR cloning. pCMV-C/DdBB and pCMV-C/Dtag contained artificial C/D snoRNAs inserted in the second intron of the β-globin gene (sequence available upon

request). pHIV-MINX-C/D contained a promoter-less version of U3 within the MINX intron and its expression was driven by the HIV-1 promoter. pTat was co-transfected for expression of MINX mRNA in mammalian cells. Plasmids coding for hTR and U64 were gift from T Kiss. peGFP-Fibrillarin was a gift from M Dundr. Nop58Ct (aa 401–529) and Nop58ΔCt + NLS (aa 1–401 + NLS) were PCR cloned and inserted into the pL30-GFP and pGEX2T vectors using Gateway technology. peGFP-Tgs1 was described previously (Verheggen *et al*, 2002). Fragments of Tgs1 were generated by PCR amplification (Supplementary Figure S4) and cloned in pSpOII-GFP plasmids or pGEX2T using Gateway technology. GFP-Tgs1 NES1mut, NES2mut, NES1Δ4 and NES2Δ5 (Supplementary Table S1) were generated by oligonucleotide-directed mutagenesis using the QuickChange II Kit (Stratagene).

Cell culture and transfections

HeLa and U2OS cells were cultured in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal calf serum (FCS) and penicillin-streptomycin. Cells were transfected according to the calcium phosphate procedure as previously described (Verheggen *et al*, 2002). Stable U2OS cells expressing GFP-Tgs1 LF, GFP-Tgs1 SF or GFP-Tgs1 NES2Δ5 were made by co-transfecting the corresponding plasmid with pCMV-Hygro-Tk. Stable isogenic HeLa Flp-in cells expressing 3xFlag-Tgs1 LF and SF were made using the Flp recombinase. Selections were done in hygromycin. Individual clones were picked and selected for proper GFP or 3xFlag expression as judged by microscopy and western blotting.

Immunofluorescence and in situ hybridization

Immunofluorescence, FISH and microinjection experiments were performed as previously described (Samarsky *et al*, 1998; Boulon *et al*, 2004). When indicated, cells were treated with LMB (7 nM for 2 h) before microinjection, and WGA (2.5 mg/ml) was coinjected with RNAs. Cells were fixed in 4% paraformaldehyde 2 h after microinjection, permeabilized in 0.1% Triton X-100 in PBS, and CB were visualized using monoclonal anti-coilin antibodies. For heterocaryon experiments, see Supplementary data. Homocaryons of HeLa cells were also done by the same method to evaluate shuttling properties of Tgs1 isoforms (Supplementary Figure S3).

Co-immunoprecipitation and western blotting

Cells were extracted with HNTG (20 mM Hepes pH 7.9, 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, complete anti-protease cocktail from Roche) and incubated with Protein G-sepharose beads (Amersham) coupled to the indicated antibody or with GFP-Trap beads (Chromotech). When indicated, cells were fractionated to purify nuclei (Trinkle-Mulcahy *et al*, 2008), which were then lysed with HNTG. For IP, the mouse monoclonal antibody 15A2 against recombinant human Tgs1 or the monoclonal anti-fibrillarin antibody 72B9 were used. Protein G-sepharose beads alone were used as a control. Beads were washed with HNTG and resuspended in Laemmli buffer and analysed by SDS-PAGE. Anti-Tgs1 polyclonal antibodies were already described (Verheggen *et al*, 2002). Anti-Nop58 antibodies were raised in rabbit against the peptide KKLQEVDSLWKEFETPEK (aa 20–37) and purified using the same peptide.

RNA analysis

To study RNA-protein interactions, IP were done from cell extracts prepared in HNTG, which were then incubated with beads coupled to

anti-GFP or anti-Tgs1 mouse monoclonal antibodies. For controls, beads without antibodies were used, except for the microarray analysis where the control was the parental HeLa cells that did not express any tagged Tgs1 isoform. Beads were washed four times and RNAs were extracted using Trizol (Invitrogen). RNAs were then analysed with RPAIII kit (Ambion) for RNase protection assay, with a probe covering nucleotides +98 to +236 of rU3B.7 (+1 is the first nucleotide of mature U3). RNA from IP were also analysed by qRT-PCR according to Lutfalla *et al* (2006) or on microarrays as described in Supplementary data.

In vitro binding assay

Recombinant His-CRM1 was produced in Tg1 bacteria transformed with pQE-CRM1 (gift from C Petosa) and purified on NTA beads (Qiagen) after lysis in 25 mM Hepes pH 7.5, 400 mM NaCl, 5 mM B-mercaptoethanol and 10 mM imidazole. hRanQ69L-GTP was produced in BL21 bacteria according to Fornerod *et al* (1997). Recombinant Tgs1 and Nop58Ct were produced as GST fusions in BL21 bacteria and purified as already described (Mouaikel *et al*, 2003). Alternatively, Tgs1 was produced in rabbit reticulocyte lysate (Promega) in the presence of ³⁵S-methionine. Binding assays were performed in interaction buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 1.5 mM MgCl₂, 8% glycerol, 1% NP40) and beads were rinsed four times with washing buffer (20 mM Tris-HCl pH 7.5, 200 mM KCl, 5 mM MgCl₂, 8% glycerol, 1% NP40) and pellets were analysed by SDS-PAGE.

Supplementary data

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

Acknowledgements

We wish to thank Pr. J Reddy for full-length PIMT (Tgs1) cDNA clone, N Watkins for the gift of U3tag plasmids and anti-Nop56 and Nop58 antibodies, B Jady and T Kiss for pHTR plasmid, A Lamond for anti-coilin antibodies, and M Fornerod and C Petosa for their help in producing recombinant CRM1 and Ran. We are grateful to I Jariel for advice on heterocaryon assays. We also thank people who helped this work: H Neel, M-C Robert, F Maloum, O Zouani and E Granier. We thank B Westman for critical reading of the manuscript. Grants were from l'ARC, La Ligue Contre Le Cancer and ANR. Fellowships for SB were from Ministère Français de la Recherche and for CG from Association Française contre les Myopathies.

Author contributions: Bérangère Pradet-Balade and Cyrille Girard have equally contributed to perform the main experiments. They were helped by Séverine Boulon and Céline Verheggen for IP, Conception Paul for microinjections and Karim Azzag for *in situ* localizations of snoRNAs. Design of the experiments was done together by Edouard Bertrand and Céline Verheggen, and they also wrote the manuscript, with help from Remy Bordonné.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Arnaoutov A, Azuma Y, Ribbeck K, Joseph J, Boyarchuk Y, Karpova T, McNally J, Dasso M (2005) Crm1 is a mitotic effector of Ran-GTP in somatic cells. *Nat Cell Biol* **7**: 626–632
- Baserga SJ, Gilmore-Hebert M, Yang XW (1992) Distinct molecular signals for nuclear import of the nucleolar snRNA, U3. *Genes Dev* **6**: 1120–1130
- Boulon S, Marmier-Gourrier N, Pradet-Balade B, Wurth L, Verheggen C, Jady BE, Rothe B, Pescia C, Robert MC, Kiss T, Bardoni B, Krol A, Branlant C, Allmang C, Bertrand E, Charpentier B (2008) The Hsp90 chaperone controls the biogenesis of L7Ae RNPs through conserved machinery. *J Cell Biol* **180**: 579–595
- Boulon S, Verheggen C, Jady BE, Girard C, Pescia C, Paul C, Ospina JK, Kiss T, Matera AG, Bordonne R, Bertrand E (2004) PHAX and CRM1 are required sequentially to transport U3 snoRNA to nucleoli. *Mol Cell* **16**: 777–787
- Chari A, Paknia E, Fischer U (2009) The role of RNP biogenesis in spinal muscular atrophy. *Curr Opin Cell Biol* **21**: 387–393
- Chekulaeva M, Filipowicz W (2009) Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol* **21**: 452–460
- Cheng Y, Dahlberg JE, Lund E (1995) Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport. *Science* **267**: 1807–1810

- Colau G, Thiry M, Leduc V, Bordonne R, Lafontaine DL (2004) The small nucleolar RNA cap trimethyltransferase is required for ribosome synthesis and intact nucleolar morphology. *Mol Cell Biol* **24**: 7976–7986
- Fok V, Friend K, Steitz JA (2006) Epstein-Barr virus noncoding RNAs are confined to the nucleus, whereas their partner, the human La protein, undergoes nucleocytoplasmic shuttling. *J Cell Biol* **173**: 319–325
- Fornerod M, Ohno M, Yoshida M, Mattaj IW (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**: 1051–1060
- Gallardo F, Olivier C, Dandjinou AT, Wellinger RJ, Chartrand P (2008) TLC1 RNA nucleo-cytoplasmic trafficking links telomerase biogenesis to its recruitment to telomeres. *EMBO J* **27**: 748–757
- Girard C, Verheggen C, Neel H, Cammas A, Vagner S, Soret J, Bertrand E, Bordonne R (2008) Characterization of a short isoform of human Tgs1 hypermethylase associating with small nucleolar ribonucleoprotein core proteins and produced by limited proteolytic processing. *J Biol Chem* **283**: 2060–2069
- Hirose T, Shu MD, Steitz JA (2003) Splicing-dependent and -independent modes of assembly for intron-encoded box C/D snoRNPs in mammalian cells. *Mol Cell* **12**: 113–123
- Izaurrealde E, Lewis J, Gamberi C, Jarmolowski A, McGuigan C, Mattaj IW (1995) A cap-binding protein complex mediating U snRNA export. *Nature* **376**: 709–712
- Kiss T (2002) Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. *Cell* **109**: 145–148
- Lemm I, Girard C, Kuhn AN, Watkins NJ, Schneider M, Bordonne R, Luhrmann R (2006) Ongoing U snRNP biogenesis is required for the integrity of Cajal bodies. *Mol Biol Cell* **17**: 3221–3231
- Leverette RD, Andrews MT, Maxwell ES (1992) Mouse U14 snRNA is a processed intron of the cognate hsc70 heat shock pre-messenger RNA. *Cell* **71**: 1215–1221
- Lyman SK, Gerace L, Baserga SJ (1999) Human Nop5/Nop58 is a component common to the box C/D small nucleolar ribonucleoproteins. *RNA* **5**: 1597–1604
- Lutfalla G, Uze G (2006) Performing quantitative reverse-transcribed polymerase chain reaction experiments. *Methods Enzymol* **410**: 386–400
- Matera AG, Shpargel KB (2006) Pumping RNA: nuclear bodybuilding along the RNP pipeline. *Curr Opin Cell Biol* **18**: 317–324
- Matera AG, Terns RM, Terns MP (2007) Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat Rev Mol Cell Biol* **8**: 209–220
- Mouaikel J, Narayanan U, Verheggen C, Matera AG, Bertrand E, Tazi J, Bordonne R (2003) Interaction between the small-nuclear-RNA cap hypermethylase and the spinal muscular atrophy protein, survival of motor neuron. *EMBO Rep* **4**: 616–622
- Mouaikel J, Verheggen C, Bertrand E, Tazi J, Bordonne R (2002) Hypermethylation of the cap structure of both yeast snRNAs and snoRNAs requires a conserved methyltransferase that is localized to the nucleolus. *Mol Cell* **9**: 891–901
- Narayanan A, Speckmann W, Terns R, Terns MP (1999) Role of the box C/D motif in localization of small nucleolar RNAs to coiled bodies and nucleoli. *Mol Biol Cell* **10**: 2131–2147
- Ohno M, Segref A, Bachi A, Wilm M, Mattaj IW (2000) PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation. *Cell* **101**: 187–198
- Peculis BA, Steitz JA (1993) Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the *Xenopus* oocyte. *Cell* **73**: 1233–1245
- Pomeranz Krummel DA, Oubridge C, Leung AK, Li J, Nagai K (2009) Crystal structure of human spliceosomal U1 snRNP at 5.5 Å resolution. *Nature* **458**: 475–480
- Reichow SL, Hamma T, Ferre-D'Amare AR, Varani G (2007) The structure and function of small nucleolar ribonucleoproteins. *Nucleic Acids Res* **35**: 1452–1464
- Samarsky DA, Fournier MJ, Singer RH, Bertrand E (1998) The snoRNA box C/D motif directs nucleolar targeting and also couples snoRNA synthesis and localization. *EMBO J* **17**: 3747–3757
- Seto AG, Zaug AJ, Sobel SG, Wolin SL, Cech TR (1999) *Saccharomyces cerevisiae* telomerase is an Sm small nuclear ribonucleoprotein particle. *Nature* **401**: 177–180
- Simonovic M, Steitz TA (2009) A structural view on the mechanism of the ribosome-catalyzed peptide bond formation. *Biochim Biophys Acta* **1789**: 612–623
- Sleeman J (2007) A regulatory role for CRM1 in the multi-directional trafficking of splicing snRNPs in the mammalian nucleus. *J Cell Sci* **120**(Part 9): 1540–1550
- Staley JP, Woolford Jr JL (2009) Assembly of ribosomes and ribonucleosomes: complex ribonucleoprotein machines. *Curr Opin Cell Biol* **21**: 109–118
- Terns MP, Dahlberg JE (1994) Retention and 5' cap trimethylation of U3 snRNA in the nucleus. *Science* **264**: 959–961
- Terns MP, Grimm C, Lund E, Dahlberg JE (1995) A common maturation pathway for small nucleolar RNAs. *EMBO J* **14**: 4860–4871
- Terns MP, Terns RM (2002) Small nucleolar RNAs: versatile trans-acting molecules of ancient evolutionary origin. *Gene Expr* **10**: 17–39
- Trinkle-Mulcahy L, Boulon S, Lam YW, Urcia R, Boisvert FM, Vandermoere F, Morrice NA, Swift S, Rothbauer U, Leonhardt H, Lamond A (2008) Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *J Cell Biol* **183**: 223–239
- Tyc K, Steitz JA (1989) U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus. *EMBO J* **8**: 3113–3119
- Verheggen C, Lafontaine DL, Samarsky D, Mouaikel J, Blanchard JM, Bordonne R, Bertrand E (2002) Mammalian and yeast U3 snoRNPs are matured in specific and related nuclear compartments. *EMBO J* **21**: 2736–2745
- Verheggen C, Mouaikel J, Thiry M, Blanchard JM, Tollervey D, Bordonne R, Lafontaine DL, Bertrand E (2001) Box C/D small nucleolar RNA trafficking involves small nucleolar RNP proteins, nucleolar factors and a novel nuclear domain. *EMBO J* **20**: 5480–5490
- Wahl MC, Will CL, Luhrmann R (2009) The spliceosome: design principles of a dynamic RNP machine. *Cell* **136**: 701–718
- Watkins NJ, Lemm I, Ingelfinger D, Schneider C, Hossbach M, Urlaub H, Luhrmann R (2004) Assembly and maturation of the U3 snoRNP in the nucleoplasm in a large dynamic multiprotein complex. *Mol Cell* **16**: 789–798
- Watkins NJ, Lemm I, Luhrmann R (2007) Involvement of nuclear import and export factors in U8 box C/D snoRNP biogenesis. *Mol Cell Biol* **27**: 7018–7027
- Yoneda Y, Imamoto-Sonobe N, Yamaizumi M, Uchida T (1987) Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. *Exp Cell Res* **173**: 586–595
- Zemp I, Kutay U (2007) Nuclear export and cytoplasmic maturation of ribosomal subunits. *FEBS Lett* **581**: 2783–2793
- Zhao R, Kakiyama Y, Gribun A, Huen J, Yang G, Khanna M, Costanzo M, Brost RL, Boone C, Hughes TR, Yip CM, Houry WA (2008) Molecular chaperone Hsp90 stabilizes Pih1/Nop17 to maintain R2TP complex activity that regulates snoRNA accumulation. *J Cell Biol* **180**: 563–578
- Zillmann M, Zapp ML, Berget SM (1988) Gel electrophoretic isolation of splicing complexes containing U1 small nuclear ribonucleoprotein particles. *Mol Cell Biol* **8**: 814–821