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TOR regulates late steps of ribosome maturation in the nucleoplasm via Nog1 in response to nutrients

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The protein kinase TOR (target of rapamycin) controls several steps of ribosome biogenesis, including gene expression of rRNA and ribosomal proteins, and processing of the 35S rRNA precursor, in the budding yeast *Saccharomyces cerevisiae*. Here we show that TOR also regulates late stages of ribosome maturation in the nucleoplasm via the nuclear GTP-binding protein Nog1. Nog1 formed a complex that included 60S ribosomal proteins and pre-ribosomal proteins Nop7 and Rlp24. The Nog1 complex shuttled between the nucleolus and the nucleoplasm for ribosome biogenesis, but it was tethered to the nucleolus by both nutrient depletion and TOR inactivation, causing cessation of the late stages of ribosome biogenesis. Furthermore, after this, Nog1 and Nop7 proteins were lost, leading to complete cessation of ribosome maturation. Thus, the Nog1 complex is a critical regulator of ribosome biogenesis mediated by TOR. This is the first description of a physiological regulation of nucleolus-to-nucleoplasm translocation of pre-ribosome complexes.

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Introduction

Ribosome biosynthesis involves the synthesis, maturation and assembly of rRNAs and ribosomal proteins (RPs) (Kressler *et al*, 1999; Venema and Tollervey, 1999; Lafontaine and Tollervey, 2001). In eukaryotes, 5.8S, 18S and 25S rRNAs are transcribed as a single, large 35S precursor consisting of noncoding sequences and then modified (by methylation and pseudouridylation) and digested by nucleases. RPs are synthesized in the cytoplasm and are imported into the nucleus and targeted to the nucleolus, where they are first assembled with other RPs and rRNA, forming a large 90S pre-ribosome complex containing components of 60S and 40S ribosomes. After processing, this complex is separated into 66S (precursor of 60S ribosome) and 43S (precursor of 40S ribosome) intermediates and they are relocated to the nucleoplasm as assembly progresses. Subsequently, the RPs/rRNA complex is exported from the nucleus through nuclear membrane pores, and finally undergoes late stages of maturation in the cytoplasm. Numerous nucleolar/nuclear proteins are involved in these pathways (Fromont-Racine *et al*, 2003; Tschochner and Hurt, 2003).

Ribosome biogenesis is a highly energy-consuming process that stops upon nutrient starvation. It is regulated by the conserved protein kinase TOR (target of rapamycin), a gigantic ATM-family protein. TOR was first identified in the budding yeast *Saccharomyces cerevisiae*, but its orthologs are widely distributed in eukaryotic organisms, including humans. *S. cerevisiae* has two highly homologous TOR proteins: Tor1 and Tor2 (Kunz *et al*, 1993; Helliwell *et al*, 1994). The yeast TOR regulates various cellular events, including ribosome biogenesis, in response to nutrient availability (Schmelzle and Hall, 2000; Rohde *et al*, 2001; Crespo and Hall, 2002). TOR upregulates transcription of rRNA and mRNA for RPs in both yeast and mammals (Zaragoza *et al*, 1998; Cardenas *et al*, 1999; Hardwick *et al*, 1999; Powers and Walter, 1999; Hannan *et al*, 2003; Tsang *et al*, 2003; Claypool *et al*, 2004; Mayer *et al*, 2004). In addition, the specific TOR inhibitor rapamycin rapidly inhibits an early step in ribosome maturation, 35S rRNA processing, in yeast (Powers and Walter, 1999), indicating that TOR also controls ribosome maturation steps in the nucleolus. However, it is unknown whether late steps of ribosome maturation in the nucleoplasm are physiologically regulated and whether TOR is involved in the regulation.

Here we show that the late stage of ribosome maturation in the nucleoplasm also requires TOR activity because of the effect of TOR on the nuclear GTP-binding protein Nog1. Upon nutrient starvation or rapamycin treatment, Nog1-containing complex is tethered to the nucleolus, and intranuclear transport of pre-60S complexes is consequently inhibited. We propose that the Nog1 complex is a critical regulator of ribosome maturation in response to nutrient availability.

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Results

Nog1 is entrapped in the nucleolus by TOR inactivation

It has been reported that TOR controls rRNA expression by controlling the localization of RNA polymerase I, as indicated by the fact that RNA polymerase I is dispersed from the nucleolus to the whole nucleus upon rapamycin treatment (Tsang *et al*, 2003). Thus, TOR activity is required for the nucleolar localization of RNA polymerase I. In addition, it is known that TOR controls the processing of rRNA (Powers and Walter, 1999). Therefore, we suspected that TOR also regulates pre-ribosomal proteins involved in ribosome maturation in the nucleolus and the nucleoplasm. To test this idea, we examined the effects of rapamycin on the localization of various pre-ribosomal proteins. Among them, we found that the GTP-binding protein Nog1 (Park *et al*, 2001) was specifically relocated in response to rapamycin. Nog1 is essential for viability and is conserved from yeast and humans (see Supplementary Figure 3). Nog1 was localized throughout the nucleus (Figure 1A, control), as reported by a proteome-wide project (Huh *et al*, 2003) (data are available on the web; http://yeastgfp.ucsf.edu/). This distribution was quite different from that of the nucleolar marker Nop1. Thus, Nog1 is localized in the nucleus, and not only in the nucleolus, consistent with the notion that Nog1 is involved in both early and late stages of 60S ribosome maturation (see below). However, Nog1 was immediately concentrated in the nucleolus upon rapamycin treatment (Figure 1A, rapamycin, see also Figure 1B). This effect was abrogated when the rapamycin-resistant mutant *TOR1-1* strain was used (Supplementary Figure 1), demonstrating that this nucleolar concentration of Nog1 by rapamycin specifically results from TOR inactivation.

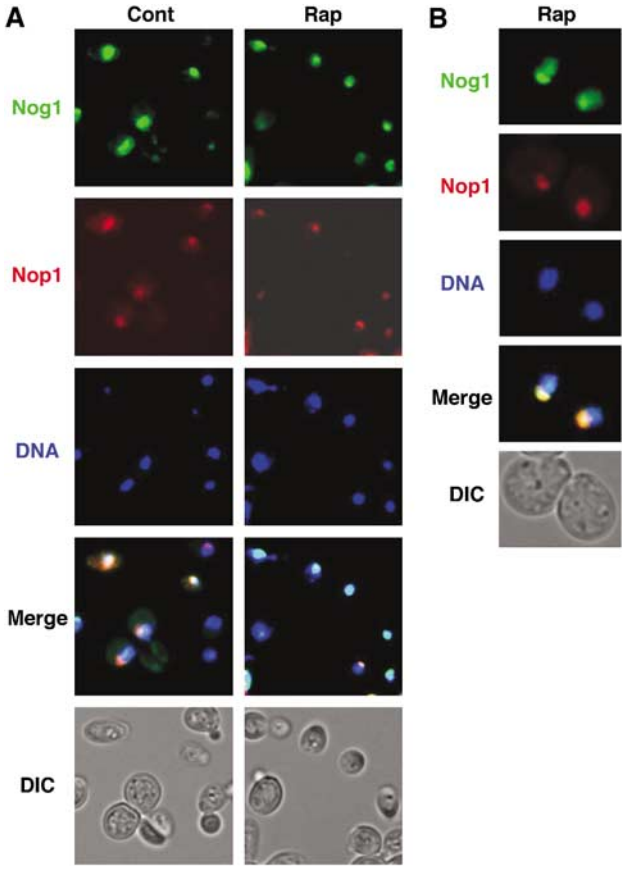

Figure 1 TOR inactivation causes Nog1 accumulation in the nucleolus. (**A**) Nog1 is accumulated in the nucleolus upon rapamycin treatment. Cells of a strain expressing *NOG1-GFP* (SCU888) with plasmid pSCU632 (pDsRed-NOP1) were treated with rapamycin (200 ng/ml) for 30 min (Rap). GFP (green), DsRed (red) and DAPI (blue) signals were observed in cells. Nomarski images (DIC) were also recorded. Cells not treated with rapamycin were used as the control (Cont). (**B**) Enlarged images of rapamycin-treated cells.

A similar nucleolar concentration was observed upon nitrogen and carbon starvation treatments (Figure 2). We called this event 'STING' (*st*arvation-*i*nduced *Nog*1 entrapment in the nucleolus). Conversely, when depleted nutrients were returned to the starved cells, Nog1 again became dispersed throughout the nucleus (Figure 2). Thus, Nog1 localization in the nucleus is reversibly regulated depending on carbon and nitrogen availabilities. In addition, the re-distribution of Nog1 throughout the nucleus after the addition of nutrients to the starved cells was completely inhibited by rapamycin (Figure 2). These findings demonstrate that TOR activity is required for the liberation of Nog1 from the nucleolus under favorable nutrient conditions and that TOR inactivation causes STING. STING is definitely caused by nutrient starvation, and is not merely a result of cessation of 60S ribosome production *per se*, as shown by the following observations. First, heat shock and hypertonic stress, which repress ribosome biogenesis (Ghoshal and Jacob, 1996; Van Nieuwenhoven *et al*, 2001) (Nanduri and Tartakoff, 2001), did not cause STING (Supplementary Figure 2A and B). Hypertonic stress causes relocation of various nucleolar proteins to the cytoplasm (Nanduri and Tartakoff, 2001). Similarly, Nog1 was relocated to the cytoplasm in response to hypertonic stress in some cells. Second, defects in different steps of 60S ribosome biogenesis in the nucleus in temperature-sensitive mutant strains *noc1-6* (early step) and *noc3-1* (late step) (Milkereit *et al*, 2001) failed to cause Nog1 accumulation in the nucleolus (Supplementary Figure 2C).

Nog1 is involved in the late stages of ribosome biogenesis in the nucleoplasm

Nog1 is essential for 60S ribosome maturation, at least for 35S rRNA processing, an early step of ribosome biosynthesis (Jensen *et al*, 2003; Kallstrom *et al*, 2003; Saveanu *et al*, 2003). However, the fact that Nog1 is distributed throughout the nucleus suggests that Nog1 is involved in late-stage maturation of the 60S ribosome in the nucleoplasm. In fact, Nog1 is associated with the Arx1 complex, a complex involved in the late stages of 60S ribosome maturation in the nucleoplasm, and with the nuclear pore complex (Rout *et al*, 2000; Allen *et al*, 2001; Nissan *et al*, 2002). In contrast, Nog1 is not found in the Kre35 complex, which is the last pre-60S complex to be distributed in the cytoplasm (Nissan *et al*, 2002). If the function of the pre-ribosomal proteins involved in the late stages of 60S ribosome maturation in the nucleoplasm is blocked, the green fluorescent protein (GFP)-tagged 60S RP Rpl25 (Rpl25-GFP) is accumulated in the nucleus, and not only in the nucleolus (Gadal *et al*, 2001; Milkereit *et al*, 2001). Three thermosensitive mutants of *NOG1* (*nog1-ts*), *nog1-11*, *nog1-12* and *nog1-13*, were isolated using the plasmid-shuffling method (see Materials and methods) (Figure 3A). They have several mutation sites at the con-

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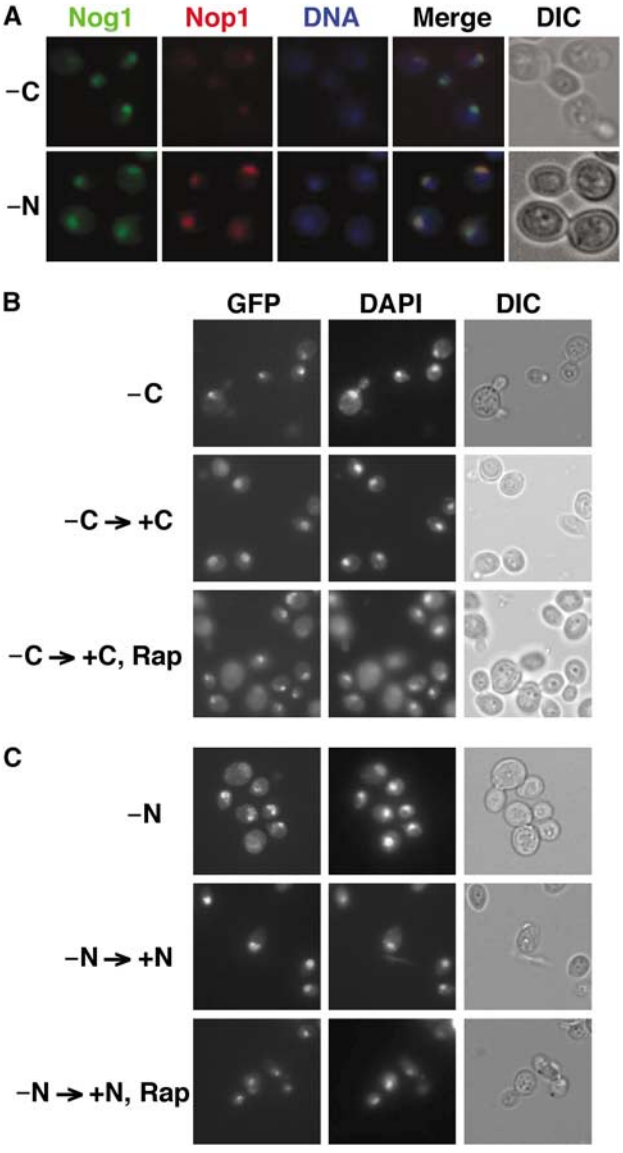

Figure 2 Nutrient starvation causes Nog1 accumulation in the nucleolus. (**A**) Cells of a strain expressing *NOG1-GFP* (SCU888) with plasmid pSCU632 (pDsRed-NOP1) were transferred to carbon- (−C) or nitrogen-deprived (−N) medium for 30 min (−C). GFP (green), DsRed (red) and DAPI (blue) signals were observed in cells. (**B**) Nog1 is accumulated in the nucleolus upon carbon starvation. Cells of a strain expressing *NOG1-GFP* (SCU888) were transferred to carbon-deprived medium for 30 min (−C) and then re-supplemented with carbon in the presence (−C→ + C, Rap) or absence (−C→ + C) of rapamycin for 60 min. GFP and DAPI signals were observed in cells. (**C**) Nog1 is accumulated in the nucleolus upon nitrogen starvation. The same strain was transferred to nitrogen-deprived medium for 30 min (−N) and then re-supplemented with nitrogen in the presence (−N→ + N, Rap) or absence (−N→ + N) of rapamycin for 60 min. GFP and DAPI signals were observed in cells.

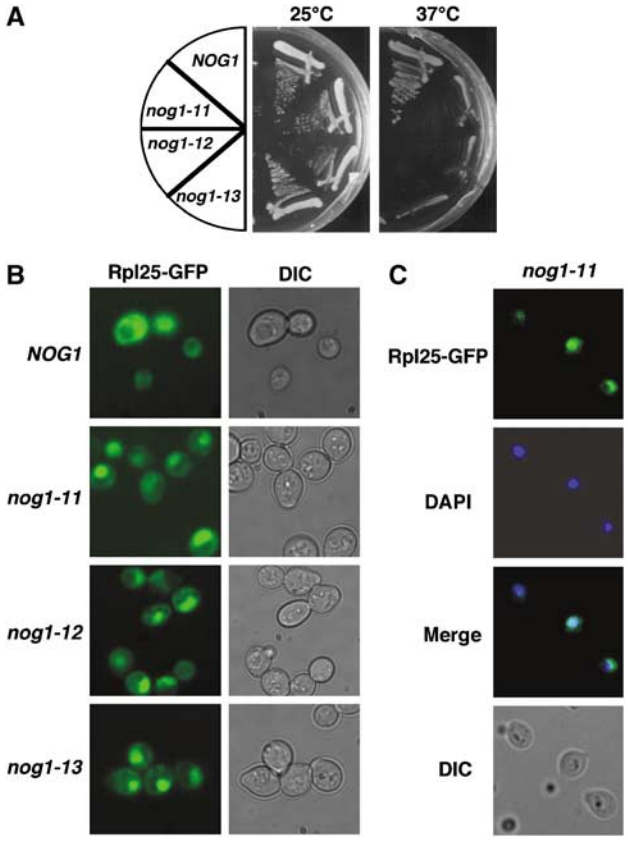

Figure 3 Pre-60S complex was accumulated in the nucleus as a result of Nog1 dysfunction. (**A**) Temperature-sensitive (ts) growth phenotype of *nog1-ts* mutants. The wild-type *NOG1* (SCU801) and *nog1* temperature-sensitive *nog1-11* (SCU823), *nog1-12* (SCU824) and *nog1-13* (SCU825) cells were incubated on plates at 25°C for 3 days or at 37°C for 2 days. (**B**) Pre-60S complex was accumulated in the nucleus of *nog1* temperature-sensitive mutants. Strains *NOG1* (SCU783), *nog1-11* (SCU823), *nog1-12* (SCU824) and *nog1-13* (SCU825), which harbor a plasmid expressing GFP-Rpl25 (pSCU615), were incubated at 37°C for 8 h and GFP signals were observed. (**C**) Strain *nog1-11* (SCU823) harboring plasmid pGFP-RPL25 (pSCU615) was incubated at 37°C for 5 h and stained for GFP (Rpl25; green) and DAPI (blue).

served amino acids (Supplementary Figure 3). Rpl25-GFP was accumulated in the nucleus, but not in the nucleolus, in these *nog1-ts* cells at restrictive temperatures (Figure 3B and C), as reported previously using Nog1-depleted cells (Saveanu *et al*, 2003). This nucleolar accumulation of Rpl25-GFP in *nog1-ts* cells is similar to that of *noc3-1*, which is defective in the late step of 60S ribosome biogenesis in the nucleoplasm, but not that of *noc1-1*, which is defective in the early step of 60S ribosome biogenesis in the nucleolus (Milkereit *et al*, 2001). Thus, Nog1 seems to be involved in 60S ribosome biogenesis at various stages up to the export of 60S pre-ribosomal particles from the nucleus. The notion that Nog1 is also required for 60S ribosome biogenesis in the nucleoplasm suggests that this maturation in the nucleoplasm would be largely repressed by Nog1 entrapment in the nucleolus, when STING occurs. Interestingly, it was reported that Rpl25-GFP was concentrated in the nucleolus in another *nog1-ts* mutant, *nog1-3* (Kallstrom *et al*, 2003), consistent with the roles of Nog1 in the early step of 60S ribosome maturation in the nucleolus. The mutant cells might be mainly defective in the early step of the dual roles of Nog1.

TOR regulates the nucleolus-to-nucleoplasm transfer of 60S pre-ribosomes

A number of 60S RPs are associated with Nog1 (Saveanu *et al*, 2003). When Nog1 is tethered to the nucleolus by TOR inactivation, are these 60S RPs still associated with Nog1? If so, transfer of the 60S pre-ribosomal complex from the nucleolus to the nucleoplasm should be completely inhibited.

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Alternatively, Nog1 may be specifically dissociated from the precursor and may stay at the nucleolus, and the 60S ribosome maturation in the nucleoplasm would still continue without Nog1 after TOR inactivation. To discriminate between these possibilities, we purified the Nog1 complex from extracts of cells treated with and without rapamycin by the tandem affinity purification (TAP) protocol (Puig *et al*, 2001) and compared the components. Nog1 was previously purified from cells grown under favorable nutrient conditions, and a large number of Nog1-associated ribosomal and pre-ribosomal proteins were reported (Saveanu *et al*, 2003). However, these proteins include ones present at stoichiometrically much lower levels as compared with Nog1, for example the nucleolar protein Nop1, which may be transiently and/or weakly associated with Nog1. In order to seek for proteins tightly binding to Nog1, namely the proteins contained in the same complex with Nog1, and to see the effects of rapamycin on them, we examined gels with light silver staining to detect proteins under conditions in which the images of the protein bands were not saturated. As a result, we identified many 60S ribosome subunit proteins, and pre-ribosomal proteins involved in 60S ribosome biogenesis, including Nog1 itself, Nop7 (also referred to as Yph1), Rlp24, Nsa1, Has1, Nop2 and Erb1, in the Nog1 complex of cells grown in the rapamycin-free medium (Figure 4, lane a), which were found in the previous work (Saveanu *et al*, 2003). Importantly, these components of the Nog1 complex, including 60S RPs, were not lost after STING occurred upon rapamycin treatment (Figure 4, lane b). The fact that the Nog1 complex does not lose 60S RPs after tethering of Nog1 to the nucleolus by TOR inactivation demonstrates that STING inhibits the nucleolus–nucleoplasm transfer of the 60S pre-ribosome. Namely, TOR functions in a late step of 60S ribosome maturation, the nucleolus–nucleoplasm transfer of the 60S pre-ribosome, by promoting the release of the Nog1 complex from the nucleolus. The experiment performed here was an indirect method to determine the localization of the pre-60S complex, and it cannot exclude the possibility that there is Nog1-independent maturation of the 60S pre-ribosome in the nucleoplasm that takes place after TOR inactivation.

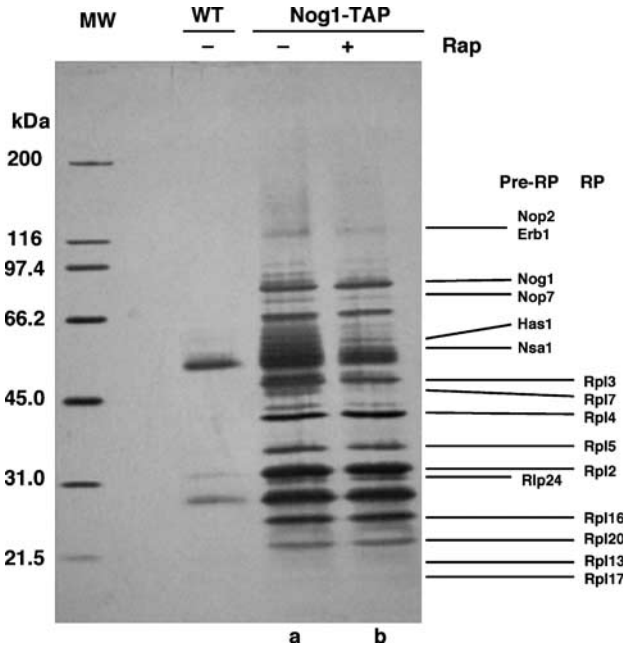

Figure 4 Identification of components of the Nog1 complex. Cells of a strain expressing *NOG1-TAP* (SCU889) were harvested before (lane a) and 30 min after rapamycin (200 ng/ml) treatment (lane b). Nog1-associated proteins extracted from cells were purified according to the TAP purification protocol, and were separated by SDS–PAGE followed by silver staining. Wild-type strain TB50a (SCU425) was used as the negative control. Ribosomal (RP) and pre-ribosomal (Pre-RP) proteins were identified by mass spectrometric analysis.

Nop7 functions together with Nog1

As mentioned above, Nop7 is a Nog1-associated protein. Reciprocally, Nog1 is found in the Nop7 complex (Harnpicharnchai *et al*, 2001). Nop7 is essential for 60S ribosome maturation and its loss of function leads to defects similar to those resulting from Nog1 depletion (Adams *et al*, 2002; Du and Stillman, 2002; Oeffinger *et al*, 2002). These facts suggest a functional interaction between Nog1 and Nop7. In fact, overexpression of *NOP7* suppressed the thermosensitive growth defects of the *nog1-11* and *nog1-13*, but not *nog1-12*, mutant strains at restrictive temperatures (Figure 5A and data not shown). Reciprocally, the growth defect of the temperature degron *nop7* (*yph1-td*) strain (Du and Stillman, 2002) at permissive (25°C) and semipermissive (34°C) temperatures was ameliorated by overexpression of *NOG1* (Figure 5B). These findings indicate that there is a functional interaction between Nog1 and Nop7. It is most likely that Nog1 functions together with Nop7 in the same complex in the regulation of 60S ribosome maturation.

Nop7 and Rlp24 show the STING phenotype in response to rapamycin

Nucleolar entrapment by rapamycin is a useful criterion to identify the tightly bound components of the Nog1 complex. Nop7 was localized in both the nucleolus and the nucleoplasm (Figure 5C), as shown previously (Huh *et al*, 2003). However, it was accumulated in the nucleolus when rapamycin was added (Figure 5C), like Nog1. Rlp24 is required for early and late stages of 60S ribosome biogenesis and it genetically and physically interacts with Nog1 (Harnpicharnchai *et al*, 2001; Saveanu *et al*, 2003). Has1, which is required for 40S ribosome maturation (Emery *et al*, 2004), is associated not only with Nog1 but also Nop7 (Bassler *et al*, 2001; Harnpicharnchai *et al*, 2001; Gavin *et al*, 2002; Ho *et al*, 2002). Rlp24 was present diffusely throughout the nucleus under normal conditions but it was concentrated in the nucleolus by rapamycin (Figure 6A), like Nog1 and Nop7. In contrast, Has1 and Nop2 were concentrated in the nucleolus even under favorable nutrient conditions (Figure 6 and Supplementary Figure 6C), as described previously (de Beus *et al*, 1994; Huh *et al*, 2003), and failed to show obvious STING phenotypes. Therefore, it is likely that Nog1, Nop7 and Rlp24, but Nop2 nor Has1, are tightly bound components of the same complex.

Many other pre-ribosomal proteins were also reported to physically interact with Nog1 and Nop7 (Bassler *et al*, 2001; Harnpicharnchai *et al*, 2001; Fatica *et al*, 2002; Gavin *et al*, 2002; Ho *et al*, 2002). We checked the localization of some of them. Tif6 and Bud20 showed the STING phenotype in response to rapamycin treatment (Supplementary Figures 4 and 5), suggesting that these proteins are also tight compo-

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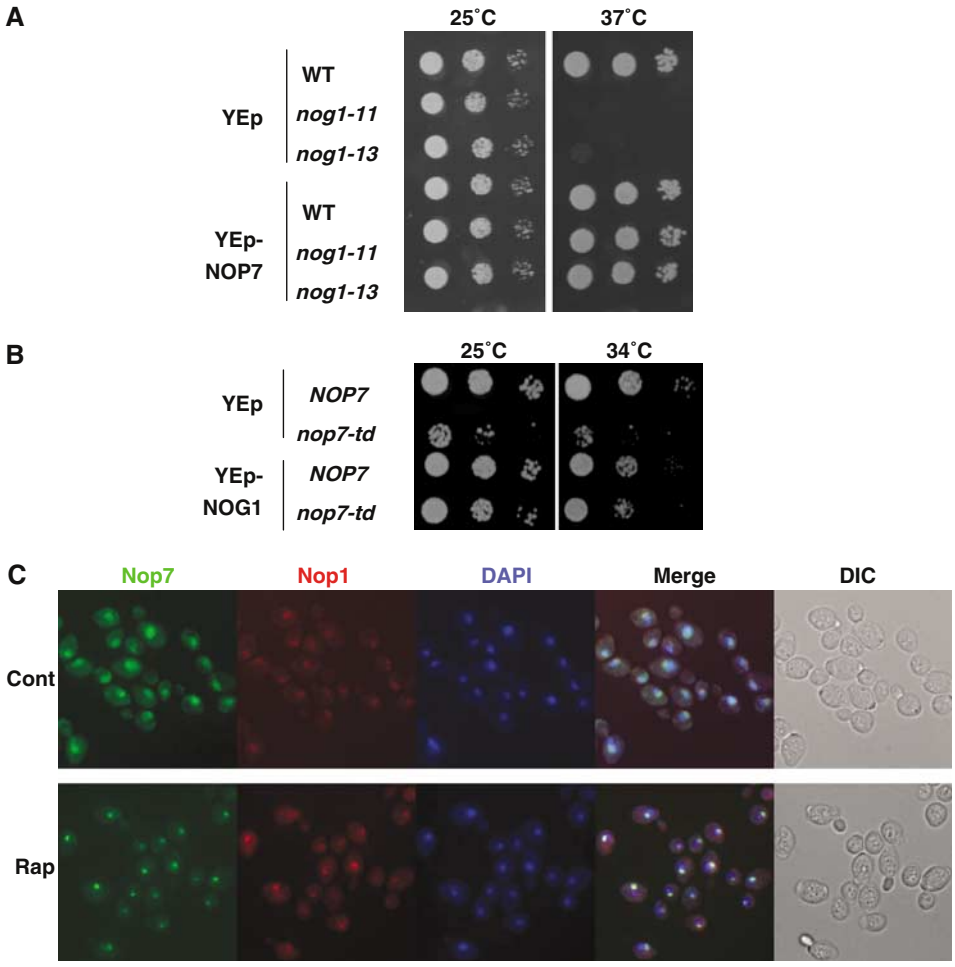

Figure 5 Nop7 functions together with Nog1. (**A**) Overexpression of *NOP7* suppresses thermosensitivity of *nog1-ts* strains. Strains *nog1-11* (SCU823), *nog1-13* (SCU825) and the wild-type *NOG1* (SCU822) were transformed with a plasmid for overexpression of *NOP7* (pSCU674) or with the empty vector (pSCU154). Serially diluted cells of each strain were spotted from the left to the right on YPAD plates. Plates were cultured at 25°C for 2 days or at 37°C for 1 day. (**B**) Overexpression of *NOG1* suppresses thermosensitivity of *nop7-td* strains. *nop7-td* (SCU89) and the wild-type *NOP7* (SCU88) strains were transformed with a plasmid for overexpression of *NOG1* (pSCU388) or with the empty vector (pSCU379). Serially diluted cells of each strain were spotted from left to right on YPAD plates. Plates were cultured at 25°C for 2 days or at 34°C for 1 day. (**C**) Nop7 shows the STING phenotype. Cells of a strain expressing *NOP7-GFP* (SCU888) harboring a plasmid expressing *DsRed-NOP1* (pSCU618) were treated with rapamycin (200 ng/ml, Rap) for 60 min. GFP (Nop7, green), RFP (Nop1, red) and DAPI (blue) signals were observed. Nontreated cells were used as the control (Cont).

nents of the Nog1 complex. In contrast, the Nog1-associated proteins Ssf1 and Nsa3 were concentrated in the nucleolus even under normal conditions (Supplementary Figure 6A and B), as described previously (de Beus *et al*, 1994; Huh *et al*, 2003). Thus, it is unlikely that Ssf1 and Nsa3 are components of the Nog1 complex relocated in response to TOR activity.

A rapamycin-resistant NOG1 mutant strain is resistant to STING

If Nog1 is a critical regulator of STING, we might be able to isolate mutant *NOG1* strains that show no STING even after TOR inactivation. Such mutant cells should be resistant to rapamycin, because the cessation of ribosome biogenesis in response to rapamycin would be impaired. To test this, we tried to isolate rapamycin-resistant alleles of *NOG1*. We successfully isolated a rapamycin-resistant *NOG1* mutant strain (*NOG1-R1*) (Figure 7A). This strain did not grow on medium containing a high concentration of rapamycin (200 ng/ml), although rapamycin-resistant *TOR1* allele *TOR1-1* can grow under the same conditions (data not shown). This weak resistance to rapamycin of *NOG1-R1* would be reasonable, because rapamycin inhibits not only ribosome biogenesis but also various other events regulated by TOR.

We investigated whether STING was induced by rapamycin in the *NOG1-R1* cells. Nog1 was accumulated in the nucleolus 60 min after treatment with 50 ng/ml of rapamycin, whereas Nog1-R1 protein was still distributed throughout the nucleus under the same conditions (Figure 7B). Thus, this mutant Nog1 is not subject to regulation by TOR and the Nog1 complex continues to undergo 60S ribosome maturation under TOR-inactive conditions. This demonstrates that Nog1 is a key factor downstream of TOR regulating release of the Nog1 complex from the nucleolus to the nucleoplasm, and that switching of the Nog1 complex-mediated ribosome biosynthesis may account for cell growth regulation in response to nutrient availability.

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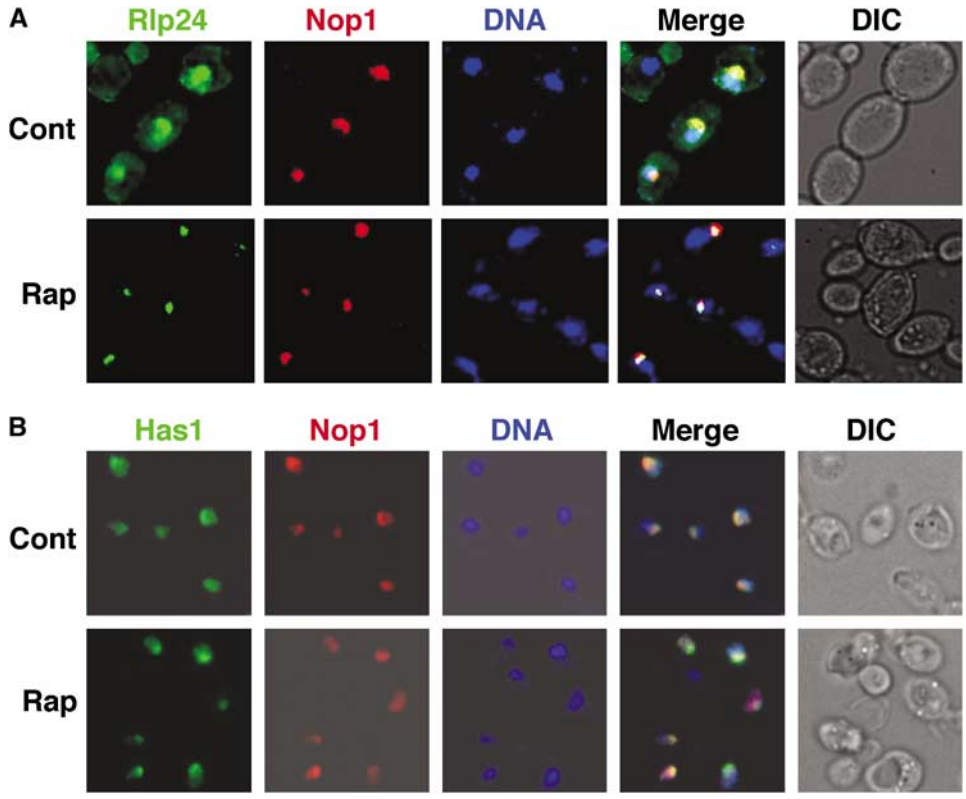

Figure 6 Effects of rapamycin on localization of Rpl24 and Has1. (**A**) Strain SCU7 harboring a plasmid expressing *RPL24-Myc13GFP* (pSCU771) and *DsRed-NOP1* (pSCU618) was treated with rapamycin (200 ng/ml, Rap) for 60 min, and GFP (Rlp24, green), RFP (Nop1, red) and DAPI (blue) signals were examined. Nontreated cells were used as the control (Cont). (**B**) Cells expressing *HA2-HAS1* (SCU1285) were treated with rapamycin (200 ng/ml, Rap) for 60 min and stained for HA (Has1; green), Nop1 (red) and DAPI (blue).

TOR upregulates protein levels of Nog1 and Nop7

Genome-wide microarray analysis suggests that TOR inactivation represses gene expression of pre-ribosomal proteins, including *NOG1* and *NOP7* (Cardenas *et al*, 1999; Hardwick *et al*, 1999). We confirmed by Northern blotting that *NOG1* expression was largely reduced by rapamycin (Figure 8A). Similar decreases occurred upon nitrogen and carbon starvation. These observations indicate that *NOG1* expression is repressed by nutrient starvation, via TOR inactivation. Furthermore, Nog1 protein was reduced 60 min after rapamycin treatment and it was undetectable after 120 min, unlike Nop1 (Figure 8B). The decrease in Nog1 protein induced by rapamycin may reflect repression of the *NOG1* gene.

We also found that Nop7 protein became undetectable in response to rapamycin. Thus, the Nog1 complex components Nog1 and Nop7 are lost faster than Nop1 after TOR inactivation. Consistently, it has been reported that nutrient starvation accompanied by long-term culturing decreased Nop7 more markedly than Nop1 (Du and Stillman, 2002). Thus, TOR inactivation repressed the Nog1 complex-mediated ribosome maturation step by two mechanisms: STING as a fast response, and thereafter elimination of the Nog1 complex as a slow response. Thus, TOR activity is required for the Nog1 complex release from the nucleolus and for maintenance of the Nog1 complex.

There are two possible mechanisms that might account for why Nog1 was rapidly lost in the cells after rapamycin treatment. The first is that TOR inactivation also stimulates Nog1 degradation. Alternatively, the turnover of Nog1 is fast even under favorable nutrient conditions, and the loss of Nog1 protein after rapamycin treatment results from shut-off of *NOG1* expression. To discriminate between these possibilities, we examined the Nog1 protein level after shut-off of the expression of Nog1 under the control of the *GAL* promoter (by addition of glucose) in the presence or absence of rapamycin. We observed a decrease in the Nog1 level after expression shut-off (Figure 8C), indicating that Nog1 is susceptible to degradation under favorable nutrient conditions. However, we did not observe a stimulation of the decrease in the presence of rapamycin, indicating that Nog1 degradation is not accelerated by TOR inactivation (Figure 9).

Rapamycin hypersensitivity and Nog1 instability in the presence of rapamycin in nog1-ts strains

In contrast to the *NOG1-R1* strain, some *nog1-ts* strains might exhibit rapamycin-hypersensitive growth because of impairment of Nog1 function. Indeed, *nog1-11* and *nog1-12* were rapamycin hypersensitive (Figure 8B). As TOR regulates STING and the Nog1 protein level, one would suspect that these mutants are susceptible to one or both of the STING and the decrease in Nog1 at the restrictive temperature. The Nog1 protein levels of three *nog1-ts* strains were the same as that of the wild-type strain at a permissive temperature (25°C; Figure 8C). Similar results were also obtained even after transfer of the cells to a restrictive temperature (37°C). However, when rapamycin was simultaneously added to cells at transfer to the restrictive temperature, Nog1 was largely lost in the *nog1-ts* mutants, whereas the wild-type Nog1 was still maintained. This indicates that the stability of Nog1 is specifically lowered by rapamycin in these mutants at

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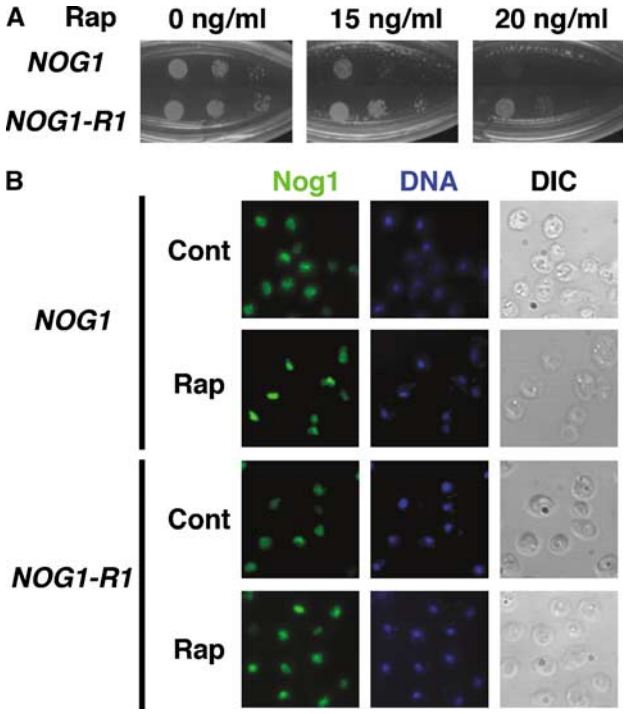

Figure 7 Rapamycin-resistant *NOG1-R1* strain is defective in STING. (**A**) Rapamycin-resistant growth of the *NOG1-R1* mutant strain. Serially diluted cells of the wild-type *NOG1* (SCU801) and *NOG1-R1* (SCU1287) strains were spotted from left to right on YPAD plates containing various concentrations of rapamycin at 30°C for 3 days. (**B**) *NOG1-R1* cells are defective in STING. *NOG1* (SCU801) and *NOG1-R1* (SCU1287) strains were treated with 50 ng/ml rapamycin for 60 min. Both types of cells expressed Nog1-HA3 and were stained for HA (Nog1; green) and DAPI (blue).

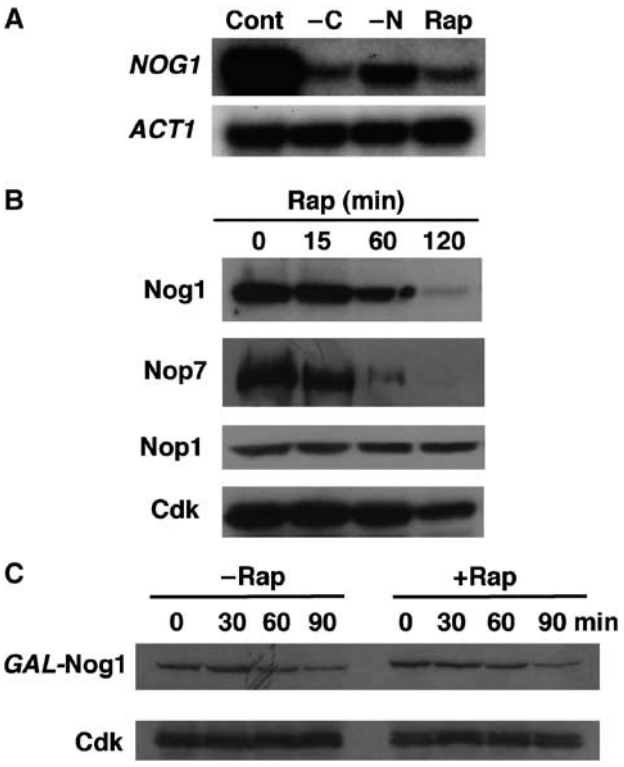

Figure 8 TOR regulates concentration of the Nog1 complex proteins. (**A**) *NOG1* expression is upregulated by TOR. Cells (SCU7) were treated with rapamycin (200 ng/ml) or transferred to medium without a nitrogen or carbon source for 15 min. *ACT1* was used as the loading control. (**B**) Changes in protein levels of Nog1, Nop7 and Nop1 after rapamycin treatment. *NOP7-myc13* strain (SCU799) with plasmid pHAC33-NOG1 (pSCU387) was treated with rapamycin (200 ng/ml). Whole-cell extracts were subjected to Western blotting using anti-HA, anti-Myc, anti-Nop1 and anti-cyclin-dependent kinase (Cdk) antibodies. Cdk was used as the loading control. (**C**) Changes in protein levels of Nog1 after shut-off of *NOG1* expression in the presence or absence of rapamycin. The wild-type cells (SCU425) harboring plasmid pGAL-HA-NOG1 (pSCU679) were pre-cultured in galactose-based medium for expressing *HA-NOG1* and then supplemented with glucose for shut-off of *NOG1* expression (at time 0). Rapamycin (200 ng/ml) was added simultaneously to cultures (+Rap). Cells not treated with rapamycin were prepared as the control (−Rap).

restrictive temperature, although the rate of degradation of the wild-type Nog1 protein is not stimulated by TOR inactivation. It is likely that these mutant proteins may be susceptible to being recognized by protein degradation machineries at restrictive temperatures under TOR-inactive conditions. On the other hand, we observed no defect in Nog1 distribution, namely no STING, at the restrictive temperature in these *nog1-ts* strains (Supplementary Figure 7), and STING upon TOR inactivation (data not shown).

Discussion

Here we have shown evidence that Nog1 is involved in late stages of 60S ribosome biogenesis in the nucleoplasm, in addition to its known involvement in the early stages of 60S ribosome biogenesis in the nucleolus. Moreover, we have suggested that Nog1, Nop7 and Rlp24 are tight components of the same complex. The latter two proteins showed localization changes similar to that of Nog1 in response to TOR activity. Therefore, we postulate that this Nog1 complex is involved in late stages of 60S ribosome maturation in the nucleoplasm in response to TOR activity. TOR regulates ribosome biogenesis at various steps: gene expression of rRNA and RPs, 35S rRNA processing (see Introduction), and transfer of the pre-ribosome from the nucleolus to the nucleoplasm (this study). The pre-60S complex is trapped in the nucleolus by TOR inactivation, via STING. Late factors involved in ribosome maturation normally cause more of an accumulation of Rpl25-GFP in the nucleoplasm, whereas earlier factors cause more of an accumulation in the nucleolus (Milkereit *et al*, 2001; Nissan *et al*, 2002; Tschochner and Hurt, 2003). Rpl25-GFP was accumulated in the nucleus, but not the nucleolus, in Nog1-defective and -depleted cells, but we cannot exclude the possibility that abortive assembly intermediates might no longer be actively retained in the nucleolus and 'leak out' into the nucleoplasm. We also cannot rule out the possibility that nucleoplasm-localized Nog1 may be simply diffused from the nucleolus. However, even if Nog1 is only involved in early ribosome maturation in the nucleolus under favorable nutrient conditions, Nog1 together with Nop7 and Rlp24 entraps many RPs in the nucleolus by STING after TOR inactivation, suggesting that Nog1 is a regulator of the transfer of pre-ribosomes under nutrient-starved conditions.

Three other nuclear GTP-binding proteins are known to be required for ribosome maturation: Nug1 (Nucleolar GTPase 1), Nog2/Nug2 (Nog2: Nuclear/NucleOlar GTP-binding

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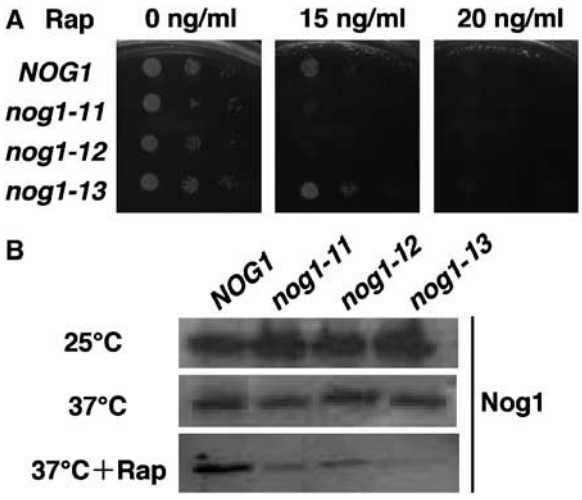

Figure 9 Rapamycin sensitivities and Nog1 protein levels of *nog1-ts* mutants. (**A**) Rapamycin-sensitive growth of *nog1-11* and *nog1-12*. Serially diluted cells of *NOG1* (SCU801), *nog1-11*, *nog1-12* and *nog1-13* strains were spotted from left to right on YPAD plates containing various concentrations of rapamycin at 30°C for 3 days. (**B**) Nog1 protein level of *nog1-ts* is decreased after shift to restrictive temperature in the presence of rapamycin. Each *nog1-ts* strain grown at 25°C was shifted to 37°C in the presence or absence of rapamycin (200 ng/ml) and the amount of Nog1 protein was determined by Western blotting using anti-HA antibody.

protein 2; involved in 60S ribosome maturation) and Bms1 (involved in 40S ribosome biogenesis) (Bassler *et al*, 2001; Gelperin *et al*, 2001). We examined the effect of rapamycin on their localization. Under normal conditions, Nug1 and Bms1 were localized to the nucleus with a higher concentration in the nucleolus (Supplementary Figure 8A and C), as previously shown (Bassler *et al*, 2001; Huh *et al*, 2003), and their nucleolar concentrations were not obviously enhanced by rapamycin. In contrast, Nog2 exhibited localization patterns similar to those of Nog1 in the presence and absence of rapamycin (Supplementary Figure 8B). Thus, it is unlikely that nucleolar entrapment by TOR inactivation is a unique feature of Nog1. It is intriguing that these NOG proteins cooperatively regulate the late step of ribosome maturation in the nucleoplasm.

TOR upregulates the gene expression of pre-ribosomal proteins (this study) (Cardenas *et al*, 1999; Hardwick *et al*, 1999). Furthermore, TOR activity is required for the maintenance of Nog1-associated proteins, because we found the specific loss of Nog1 and Nop7 (as compared with Nop1) after TOR inactivation. Nog1 and Nop7 were lost after repression of gene expression in the presence of rapamycin. Nog1 is a relatively unstable protein (Figure 8C). It is probable that Nop7 is also unstable. It is known that long-term starvation of cells delays restarting of growth after the administration of nutrients. Starvation-induced Nog1 complex loss is probably one of the reasons for the delay of the restarting of ribosome biogenesis, and therefore re-growth, when nutrients are re-added.

How does TOR control the Nog1 complex? TOR mainly exists in the cytoplasm, and a very small amount of TOR is present in the nucleus (Wedaman *et al*, 2003). In the case of mammalian cells, mammalian TOR (mTOR) is shuttled between the nucleus and the cytoplasm. mTOR is exported from the nucleus by the exportin Crm1, and loss of function of Crm1 leads to the nuclear accumulation of mTOR (Kim and Chen, 2000). One could suspect that yeast TOR possesses the same features, although there has been no report demonstrating this. If TOR indeed behaves like mTOR, TOR may directly regulate the Nog1 complex distribution in the nucleus by regulating the phosphorylation status of Nog1-associated proteins or a putative anchoring nucleolar factor. Yeast possesses the ortholog Crm1/Xpo1, but no nuclear accumulation of TOR was found in *xpo1-1* cells at non-permissive temperatures (our unpublished data), although other exportins may be responsible for TOR export. Alternatively, TOR may indirectly regulate the Nog1 complex localization via a downstream factor of TOR, for example, nuclear protein kinases downstream of TOR, Yak1 and Rim15 (Pedruzzi *et al*, 2003; Schmelzle *et al*, 2004).

On the other hand, the dual-specificity protein phosphatase Yvh1 (yeast vaccinia VH1 homolog) physically interacts with Nop7, and overproduction of Nop7 suppresses the slow growth in a *yvh1* disruptant, although Nop7 seems not to be a substrate of Yvh1 (Sakumoto *et al*, 2001). This suggests that Yvh1 is related to regulation of the Nog1 complex. Furthermore, *YVH1* overexpression suppressed the thermosensitive growth of some of the *nog1* mutants (our unpublished data). It is likely that Yvh1 activity is required for the Nog1 complex function under nutrient-rich conditions. It is currently unclear whether Yvh1 and TOR independently regulate the Nog1 complex function or whether Yvh1 acts as a downstream factor of TOR.

It is also unknown how Nog1 is trapped in the nucleolus under starvation conditions. The nucleolus serves as a storage depot for various important regulatory factors, including Mdm2, Pch2, Sir2 and Cdc14 (Visintin and Amon, 2000). The yeast protein phosphatase Cdc14 is sequestered in the nucleolus until metaphase, but it is released by the FEAR (for Cdc fourteen early anaphase release) network at early anaphase and then by the MEN (for mitotic exit network) at telophase (D'Amours and Amon, 2004). Its nucleolar-tethering factors are Net1 and Fob1 (Stegmeier *et al*, 2004). When Cdc14 is released from Net1, both proteins are hyperphosphorylated by cyclin B–Cdk (cyclin-dependent kinase) (Azzam *et al*, 2004). Interestingly, it was reported that Nog1 interacts with Fob1 in a genome-wide yeast two-hybrid assay (Uetz *et al*, 2000). It will be important to test whether Fob1 is involved in STING. It is likely that TOR signaling regulates STING by controlling the phosphorylation status of component(s) of the Nog1 complex and/or the putative nucleolus-tethering factor(s).

The TOR system and the Nog1 complex components (Nog1, Nop7 and Rlp24) are highly conserved from yeast to vertebrates. The vertebrate Nop7 homolog pescadillo (PES1) is also involved in 60S ribosome maturation (Lerch-Gaggl *et al*, 2002; Lapik *et al*, 2004). The vertebrate Nog1 homolog NGB/CRFG is less well characterized. NBG expression in the outer medulla of the kidney is reduced in a renal disease mouse model (Laping *et al*, 2001), but the function of NBG is unknown. It is most likely that TOR-regulated ribosome maturation via the Nog1 complex is also conserved among various eukaryotic species. Although the molecular mechanisms of ribosome maturation control in response to nutrient availability are largely unknown in mammals at present, we believe that the findings of our study are suggestive and in accord with similar studies in mammalian cells.

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Materials and methods

Strains, plasmids and media

S. cerevisiae strains and plasmids used are listed in Supplementary Tables 1 and 2, respectively. Gene disruption and genomic C-terminal tagging of GFP, 3 × hemagglutin A (HA), 13 × Myc and TAP tags were conducted by the one-step PCR-mediated method (Longtine *et al*, 1998; Puig *et al*, 2001). The resultant tagged Nog1 proteins are all functional, as shown by the fact that they suppressed the lethality of *nog1*-deleted cells. Detailed methods of the construction of strains and plasmids are available on request. Glucose-based YPAD (YPD containing 0.01% adenine) and synthetic minimal medium (SD) complemented with the appropriate nutrients for plasmid maintenance were prepared in standard ways. YPGalR and SGalR are identical to YPAD and SD except that they contain 1% galactose and 1% raffinose instead of 2% glucose. For carbon depletion, logarithmically growing cells cultured in SD medium were transferred to a glucose-free SD medium. For nitrogen depletion, logarithmically growing cells cultured in SD medium were transferred to SD medium without nitrogen sulfate and amino acids.

Construction of thermosensitive nog1 mutants

Deletion of *NOG1* was performed by the one-step PCR-mediated gene disruption method (Longtine *et al*, 1998) using diploid SCU165 strain. A *URA3*-marked plasmid, pNOG1-HA3 (pSCU387), was transformed into the resulting strains. Haploid cells *nog1*::*HIS3* [pHAC33-NOG1] (SCU783) obtained from the transformants were used for the plasmid shuffling. We added the C-terminal HA3 tag to Nog1 protein to enable easy assessment of the protein expression status thereafter. A *LEU2*-marked plasmid, pHAC111, cut with *Bam*H1/*Sal*1 and a *NOG1* fragment (promoter plus ORF) amplified using a mutagenesis PCR kit (Clontech) were simultaneously transformed into SCU783 cells to construct pHAC111-nog1-ts (temperature sensitive) by the homologous recombination method. After 5 days of culture on SD-Leu (SD-L) plates, transformants were replica-plated to SD-L plus 5-fluoro-orotic acid to eliminate the *URA3*-marked plasmid pHAC33-NOG1. As a result, three thermosensitive *nog1* strains (viable at 25°C and lethal at 37°C) were isolated.

Western blot analysis

Cells (a 10-ml culture in SD grown to mid-log phase) were lysed in 100 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA and 1% NP-40) by vortexing 4 × 30 s with glass beads, using a FastPrep machine (Savant Instruments). Lysis buffer contained a protease inhibitor cocktail (Complete™, Roche) and protein phosphatase inhibitors (10 mM NaF, 10 mM *p*-nitrophenylphosphate, 10 mM sodium pyrophosphate and 10 mM β-glycerolphosphate). Crude extracts were cleared by a 5-min, 1500 *g* spin and the supernatant was used for Western blot analysis as described in our previous report (Beck *et al*, 1999) using anti-HA mouse monoclonal antibody (16B12, BAbCo), anti-Myc mouse monoclonal antibody (9E10, Santa Cruz), anti-cyclin-dependent kinase (Cdk) rabbit polyclonal antibody (anti-PSTAIRE, Santa Cruz) and anti-Nop1 mouse monoclonal antibody (a gift from John Aris) (Henriquez *et al*, 1990).

Nog1 purification

Cells expressing a TAP-tagged or untagged (mock purification) version of Nog1 were grown in 1 l of YPAD to $OD_{600} = 1.5$ and harvested by centrifugation. The cells were lysed in 18 ml of lysis buffer plus protease inhibitors with glass beads using a Biospecs Beadbeater. Cell lysates were cleared by a 5-min, 500 *g* spin and passed through 500 μl of CL-4B Sepharose (Amersham Pharmacia Biotech). Thereafter, TAP of TAP-tagged Nog1 was performed according to the original protocol (Puig *et al*, 2001). Calmodulin bead-bound proteins were used for MASS spectrometric analysis

MASS spectrometry

Silver-stained proteins separated by 1D gel electrophoresis were digested as described previously (Puig *et al*, 2001). Peptides to be subjected to MALDI-TOF analysis were desalted on C18 ZipTips (P10 tip size, Millipore) with 1.5 μl of 80% AcCN and 0.1% TFA containing 1 mg/ml α-cyano-4-hydroxycinnamic acid (Aldrich). Five hundred nanoliters of the eluate was deposited onto anchor spots of a Scout 400 m/36 sample support (Bruker Daltonik). Mass spectra were recorded using a Bruker Scout 26 Reflex III instrument (Bruker Daltonik). For database analysis, all spectra recorded for tryptic peptides were compared to the *S. cerevisiae* protein database using the Mascot search program (www.matrixscience.com).

Northern blot analysis

RNA isolation and Northern blot analysis were performed as described (Crespo *et al*, 2002). For probes, fragments of *NOG1*, *NOP1* and *ACT1* were amplified by PCR.

Indirect immunofluorescence

Logarithmically growing cells were fixed with formaldehyde and digested with Zymolyase as described (Beck *et al*, 1999). HA-tagged proteins and Nop1 were detected with a polyclonal rabbit anti-HA (clone Y11; Santa Cruz Biotechnology), a polyclonal rabbit anti-GFP (a gift from Pamela Silver) and a monoclonal mouse anti-Nop1 antibody (a gift from John Aris). The samples were then treated with Cy3-conjugated and Alexia 488-conjugated secondary antibody (Molecular Probes Inc.) and 4′,6-diamidino-2-phenylindole (DAPI) as described (Beck *et al*, 1999). Washed cells were viewed using an Olympus IX71-23FL/S microscope (× 100 objective) and a cooled charge-coupled device camera (ORCA-ER-1, Hamamatsu Photonics) connected to a Scanalytics Image Processor Lumina-Vision (Mitani Corp., Tokyo, Japan).

GFP and red fluorescent protein observations

Logarithmically growing cells expressing GFP- and/or red fluorescent protein (RFP) (DsRed)-tagged proteins were fixed with 70% ethanol for 30 s. After washing with distilled water, cells were stained with DAPI at 1 μg/ml for 10 min. Washed cells were viewed as mentioned in the section Indirect immunofluorescence.

Nucleotide sequencing

The nucleotide sequence was determined with a DTCS Quick Start Kit (Beckman Coulter) and an automatic DNA sequencer (model CEQ8000; Beckman Coulter) according to the manufacturer's instructions.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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