The EMBO Journal (2008) 27, 748–757 | © 2008 European Molecular Biology Organization | All Rights Reserved 0261-4189/08
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THE EMBO JOURNAL

TLC1 RNA nucleo-cytoplasmic trafficking links telomerase biogenesis to its recruitment to telomeres

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The yeast telomerase holoenzyme, which adds telomeric repeats at the chromosome ends, is composed of the *TLC1* RNA and the associated proteins Est1, Est2 and Est3. To study the biogenesis of telomerase in endogenous conditions, we performed fluorescent *in situ* hybridization on the native *TLC1* RNA. We found that the telomerase RNA colocalizes with telomeres in G1- to S-phase cells. Strains lacking any one of the Est proteins accumulate *TLC1* RNA in their cytoplasm, indicating that a critical stage of telomerase biogenesis could take place outside of the nucleus. We were able to demonstrate that endogenous *TLC1* RNA shuttles between the nucleus and the cytoplasm, in association with the Crm1p exportin and the nuclear importins Mtr10p–Kap122p. Furthermore, nuclear retention of the *TLC1* RNA is impaired in the absence of yKu70p, Tel1p or the MRX complex, which recruit telomerase to telomeres. Altogether, our results reveal that the nucleo-cytoplasmic trafficking of the *TLC1* RNA is an important step in telomere homeostasis, and link telomerase biogenesis to its recruitment to telomeres.

The EMBO Journal (2008) **27,** 748–757. doi:10.1038/emboj.2008.21; Published online 14 February 2008
Subject Categories: membranes & transport; RNA
Keywords: Crm1p; FISH; nucleo-cytoplasmic shuttling; telomerase; *TLC1* RNA

Introduction

Telomeres are essential chromosomal substructures constituted of DNA repeats and associated proteins. They ensure stability of chromosome ends by protecting them from non-homologous end-joining (NHEJ) and degradation. The overall length of telomeric repeat arrays is determined genetically and, in most eukaryotic cells, maintained by a ribonucleoprotein complex (RNP) called the telomerase (Kelleher *et al*, 2002; Hug and Lingner, 2006). The RNA moiety of telomerase is used as a template via reverse transcription to allow addition of DNA repeats to the telomere ends (Greider and Blackburn, 1985, 1989; Morin, 1989). In yeast, the telomerase complex is composed of the RNA molecule *TLC1*, which is used as a scaffold for the binding of a number of proteins, including three Est proteins (for ever shorter telomere) (Lundblad and Szostak, 1989; Singer and Gottschling, 1994; Zappulla and Cech, 2004). One of these, Est2p, constitutes the catalytic subunit of the holoenzyme (Counter *et al*, 1997; Lingner *et al*, 1997), whereas Est1p and Est3p are involved in regulating the activity of the telomerase (Qi and Zakian, 2000; Evans and Lundblad, 2002; Taggart *et al*, 2002). The yKu70/80 heterodimer complex also associates with the *TLC1* RNA, and is directly implicated in the recruitment of telomerase to the telomeres (Stellwagen *et al*, 2003; Fisher *et al*, 2004).

Several factors are known to regulate the activity and recruitment of telomerase at the telomeres. Among these, the yeast ATM homologue Tel1p is a central actor. Besides its role in the DNA damage checkpoint, Te1lp also regulates the association of telomerase with telomeres (Goudsouzian *et al*, 2006). In cells lacking *TEL1*, telomeres are short but stable (Lustig and Petes, 1986; Ritchie *et al*, 1999). Recently, Tel1p has been shown to be bound preferentially to short telomeres (Hector *et al*, 2007; Sabourin *et al*, 2007), allowing the recruitment of telomerase and promoting telomere elongation. Another key actor in the regulation of telomerase association with telomeres is the three-component MRX complex, consisting of Mre11p, Rad50p and Xrs2p. The MRX complex is required for homologous recombination and NHEJ during double-strand break repair of chromosomes (D'Amours and Jackson, 2002). Moreover, cells lacking any component of MRX have short telomeres, as do cells lacking Tel1p (Boulton and Jackson, 1998), and the telomeric overhangs in these cells are altered (Larrivee *et al*, 2004). Deletion of *MRE11* reduces the association of Est1p and Est2p with telomeres (Goudsouzian *et al*, 2006), and there is evidence that both Tel1p and MRX act on telomeres by the same pathway (Ritchie and Petes, 2000).

Although the *TLC1* RNA is a large polymerase II transcript with both poly(A)+ and poly(A)− forms detectable in yeast cells (Chapon *et al*, 1997; Ferrezuelo *et al*, 2002), it shares many characteristics with snRNAs. Hence, *TLC1* has a 2,2,7-trimethylguanosine (TMG) cap structure and is known to bind Sm proteins (Chapon *et al*, 1997; Seto *et al*, 1999). Thus, although most of the individual components that constitute the telomerase holoenzyme have been identified, the assembly of the RNP and its regulation *in vivo* are still poorly understood. Recent studies have reported on the trafficking of telomerase RNA in yeast (Ferrezuelo *et al*, 2002; Teixeira *et al*, 2002) and mammalian (Jady *et al*, 2006; Tomlinson *et al*, 2006) cells, but the biological significance of this phenomenon remains unclear.

Here, we show that besides their role in telomerase activity, Est1–3 and yKu proteins act independently to promote the

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Received: 24 October 2007; accepted: 21 January 2008; published online: 14 February 2008

nuclear localization of this holoenzyme. Indeed, in the absence of any single Est protein or yKu70p, the *TLC1* RNA accumulates in the cytoplasm. Yeast cells bearing a deletion of *TEL1*, *MRE11* or *XRS2*, which are defective in the recruitment of telomerase to telomeres, also show reduced nuclear accumulation of *TLC1* RNA. We defined the nucleo-cytoplasmic shuttling pathway involved in this process and found that it requires hypermethylation of the 5′ cap of *TLC1* RNA in the nucleolus, followed by nuclear export of this RNA by the Crm1p pathway, and Mtr10p–Kap122p for its nuclear import. Our study reveals a subcellular trafficking of telomerase that is essential for its biogenesis and its association with the telomeres.

Results

The TLC1 RNA colocalizes with telomeres in G1 cells

To directly visualize *in situ* the endogenous form of the *TLC1* RNA, a set of five *TLC1*-specific oligonucleotide probes coupled to Cy3 fluorophores were designed and fluorescent *in situ* hybridization (FISH) experiments were performed on a wild-type (WT) yeast strain. As shown in Figure 1A, the signal observed with the use of these probes clustered in the form of nuclear foci. To assess the specificity of these probes, the same experiment was performed in a *tlc1* strain (Figure 1A) and this resulted in little or no fluorescent signal, confirming that the probes were specific for the *TLC1* RNA. Single plane projection of a three-dimensional reconstruction of yeast cells hybridized with the *TLC1* probes showed that the *TLC1* RNA clustered into 8–10 foci inside the cell nucleus (Figure 1B), which is consistent with a normal telomere clustering in yeast (Gotta *et al*, 1996; Laroche *et al*, 2000). To determine if the observed localization of *TLC1* RNA foci was associated with nuclear telomere clustering, the detection of *TLC1* RNA by FISH was coupled with immunofluorescence (IF) against a 13xMyc-tagged Rap1 protein, a telomere-binding protein commonly used for the detection of telomere position in yeast (Klein *et al*, 1992). As shown in Figure 1C, the FISH and IF signals overlap extensively and in G1–S phase of the cell cycle, 92% of the *TLC1* RNA and Rap1p foci colocalized. These results suggest that *TLC1* RNA is present at the telomeres in G1–S, at which time the catalytic subunit Est2p and the yKu complex are also associated with the telomeres (Taggart *et al*, 2002; Fisher *et al*, 2004).

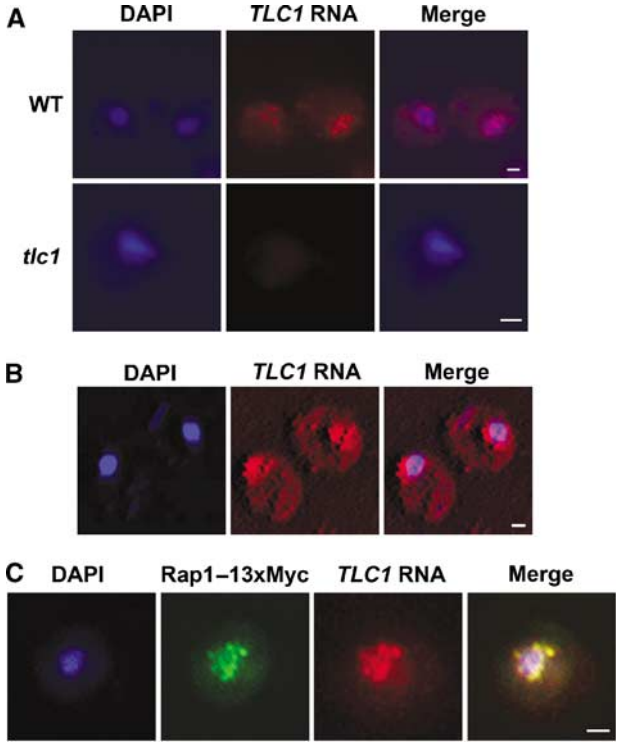

Figure 1 The *TLC1* RNA clusters into 8–10 foci inside the cell nucleus that colocalized with telomeres. (**A**) Detection of the endogenous form of the *TLC1* RNA in a WT strain (W303) using FISH assay. FISH against *TLC1* RNA in a *tlc1* strain was used as a control. DAPI, DNA staining. Scale bar = 1 μm. The same intensity levels were set for the WT and *tlc1* images. (**B**) FISH against *TLC1* RNA, followed by acquisition of a stack of 100 images reconstructed with the Metamorph program and merged with the DAPI signal. Scale bar = 1 μm. (**C**) Dual FISH against *TLC1* RNA and immunolocalization of the Rap1–13xMyc protein. Signals were deconvoluted, merged and colocalization was measured with the Metamorph program. Scale bar = 1 μm.

Est1–3 and yKu are essential for the nuclear accumulation of the TLC1 RNA

A deletion of any of the *EST* genes leads to progressive shortening of telomeres followed by growth arrest (Lundblad and Szostak, 1989). To investigate the contribution of these proteins to *TLC1* RNA sorting and telomerase holoenzyme biogenesis, the localization of this RNA was determined in strains lacking the *EST1–3* genes. As shown in Figure 2A, in strains lacking an *EST* gene, the probes for the *TLC1* RNA detected cytoplasmic foci with little signal associated with the nuclei. The proportions at which a cytoplasmic phenotype was observed are indicated in Figure 3 and reflect the penetrance of the phenotype in a global yeast population. These results show that the absence of any one of the Est proteins affected not only the activity of the telomerase but also the accumulation of the *TLC1* RNA in the nucleus. As Est1p and Est2p bind the *TLC1* RNA in the absence of each other (Hughes *et al*, 2000; Teixeira *et al*, 2002) and both accumulate in the nucleus when overexpressed (Zhou *et al*, 2000; Teixeira *et al*, 2002), overexpression of these proteins may promote the nuclear accumulation of the *TLC1* RNA by carrying this RNA in the nucleus. Therefore, overexpression of Est1 and Est2 proteins was performed to determine if they could rescue the cytoplasmic accumulation of *TLC1* RNA observed in the *est1*, *est2* and *est3* strains. As shown in Figure 3, overexpression of either *EST1* in an *est1* strain or *EST2* in an *est2* strain efficiently restored the nuclear phenotype of the *TLC1* RNA. Unexpectedly, both proteins compensated for the deletion of one another by restoring the nuclear localization of the *TLC1* RNA in the various knockout backgrounds (*EST2* in an *est1* strain or *EST1* in an *est2* strain). However, only the overexpression of *EST2* restored the nuclear localization of *TLC1* RNA in an *est3* strain (Figure 3). The reason why only Est2p and not Est1p overexpression suppresses the *est3* localization phenotype remains unclear.

Deletion of the yKu genes also leads to shorter telomeres and an alteration in the chromosomal DNA end structure, but these cells retain their ability to grow (Porter *et al*, 1996; Gravel *et al*, 1998). To determine the contribution of yKu in *TLC1* RNA nuclear sorting, FISH experiments were performed

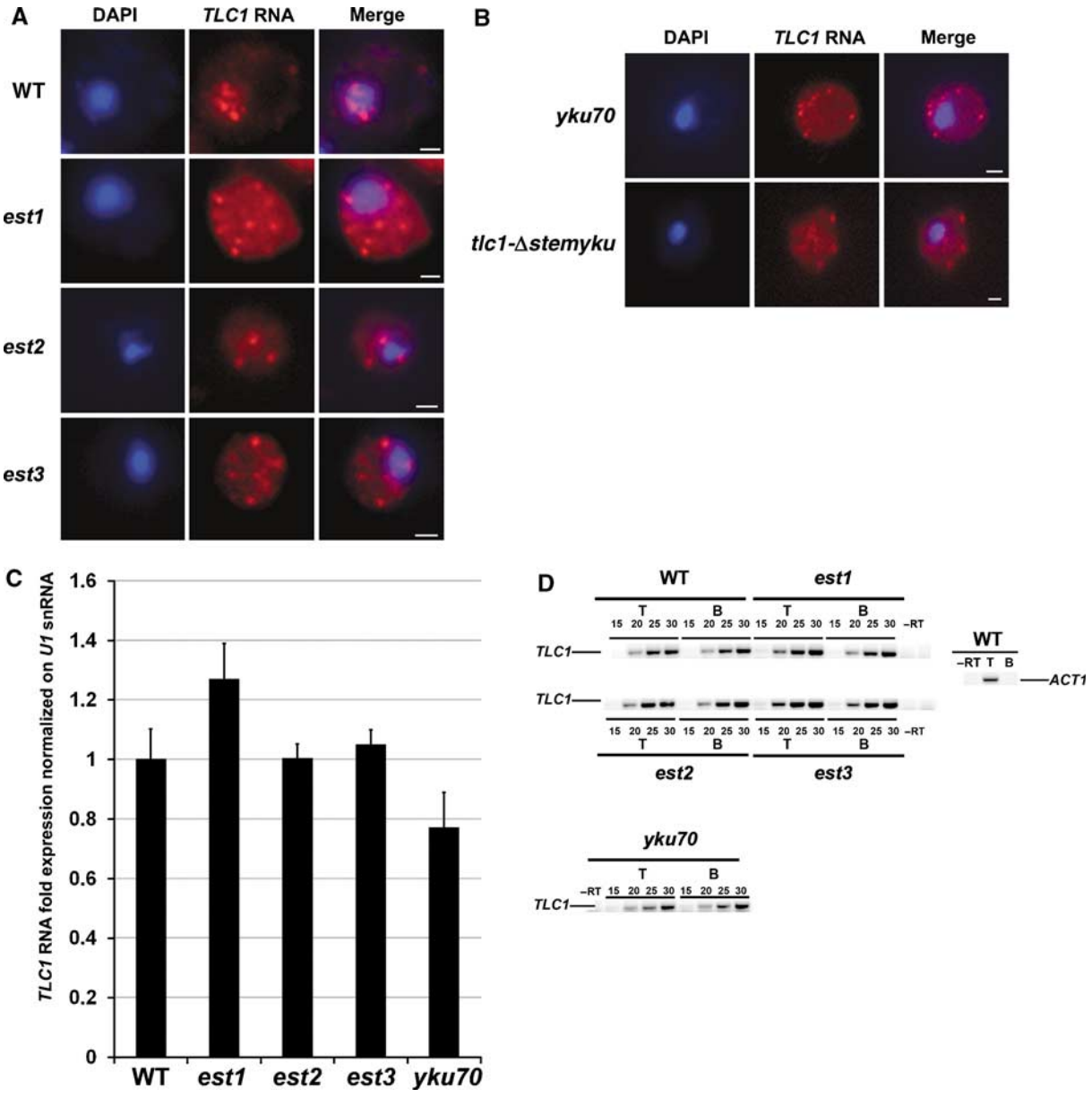

Figure 2 Deletion of *ESTs* or *YKU70* resulted in the cytoplasmic accumulation of the *TLC1* RNA. (**A**) FISH against *TLC1* RNA in WT (BY4742), *est1*, *est2* or *est3* strain. Scale bar = 1 μm. (**B**) FISH against *TLC1* RNA in *yku70* and a *tlc1-Δstemyku* strains. Scale bar = 1 μm. (**C**) *TLC1* RNA expression level measured by qPCR in the various strains. *TLC1* RNA level was normalized against that of *U1* snRNA and compared with the RNA levels in the WT strain (BY4742). (**D**) Level of TMG-capped *TLC1* RNA in the various strains measured by immunoprecipitation with an anti-TMG antibody followed by semiquantitative RT–PCR amplification of the *TLC1* RNA. Numbers indicate PCR cycles. T (total): RNA from total extract; B (bound): RNA immunoprecipitated with anti-TMG antibody. Anti-TMG immunoprecipitation and RT–PCR amplification (30 cycles) of *ACT1* mRNA served as a negative control.

on a *yku70* strain and demonstrated that the *TLC1* RNA was not retained in the cell nuclei in the absence of the yKu70/80p heterodimer (Figure 2B). To confirm this result, a strain expressing endogenous levels of a *TLC1* RNA deleted for the yKu70/80p binding stem (*tlc1-Δstemyku*) was used, and a phenotype similar to a *YKU70* deletion was observed (Figure 2B), suggesting that the binding of the *TLC1* RNA by the yKu70/80p heterodimer is necessary to ensure nuclear localization of the telomerase holoenzyme. Overexpression of *EST1* or *EST2* in a *yku70* strain could not bypass the requirement of yKu70p for the nuclear localization of *TLC1* RNA (Figure 3), suggesting that the *ESTs* and *yKU* have different roles in the nuclear localization of *TLC1* RNA.

To determine whether the *TLC1* RNA mislocalization is linked to a flawed processing or modification of *TLC1* RNA level, quantitative PCR (qPCR) experiments were performed to assess expression levels of the *TLC1* RNA in the various mutants. Deletion of either *EST* genes or *YKU70* did not significantly modify the expression level of the *TLC1* RNA (Figure 2C). To determine the state of maturation of the *TLC1* RNA found in the various mutants, immunoprecipitation of TMG-capped RNAs was performed, followed by RT–PCR of the *TLC1* RNA. The results in Figure 2D show that deletions of the *ESTs* and *YKU70* genes had little effect on the hypermethylation of the *TLC1* cap or on the modification of polyadenylation of this RNA (data not shown).

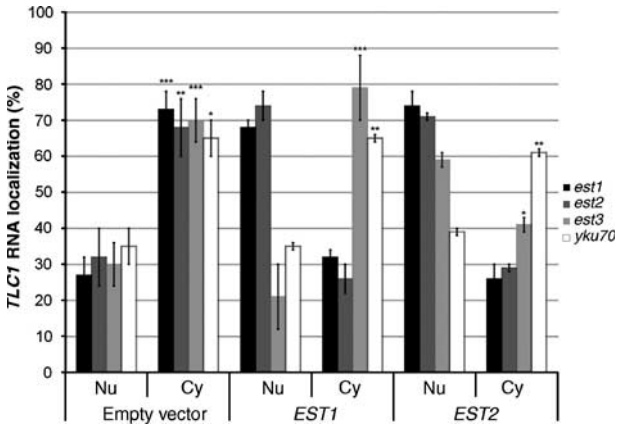

Figure 3 Overexpression of *EST1* or *EST2* in *ESTs* and *YKU70* knockout strains and their effect on the nuclear accumulation of the *TLC1* RNA. The *est1*, *est2*, *est3* or *yku70* strain was transformed with either the empty vector or a plasmid overexpressing *EST1* or *EST2*. The percentage of cells with *TLC1* RNA in the cytoplasm (Cy) or the nucleus (Nu) is shown. The data are from at least three independent experiments, where 100 cells were scored. $*P<0.01$, $**P<0.005$ and $***P<0.001$. Standard deviations compare mutants versus the WT strain (BY4742).

Association of TLC1 RNA with telomeres maintains its nuclear retention

The previous results show that at least two independent pathways may be involved in the nuclear retention of the *TLC1* RNA. One is dependent on the Est proteins, suggesting that proper assembly of the telomerase holoenzyme is required for its accumulation in the nucleus. The other pathway is dependent on the yKu heterodimer, which is involved in the recruitment of telomerase to the telomeres (Stellwagen *et al*, 2003; Fisher *et al*, 2004), suggesting that anchoring at the telomeres may be important for the nuclear retention of telomerase. To explore this possibility, mutants that affect the association of telomerase with telomeres were tested. Previous data have shown that the kinase Tel1p and the MRX complex, key mediators of DNA damage and DNA repair pathways, are both required for the recruitment of telomerase components to the telomeres (Goudsouzian *et al*, 2006). Using FISH, the distribution of *TLC1* RNA was determined in a *tel1* strain and in strains deleted of *MRE11* and *XRS2*, which encode two components of the MRX complex. As shown in Figure 4A, deletion of these genes resulted in a redistribution of *TLC1* RNA from the nucleus to the cytoplasm. Quantification of the phenotypes of these cells shows that the number of cells with *TLC1* RNA mostly in the nucleus dropped by two-fold, whereas the number of cells with *TLC1* RNA in both nucleus and cytoplasm, or mostly cytoplasmic, increased by over three-fold (Figure 4B). Combined with the results on yKu, these data suggest that nuclear retention of the telomerase holoenzyme requires its anchoring to telomeres.

Nuclear export of TLC1 RNA occurs by the Crm1p pathway

The previous experiments strongly suggest that the *TLC1* RNA has a cytoplasmic phase and thus must transit through the nuclear pore complex. To characterize the RNA export machinery required for the nuclear export of *TLC1* RNA, the distribution of this RNA was examined in strains harbouring different mutations affecting the Crm1p export pathway, the main export pathway for non-coding RNAs in yeast (Moy and Silver, 2002). Leptomycin B (LMB) was used to inhibit the binding of Crm1p to nuclear export signal-containing proteins in an LMB-sensitive strain (Y464) deleted of its *YKU70* gene, as the *TLC1* RNA was cytoplasmic in a *yku70* background. In this strain, the *TLC1* RNA accumulated in the cytoplasm before LMB exposure, but was sequestered in the nucleus following 2 h of LMB treatment (Figure 5A–C), indicating a defect in the export mechanism of *TLC1* RNA in the absence of Crm1p function. These results were confirmed using a thermosensitive form of Crm1p (*xpo1-1*) in a *yku70* background (Figure 5B and C), which demonstrates that Crm1p has a central role in the nuclear export of this RNA. Furthermore, we determined that *TLC1* RNA was exported independent of the rRNA and mRNA nuclear export machineries by using specific export mutants (Supplementary data, Supplementary Figure S1).

Hypermethylation of the 5′ cap of TLC1 *RNA requires the methyltransferase Tgs1p and occurs in the nucleolus, before the nuclear export of this RNA*

In metazoans, Crm1p is involved in the nuclear export of monomethyl guanosine (m^7G)-capped snRNAs, leading to their hypermethylation into a trimethyl guanosine (m_3G) cap in the cytoplasm before their re-import in the nucleus as snRNPs (Matera *et al*, 2007). Although the results presented above may suggest a similar pathway for the *TLC1* RNA, such a mechanism is still controversial for snRNA maturation in yeast (Hopper, 2006). To determine if the *TLC1* RNA is TMG-capped before or after its nuclear export, this RNA was retained in the nucleus by LMB treatment in the Y464*yku70* strain, immunoprecipitated with an anti-TMG antibody and detected by RT–PCR. As shown in Figure 5D, the *TLC1* RNA was detected in the pellet after immunoprecipitation, suggesting that the hypermethylation of this RNA occurs before its nuclear export. This result is supported by the observation that the *TLC1* RNA was properly capped in all *est* mutants, which blocks telomerase biogenesis at a cytoplasmic step (Figure 2D).

The modification of the m^7G cap of snRNAs and snoRNAs has been shown to depend on the methyltransferase Tgs1p in yeast (Mouaikel *et al*, 2002). To test the requirement of Tgs1p for the hypermethylation of the 5′ cap of *TLC1* RNA, an anti-TMG cap antibody was used to immunoprecipitate the *TLC1* RNA in WT and *tgs1* strains. The hypermethylated form of *TLC1* RNA can be immunoprecipitated normally in a WT strain, whereas the *TLC1* RNA can no longer be found in the bound fraction in a *tgs1* strain (Figure 6A), indicating that the *TLC1* 5′ cap is no longer hypermethylated in this mutant. Tgs1p is a nucleolar protein and a previous study has found that the deletion of *TGS1* resulted in the nucleolar accumulation of RNA substrates of this methyltransferase (Mouaikel *et al*, 2002). To determine if this cap methylation defect could have an impact on the localization of the *TLC1* RNA, FISH was performed against the *TLC1* RNA in a *tgs1* strain containing an Nop1–GFP marker to visualize the nucleolus. As shown in Figure 6B, blocking the hypermethylation of *TLC1* RNA cap induced a nucleolar accumulation of this transcript, suggesting that this maturation step of the *TLC1* RNA occurs in the nucleolus, as for other substrates of the Tgs1p.

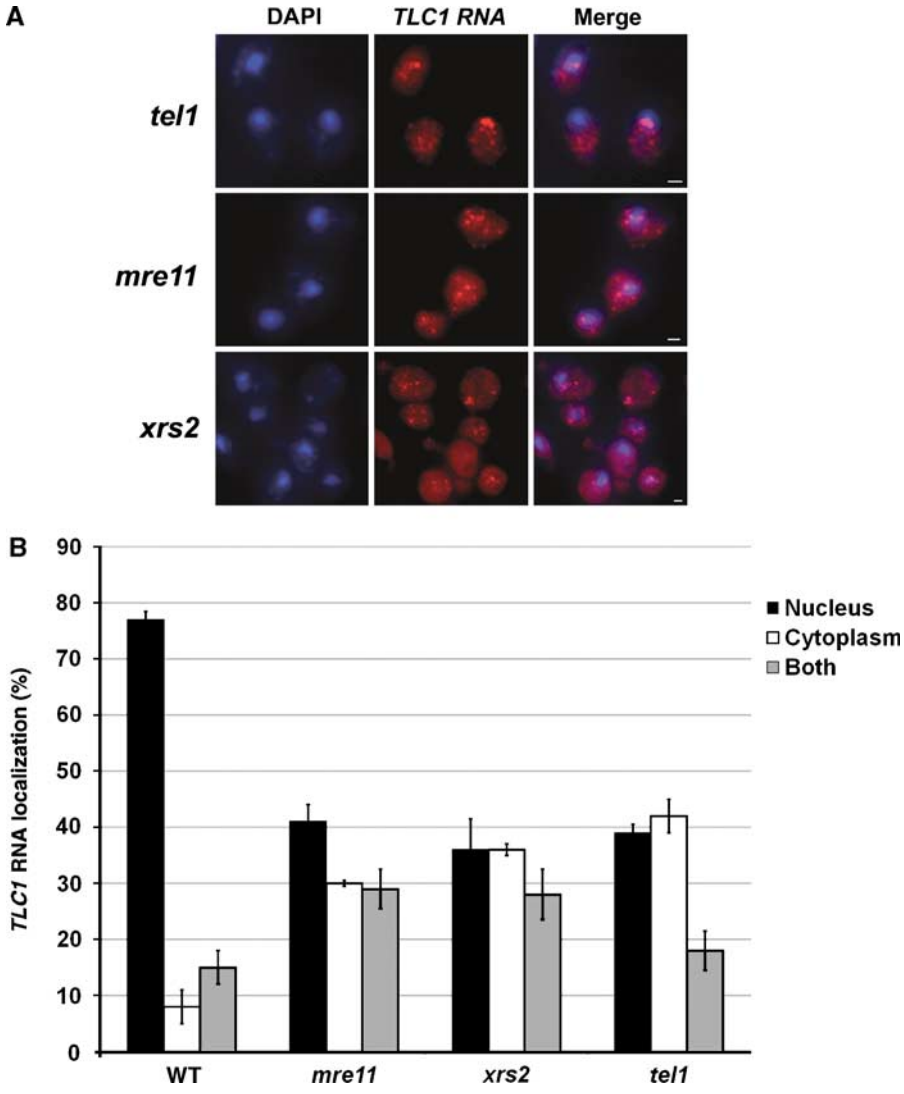

Figure 4 Deletion of *TEL1*, *MRE11* or *XRS2* decreases the nuclear accumulation of *TLC1* RNA. (**A**) FISH against *TLC1* RNA in *tel1*, *mre11* or *xrs2* strain. Scale bar = 1 μm. (**B**) Quantification of the *TLC1* RNA distribution observed in WT (BY4742), *tel1*, *mre11* or *xrs2* strain. A total of 100 cells were counted for each time point, in at least three independent experiments.

The TLC1 RNA shuttles between the nucleus and the cytoplasm, and requires the importins Mtr10p and Kap122p for its nuclear import

We have shown that the *TLC1* RNA can be found both in the nucleus and the cytoplasm of yeasts, and that its nuclear export is dependent on Crm1p. However, it is not clear if this RNA truly shuttles between these cellular compartments, or if the nuclear export observed resulted only from a defect in the anchoring of a misassembled telomerase complex at telomeres. Evidence that the *TLC1* RNA shuttles comes from the work of Teixeira and colleagues, which used an heterokaryon assay to show that overexpressed *TLC1* RNA was able to shuttle between nuclei (Teixeira *et al*, 2002). To determine if endogenous *TLC1* RNA can shuttle between the nucleus and the cytoplasm, an heterokaryon shuttling assay was also performed. A *tlc1* strain was mated with a strain containing a WT *TLC1* gene and a modified *kar1* allele (MS739). This allele allows mating of the two strains but prevents the fusion of their nuclei (Vallen *et al*, 1992). Heterokaryons were identified by morphologic criteria and the presence of two or more nuclei per cell. Even if these heterokaryons have WT alleles of the *ESTs* and *YKUs* genes, they are not like WT cells. If the *TLC1* RNA shuttles between the nucleus and the cytoplasm, it should be detected in every nucleus of an heterokaryon. As shown in Figure 7A, the endogenous *TLC1* RNA was able to shuttle from a nucleus to another in 47% of the heterokaryons observed. To eliminate the possibility that nonspecific leakage from the nucleus may be responsible for this phenotype, the non-shuttling nuclear protein Loc1p was used as a negative control in the heterokaryon assay (Long *et al*, 2001). When Loc1p–Myc was transiently expressed in the *kar1* strain before mating, it remained in only one nucleus in 100% of the heterokaryons observed (Supplementary Figure 2), suggesting that the nucleus was not leaky during the assay. Altogether, these experiments demonstrate that *TLC1* RNA normally shuttles between the nucleus and the cytoplasm.

It could be hypothesized that a shuttling *TLC1* RNA requires a component of the nuclear import machinery to ensure its normal nuclear localization. Previous data suggest that the importin Mtr10p is required for *TLC1* RNA biogenesis and nuclear accumulation (Ferrezuelo *et al*, 2002). To assess

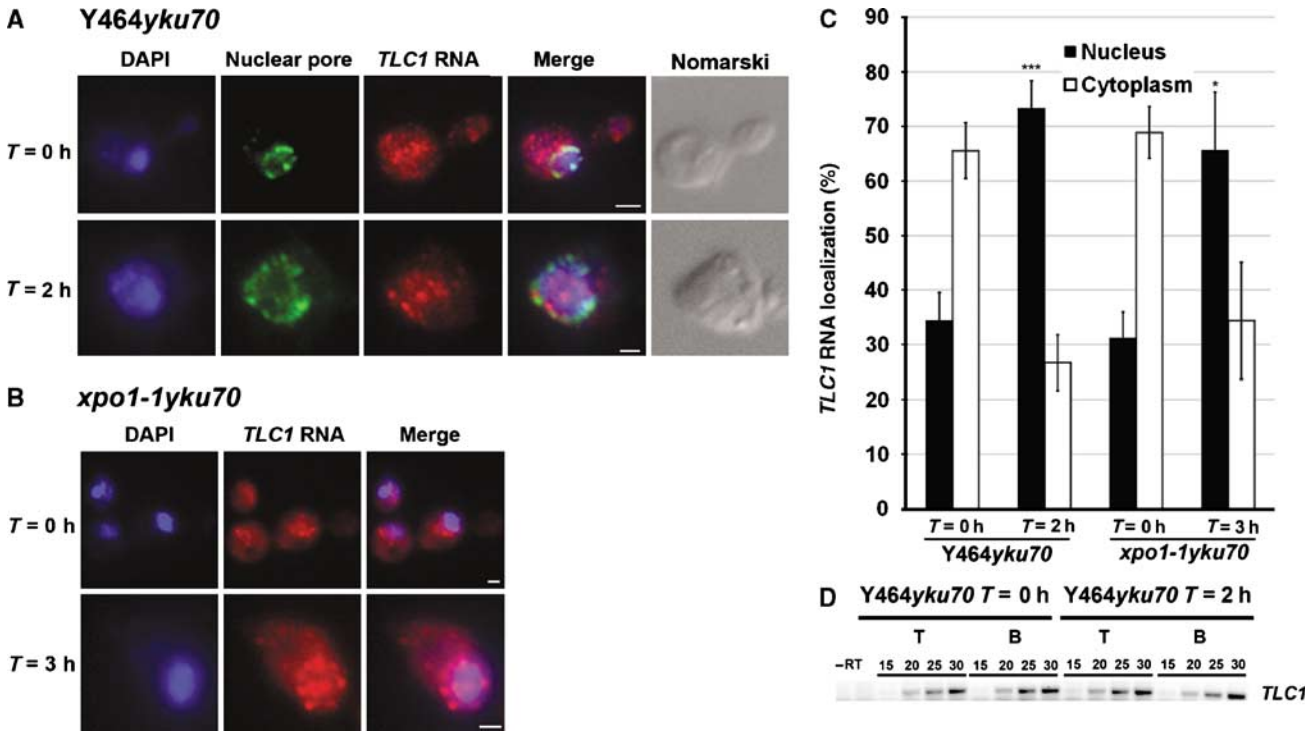

Figure 5 The *TLC1* RNA is exported from the nucleus through the Crm1p-dependent pathway. (**A**) The LMB-sensitive strain Y464*yku70* was treated with 100 ng/ml LMB for 0 or 2 h. IF against the nuclear pore complex was used to have the absolute position of the nuclear membrane. Scale bar = 1 μm. (**B**) The *xpo1-1 yku70* strain was grown at 30°C and shifted to 37°C for 3 h. FISH against the *TLC1* RNA was realized after 0 or 3 h at restrictive temperature. Scale bar = 1 μm. (**C**) Quantification of the *TLC1* RNA distribution observed in the Y464*yku70* and *xpo1-1 yku70* strains. A total of 100 cells were counted for each time point, in at least three independent experiments. $*P<0.01$ and $***P<0.001$. (**D**) Level of TMG-capped *TLC1* RNA in the Y464*yku70* strain after 0 or 2 h of LMB treatment as measured by immunoprecipitation with an anti-TMG antibody followed by semiquantitative RT–PCR amplification of the *TLC1* RNA. Numbers indicate PCR cycles. T: RNA from total extract; B: RNA immunoprecipitated with anti-TMG antibody.

the requirement of importin proteins for the nuclear import of *TLC1* RNA, a collection of importin deletion strains (either deletions or, if the gene is essential, conditional alleles) were screened for the localization of the *TLC1* RNA. Deletion of most importins did not induce a cytoplasmic accumulation of *TLC1* RNA (Figure 7B and C). However, deletion of *KAP122* and *MTR10* resulted in a significant reduction of the nuclear accumulation of the *TLC1* RNA (Figure 7B and C). Mtr10p-lacking cells express low levels of *TLC1* RNA (Ferrezuelo *et al*, 2002), a phenotype that was not observed with other mutants that promote cytoplasmic accumulation of this RNA (Figure 2C). Therefore, Mtr10p may affect *TLC1* RNA by a pathway that is independent of nuclear import. Indeed, overexpression of *TLC1* in an *mtr10* strain restored WT telomere length but did not increase its nuclear accumulation (Ferrezuelo *et al*, 2002), suggesting the presence of redundant nuclear import pathways for this RNA.

Discussion

Our study provides the first direct visualization in yeast cells of the endogenous telomerase RNA subunit, the *TLC1* RNA, of which there are only about 30 copies per cell (Mozdy and Cech, 2006). In G1 phase, *TLC1* RNA was found to cluster into 8–10 foci inside the cell nucleus, colocalizing with the bona fide telomeric protein Rap1p. Nuclear accumulation of the *TLC1* RNA depends on the proper assembly of the telomerase complex, as deleting any of the telomerase proteins (Est1–3) resulted in a mostly cytoplasmic accumulation of this RNA. Other studies have used FISH to detect the *TLC1* RNA in yeast cells (Ferrezuelo *et al*, 2002; Teixeira *et al*, 2002) and have provided evidence of a nucleo-cytoplasmic trafficking of the telomerase RNA. However, neither of these detected any defect in *TLC1* RNA distribution in *ESTs* knockout strains. As both studies used overexpressed *TLC1* RNA, this may result in the titration of a limiting factor essential for *TLC1* RNA nuclear export or for its processing (Chapon *et al*, 1997), thus promoting the nuclear retention of this RNA.

Our data on the *EST* genes suggest that part of the biogenesis of the telomerase holoenzyme occurs in the cytoplasm, which implies that the cytoplasmic *TLC1* RNA is associated with Est proteins. This is supported by a previous study that has shown that the deletion of *EST1* (which resulted in a cytoplasmic *TLC1* RNA; Figure 2A) did not affect the formation of the *TLC1*–Est2p complex *in vivo*, and vice versa (Hughes *et al*, 2000). Moreover, a *TLC1* RNA deleted of its Est1p-binding domain still maintains its association with Est2p *in vivo* (Osterhage *et al*, 2006). In the light of our results, this suggests that a cytoplasmic *TLC1* RNA is associated with Est proteins. A cytoplasmic assembly of telomerase could prevent the nuclear accumulation of misassembled telomerase complexes, which could behave like dominant negative enzymes. This may happen if the *TLC1* RNA is not properly folded and/or cannot interact with one of the Est proteins. Proper assembly of the telomerase holoenzyme may be monitored in the cytoplasm, and only mature and functional ribonucleocomplexes may be imported into the nucleus.

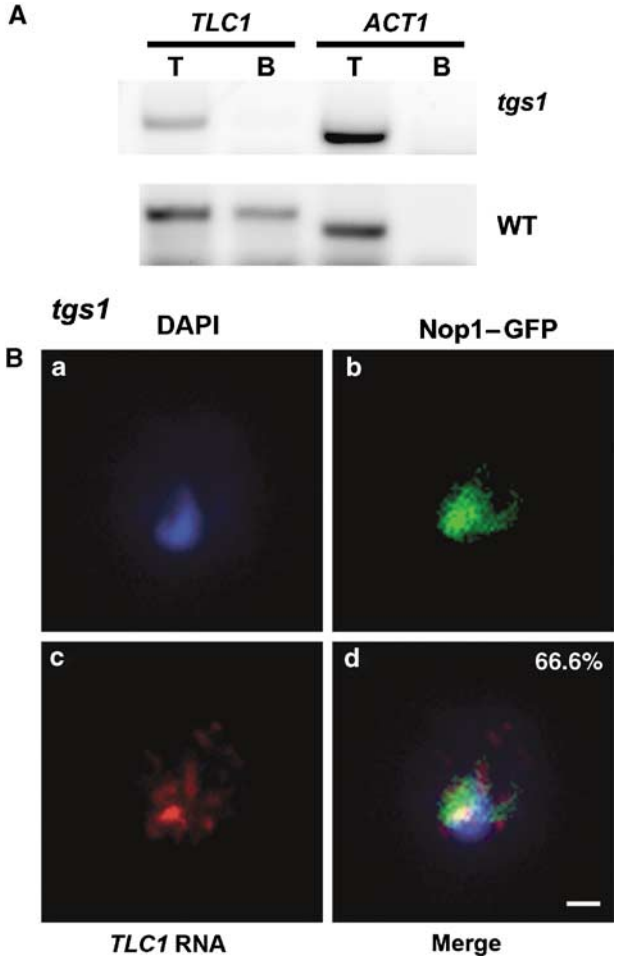

Figure 6 Tgs1p hypermethylates the 5′ cap of *TLC1* RNA in the nucleolus. (**A**) RT–PCR amplification of *TLC1* RNA from WT (W303) and *tgs1* strains. T: total RNA extract; B: immunoprecipitated TMG-capped *TLC1* RNA. The *ACT1* mRNA was used as a negative control. (**B**) Deletion of *TGS1* resulted in a nucleolar accumulation of the *TLC1* RNA. FISH experiment was performed against *TLC1* RNA in a *tgs1* strain. The Nop1–GFP was used to visualize the nucleolus. Scale bar = 1 μm. Number in panel d indicates the percentage of cells with this phenotype.

Another possibility is that only the mature telomerase holoenzyme can be retained in the nucleus by its recruitment at the telomeres. Indeed, our study also shows that the association of telomerase with telomeres is essential for maintaining this holoenzyme in the nucleus. First, disruption of *YKU70* or deletion of the stem–loop that binds the yKu70/80 heterodimer in the *TLC1* RNA results in a cytoplasmic *TLC1* RNA. As yKu is involved in the recruitment of telomerase to telomeres, this implies that tethering the telomerase to telomeres is essential for maintaining it in the nucleus. Without an association with telomeres, the telomerase possibly shuttles in and out of the nucleus by the Crm1p pathway and, at steady state, is mostly cytoplasmic. These data are supported by deletions of *TEL1* and of components of the MRX complex, both being involved in the recruitment of telomerase to telomeres, which result in a decreased nuclear accumulation of *TLC1* RNA. However, the decreased localization of *TLC1* RNA in the nucleus of *tel1*, *mre11* and *xrs2* strains is not as pronounced as in a *yku70* strain, possibly due to the role of yKu as a more direct mediator of the interaction between telomeres and the *TLC1* RNA (Stellwagen *et al*, 2003).

Based on the above results, a model for the trafficking of the telomerase can be suggested. (1) After being generated by RNA polymerase II, the *TLC1* RNA traverses the nucleolus where cap hypermethylation occurs. (2) This RNA is then exported to the cytoplasm by the Crm1p export pathway, where part of the assembly of the telomerase holoenzyme occurs (most likely the association of Est and the yKU proteins). (3) The assembled telomerase is re-imported into the nucleus by the Mtr10p or Kap122p importin pathways. (4) Nuclear retention of telomerase requires its association with the telomeres. Interestingly, the *TLC1* RNA remained nuclear in all the phases of the cell cycle in a WT background (data not shown). It would therefore appear that even if Est proteins are essential for *TLC1* RNA nuclear localization, variations in Est protein levels during the cell cycle do not disturb *TLC1* RNA nuclear accumulation. For instance, Est2p and *TLC1* RNA are present at the telomeres in G1 (Figure 1C; Taggart *et al*., 2002), when Est1p levels are extremely low (Osterhage *et al*, 2006). As the *TLC1* RNA has a long half-life (>1 h; Larose *et al*, 2007), it is possible that the *TLC1* RNA detected at the telomeres in G1 was synthesized during the previous mitosis and remained nuclear when the levels of Est1p decrease in G1. Indeed, our data suggest that it is during the biogenesis of telomerase (i.e., with newly synthesized *TLC1* RNA) that all Est proteins are required for *TLC1* RNA nuclear localization. This also fits with the observation that synthesis of *TLC1* RNA drops in G1 and increases in S phase (Chapon *et al*, 1997), like Est1p levels, suggesting a coordination between Est proteins and *TLC1* RNA for telomerase assembly and nuclear localization.

Finally, our data raise the possibility that in yeast, TMG-capped RNAs such as snRNAs, may also undergo a nucleocytoplasmic shuttling. Although there is some evidence of such shuttling (Olson and Siliciano, 2003), it is still controversial. Altogether, this study reveals another level of regulation of telomerase assembly and recruitment to telomeres, and provides a different perspective on how telomere homeostasis is achieved.

Materials and methods

Fixation of yeast cells

Fixation protocol of yeast cells has been described previously (Chartrand *et al*, 2000). All centrifugation steps were carried out at 4°C when not mentioned. The cells were grown in the appropriate media until they reached early- to mid-log phase (OD_{600} of 0.2–0.6) and then fixed with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 45 min at room temperature. The cells were harvested by centrifugation for 5 min at 3400 r.p.m. and washed twice with 10 ml of ice-cold 1 × buffer B (1.2 M sorbitol, 0.1 M potassium phosphate (pH 7.5)). The cells were resuspended in 1 ml of 1 × buffer B containing 20 mM vanadylribonucleoside complex (VRC), 28 mM β-mercaptoethanol, 0.06 mg/ml phenylmethylsulphonyl fluoride, 5 μg/ml of pepstatin, leupeptin and aprotinin and 120 U/ml RNA guard (all from Sigma) and transferred to a microtube containing dried oxalyticase produced from *Escherichia coli DH5α*. The cells were incubated at 30°C for 16–18 min to allow cell wall digestion and then harvested by centrifugation for 4 min at 3400 r.p.m. The cells were washed once with 1 ml of 1 × buffer B and resuspended in 750 μl of 1 × buffer B. A 100 μl portion of the spheroplasts was spotted on microscope coverslips coated with poly-L-lysine. The cells were incubated at 4°C for 30 min to allow the binding on the coverslip, washed once with 1 × buffer B and dehydrated in 70% ethanol in DEPC-treated water. The cells were kept at −20°C until fluorescence microscopy experiments were conducted.

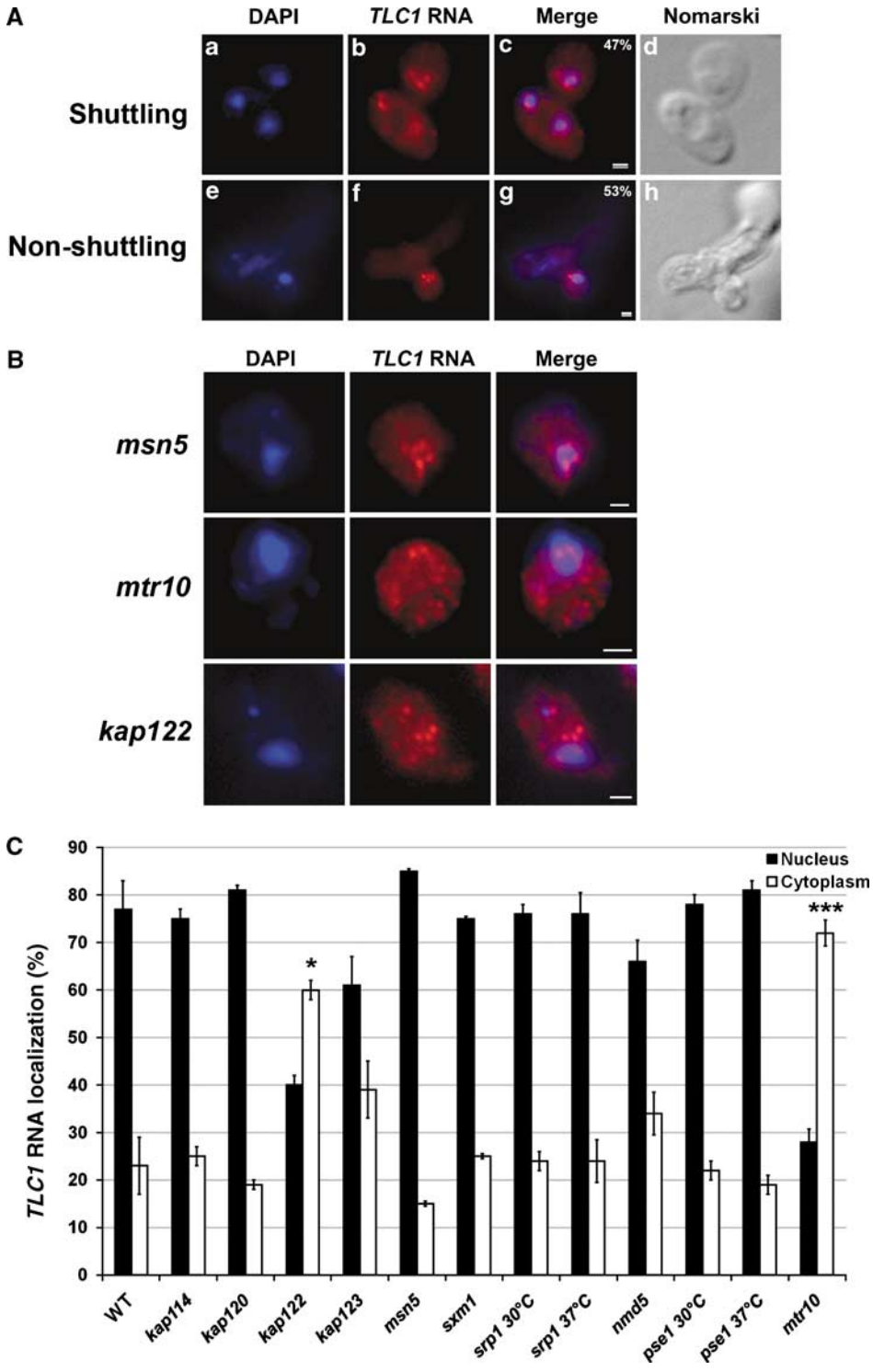

Figure 7 The *TLC1* RNA shuttles between the nucleus and the cytoplasm. (**A**) Heterokaryon shuttling assay. Heterokaryons were created by mating a *Mat*a strain deleted of the *TLC1* gene with a *Mat*α strain carrying the *kar1-1* allele and a WT *TLC1* gene (MS739). Upon mating and FISH, the distribution of the *TLC1* RNA in the nuclei of heterokaryons was determined. Although 47% of the heterokaryons contained *TLC1* RNA in every nucleus, indicating that this RNA was shuttling between the nuclei (a–c), 53% of the heterokaryons still contained *TLC1* RNA restricted to one nucleus (e–g). (**B**) Deletion of *KAP122* and *MTR10* resulted in the cytoplasmic accumulation of the *TLC1* RNA. The *TLC1* RNA showed a normal nuclear localization in the *msn5* strain. Scale bar = 1 μm. (**C**) Quantification of the *TLC1* RNA distribution observed in the various importin alpha and beta mutant strains. Distribution at permissive (30°C) and restrictive (37°C) temperature is shown for the temperature-sensitive mutants *pse1-1* and *srp1-31*. WT strain is BY4742. $^{*}P<0.01$ and $^{***}P<0.001$.

Fluorescence microscopy of fixed yeast cells and signal detection

FISH experiments using oligonucleotide DNA probes have been described previously (Chartrand *et al*, 2000). Following fixation of the yeasts, the cells were rehydrated with two washes of 2 × SSC for 5 min at room temperature. The coverslips were incubated with 2 × SSC 40% formamide for 5 min at room temperature. In parallel, 10 μl of a mix of *TLC1* probes (1 ng/μl) was mixed with 4 μl of a

5 mg/ml solution of 1:1 sonicated salmon sperm/*E. coli* tRNA (Sigma). The mix was lyophilized in a speed vacuum. The pellet was resuspended in 12 µl of 80% formamide and 10 mM sodium phosphate (pH 7.0). The 12 µl probe solution was heated at 95°C for 5 min and 12 µl of 4 × SSC, 20 mM VRC, 4 µg/µl BSA and 50 U of RNA guard was added. The probe preparation was finally dropped on a parafilm sheet and the coverslips were placed on the drop face-down. Hybridization was then carried out overnight at 37°C. Following hybridization, the cells were washed sequentially twice with 2 × SSC 40% formamide for 15 min at 37°C, once with 2 × SSC 0.1% Triton X-100 and twice with 1 × SSC. The coverslips were incubated in 1 × PBS containing diamidino phenylindole (DAPI) for 2 min and mounted on the microscope slides with mounting medium (86% glycerol, 1 mg *p*-phenylene diamine, 1 × PBS).

Dual FISH and IF protocols were conducted as described previously (Chartrand *et al*, 2000). Following the last wash of 1 × SSC of the FISH protocol, the cells were incubated with 1 × PBS 0.1% BSA, 1 × PBS 0.1% BSA 0.1% NP-40 (Sigma) and 1 × PBS 0.1% BSA for 5 min each at room temperature. The primary antibody was diluted to the appropriate concentration (ranging from 1/400 to 1/2500) in 1 × PBS containing 0.1% BSA, 20 mM VRC and 120 U/ml RNA guard. Mab414 αFG Nup (Abcam) was used to detect the position of nuclear pores. 9E10 anti-Myc was purchased from Roche. Hybridization was carried out from 2 h to overnight at 37 and 4°C, respectively. The cells were washed sequentially with 1 × PBS containing 0.1% BSA, 1 × PBS containing 0.1% BSA and 0.1% NP-40 and finally 1 × PBS containing 0.1% BSA for 15 min at room temperature. The secondary antibody was diluted in 1 × PBS containing 0.1% BSA, 20 mM VRC and 120 U/ml RNA guard (usually 1/1000) and incubated at room temperature for 60–90 min in the dark. The cells were washed with 1 × PBS containing 0.1% BSA, 1 × PBS containing 0.1% BSA and 0.1% NP-40 and finally 1 × PBS containing 0.1% BSA. The coverslips were finally incubated for 2 min in 1 × PBS containing DAPI and mounted.

Image acquisition, deconvolution and processing

All the images were acquired using a Nikon Eclipse E800 epifluorescence microscope equipped with a Nikon × 100 DIC H (1.4 NA) lens and with a Photometrics CoolSNAP fx CCD camera. Images were acquired using the Metamorph software and processed with Adobe Photoshop. For the deconvolution experiments, images were imported into the AutoDeblur software and the background was removed by autodetection. Deconvolution was performed using a PSF theoretical algorithm, ranging from 10 to 60 iterations every channel. Deconvoluted channels were then overlaid and the level of colocalization was determined using the Metamorph program. The very low level of the *TLC1* RNA does not allow the use of Photoshop scripts to process the pictures and intensity levels were adjusted manually to allow processing.

Heterokaryon-based nucleo-cytoplasmic shuttling assay

The *tlc1* and *TLC1 kar1-1* strains were grown in YEPD to an OD_{600} of 0.5. A 25 ml portion of *tlc1* strain was mated with 12.5 ml of *TLC1 kar1-1* strain in 150 ml of YEPD. After 5 h of mating, the cells were fixed and FISH experiment was performed for the *TLC1* RNA. For plate mating, 5 ml of *tlc1* strain and 2.5 ml of *TLC1 kar1-1* strain were harvested by centrifugation, washed twice with YEPD and resuspended in 300 µl of YEPD. The cells were mixed in a microtube and 200 µl was spotted on a YEPD plate. After adsorption (15 min), the plates were placed at 30°C for 5 h. After this, the cells were recovered with a cell scraper (Fisher), diluted in 50 ml of YEPD and fixed. FISH experiment was carried out as described.

As a negative control, the *kar1-1* strain was transformed with pRL134 (pGal-*LOC1-6xMyc*; kindly provided by Roy Long). Transformants were selected and grown overnight at 30°C in SD media containing 2% raffinose. Cells were diluted at OD_{600} 0.2 in 50 ml of SD media containing 2% galactose. Induction of Loc1p–Myc was carried out at 30°C for 2 h. After induction, expression of Loc1–Myc was repressed by adding glucose to a final concentration of 2% for 2 h at 30°C. *kar1-1* + pRL134 cells were mated with *tlc1* cells and processed as above.

Quantitative RT–PCR analysis

Total RNA was extracted from yeast strains using the yeast RNA miniprep protocol as described previously (Schmitt *et al*, 1990). A 2 µg portion of total RNA was reverse-transcribed using *ACT1* mRNA-, *U1* snRNA- and *TLC1* RNA-specific primers. Total RNA was resuspended in DEPC-treated water containing 2 pmol of gene-specific primers and 1 µl of 25 mM dNTP to 12 µl. The samples were heated for 5 min at 65°C and chilled on ice rapidly. A 4 µl volume of 5 × RT–PCR buffer (250 mM Tris–HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 2 µl of 1 M DTT and 1 µl of RNA guard were added to the reaction. The samples were preheated for 2 min at 42°C and 1 µl (200 U) of Revert-aid M-MuLV-RT was added to start the reaction. The samples were reverse-transcribed for 50 min at 42°C. Subsequently, gene expression level was determined using primer and probe sets from the Universal Probe Library (Roche Scientific; https://www.roche-applied-science.com). PCRs for 384-well plate formats were performed using 2 µl of cDNA samples (50 ng), 5 µl of TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA), 2 µM of each primer and 1 µM of the Universal TaqMan probe in a total volume of 10 µl. The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level programmed to an initial step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All the reactions were run in triplicate and the average values were used for quantification. The *ACT1* or *snR19* (*U1* snRNA) was used as an endogenous control. The relative quantification of target genes was determined using the rCT method. Briefly, the C_t (threshold cycle) values of target genes were normalized to an endogenous control gene (*ACT1* or *snR19*) (rCT = C_t target − C_t *ACT1* or *snR19*) and compared to each other.

Immunoprecipitation of TMG-capped RNA

The total RNA was extracted using the yeast RNA miniprep protocol as described previously (Schmitt *et al*, 1990). A 60 µg portion of total RNA was resuspended in 150 µl of DEPC-treated water and 2 µl of anti-TMG antibody (Calbiochem) was added. Extracts were incubated on a roller drum at 4°C for 2 h. Following this, 40 µl of protein-A Sepharose beads was added and the immunoprecipitation was incubated at 4°C on a roller drum for 2 h. The extracts were then spun for 4 min at 4500 r.p.m. at 4°C and washed three times for 4 min with washing buffer (25 mM HEPES (pH 7.5), 35 mM potassium chloride, 2 mM $MgCl_2$). A 200 µl volume of elution buffer (50 mM Tris (pH 8), 100 mM NaCl, 20 mM EDTA, 1% SDS) was added and incubated at 65°C for 15 min or at 95°C for 5 min. The immunoprecipitated RNA was extracted in phenol–chloroform and subjected to ethanol precipitation. The pellets were resuspended in 45 µl of DEPC-treated water and 5 µl of DNase I digestion buffer was added, following which 1 µl of DNase I was added and the samples were incubated for 1 h at 37°C to eliminate DNA contamination. The RNA was extracted with 1 volume of phenol–chloroform and ethanol precipitated. The pellet was resuspended in 30 µl of DEPC-treated water and 3 µl was used for RT–PCR. The same protocol was used for the amplification of total RNA without the antibody and bead incubation, and 1.5 µl of RNA was used in RT–PCR. PCR amplification of the reverse-transcribed product was performed for 15, 20, 25 or 30 cycles and analysed on 1.5% agarose gel.

Supplementary data

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

Acknowledgements

We thank the laboratories of P Silver, P-E Gleizes, E Hurt, R Long, V Lundblad and J Vogel for providing yeast strains and plasmids. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to PC and grant MOP74438 from the Canadian Institutes for Health Research to RJW. PC was supported by fellowships from the Fond de Recherche sur la Nature et les Technologies du Québec and the Fond de Recherche en Santé du Québec.

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