Distinct cytoplasmic maturation steps of 40S ribosomal subunit precursors require hRio2

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During their biogenesis, 40S ribosomal subunit precursors are exported from the nucleus to the cytoplasm, where final maturation occurs. In this study, we show that the protein kinase human Rio2 (hRio2) is part of a late 40S preribosomal particle in human cells. Using a novel 40S biogenesis and export assay, we analyzed the contribution of hRio2 to late 40S maturation. Although hRio2 is not absolutely required for pre-40S export, deletion of its binding site for the export receptor CRM1 decelerated the kinetics of this process. Moreover, in the absence of hRio2, final cytoplasmic 40S maturation is blocked because the recycling of several trans-acting factors and cytoplasmic 18S-E precursor ribosomal RNA (rRNA [pre-rRNA]) processing are defective. Intriguingly, the physical presence of hRio2 but not its kinase activity is necessary for the release of hEnp1 from cytoplasmic 40S precursors. In contrast, hRio2 kinase activity is essential for the recycling of hDim2, hLtv1, and hNob1 as well as for 18S-E pre-rRNA processing. Thus, hRio2 is involved in late 40S maturation at several distinct steps.

Introduction

The biogenesis of ribosomal subunits is a fundamental process, as it supplies cells with the machinery required for translation. In eukaryotes, ribosome biogenesis begins with precursor ribosomal RNA (rRNA [pre-rRNA]) transcription in nucleoli. Early ribosomal precursors are assembled from pre-rRNA, ribosomal proteins, and additional factors. These preribosomes undergo nuclear maturation in a series of remodeling and pre-rRNA processing steps before they are exported to the cytoplasm, where final maturation ensures that the two subunits reach translation competence (for reviews see Fromont-Racine et al., 2003; Tschochner and Hurt, 2003; Granneman and Baserga, 2004; Zemp and Kutay, 2007; Henras et al., 2008). Besides ribosomal proteins and rRNAs, >150 trans-acting factors are required for ribosome biogenesis. Generally, trans-acting factors are well conserved from yeast to human cells, suggesting that ribosome biogenesis is similar in these organisms. However, few of these factors have been studied in vertebrate cells, and the composition of ribosomal precursors in higher eukaryotes is largely unknown.

Along their nuclear maturation pathway, early preribosomes are separated into pre-40S and -60S particles, which are exported to the cytoplasm as independent entities. Ribosomal subunit export involves a subset of nuclear pore complex components (Hurt et al., 1999; Moy and Silver, 1999, 2002; Stage-Zimmermann et al., 2000; Gleizes et al., 2001; Bernad et al., 2006) and relies on the RanGTPase system (Hurt et al., 1999; Moy and Silver, 1999, 2002; Stage-Zimmermann et al., 2000; Gleizes et al., 2001; Trotta et al., 2003).

Nuclear export of 60S subunits depends on the RanGTP-binding exportin Xpo1p/CRM1 (Ho et al., 2000; Gadal et al., 2001; Moy and Silver, 2002; Thomas and Kutay, 2003; Trotta et al., 2003), which recognizes a Leu-rich nuclear export sequence (NES; Fornerod et al., 1997; Stade et al., 1997) in the 60S export adapter Nmd3 (Ho et al., 2000; Gadal et al., 2001; Thomas and Kutay, 2003; Trotta et al., 2003). In yeast, additional factors support pre-60S export, including the mRNA export receptor Mex67p/Mtr2p, the pre-60S component Arx1p (Bradatsch et al., 2007; Hung et al., 2008; Yao et al., 2008), and

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Abbreviations used in this paper: FRT, flippase recognition target; HeLaK, HeLa Kyoto; HeLaY, HeLa Yale; KD, kinase dead; LMB, leptomycin B; MS, mass spectrometry; NES, nuclear export sequence; pre-rRNA, precursor rRNA; rRNA, ribosomal RNA; tet, tetracycline; TetR, tet repressor; WT, wild type.

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the HEAT repeat protein Rrp12p, which, in addition, assists 40S biogenesis (Oeffinger et al., 2004).

Like pre-60S export, pre-40S export depends on a functional Xpo1p/CRM1 pathway in both yeast and vertebrate cells (Moy and Silver, 1999, 2002; Thomas and Kutay, 2003; Trotta et al., 2003). Two NES-containing trans-acting factors, Ltv1p and Dim2p, have been implicated as potential Xpo1p adapter proteins in pre-40S export in *Saccharomyces cerevisiae* (Seiser et al., 2006; Vanrobays et al., 2008), whereas export adapters serving 40S subunit export in vertebrate cells have not yet been identified.

After export to the cytoplasm, ribosomal subunit precursors need to be further matured before they can participate in translation. One important aspect in the cytoplasmic maturation of preribosomal subunits lies in the release of trans-acting factors from ribosomal precursor particles. Several such trans-acting factor recycling steps have been described for 60S biogenesis (for review see Zemp and Kutay, 2007), but how trans-acting factors are released from cytoplasmic pre-40S particles is largely unknown. In contrast to pre-60S particles, 40S precursors undergo a final cytoplasmic pre-rRNA processing event, which yields mature 18S rRNA from 20S pre-rRNA in yeast and from 18S-E pre-rRNA in human cells (Udem and Warner, 1973; Rouquette et al., 2005). It is currently not understood whether and how pre-rRNA processing is coordinated with the recycling of 40S biogenesis factors.

In yeast, Rio1p and Rio2p, two members of the RIO (right open reading frame) family of atypical protein kinases, were identified as trans-acting factors involved in late steps of 40S biogenesis (Vanrobays et al., 2001, 2003; Geerlings et al., 2003). Both proteins are essential, indicating that they do not have fully redundant functions. Still, depletion of either Rio1p or Rio2p results in the same pre-rRNA processing defect, i.e., the accumulation of 20S pre-rRNA. In contrast to Rio1p, Rio2p is a stable component of several distinct late pre-40S particles (Schafer et al., 2003). Because of conflicting data, it is not yet clear whether Rio2 is involved in pre-40S export in yeast. 20S pre-rRNA accumulated in the cytoplasm of Rio2p-depleted cells and in *rio2-1* mutant cells (Vanrobays et al., 2003; Leger-Silvestre et al., 2004), whereas the 40S subunit reporter Rps2-GFP accumulated in the nucleus of *rio2-1* mutant cells (Schafer et al., 2003).

Recently, human Rio2 (hRio2) has been shown to be required for cytoplasmic 18S-E to 18S rRNA processing, indicating that the role of Rio2 in final 18S rRNA maturation is conserved from yeast to vertebrates (Rouquette et al., 2005). In this study, we have further investigated the role of hRio2 in 40S subunit maturation in human cells. We could isolate and characterize an hRio2-containing 40S precursor, which contains 18S-E pre-rRNA and at least six trans-acting factors. Depletion of hRio2 from HeLa cells revealed that hRio2 is required for distinct steps of late 40S biogenesis. First, an NES in hRio2 plays a nonessential role in pre-40S export. Second, we demonstrate that hRio2 is required for the release of trans-acting factors from 40S precursors after export. Intriguingly, its physical presence but not its kinase activity is required for hEnp1 recycling to the nucleus. In contrast, hRio2 kinase activity is necessary for a second cytoplasmic maturation step during which hDim2, hLtv1, and hNob1 are released from 40S subunits and 18S-E to 18S rRNA processing occurs.

Results

hRio2 is a component of late 40S subunit precursors

In human cells, no pre-40S particles have been characterized to date. To test whether hRio2 is a component of 40S precursors, we first examined whether hRio2 is associated with 40S-sized complexes in cells. HeLa Kyoto (HeLaK) cell extract was centrifuged on a sucrose gradient followed by Western blot analysis. hRio2 predominantly cosedimented with pre-40S or 40S subunits (Fig. 1 A), and the same applied to hEnp1/bystin-like, the human homologue of *S. cerevisiae* Enp1p, which is a known component of pre-40S particles in yeast (Schafer et al., 2003). These results suggest that both proteins are indeed present in 40S precursors. To isolate hRio2-associated particles, we immunoprecipitated hRio2 from pooled 40S fractions using an hRio2-specific antibody. Immunoprecipitation of the pre-60S component hNmd3 served as a negative control. Several proteins specifically coprecipitated with hRio2 (Fig. 1 B) and were identified by mass spectrometry (MS) as hTsr1, hRio2, hLtv1, hEnp1, hNob1, and hDim2. The yeast homologues of all of these factors are known components of pre-40S particles (Schafer et al., 2003). Moreover, we detected ribosomal proteins of the small but not the large subunit, which is consistent with the hypothesis that the isolated particle corresponds to a 40S precursor. A few additional MS hits like tubulin or vimentin were considered to be unspecific contaminants. Using antibodies raised against hEnp1, hLtv1, and hRps3, we could confirm the MS results by Western blotting (Fig. 1 C), as all three proteins coprecipitated with hRio2. Furthermore, we analyzed the RNA content of the isolated particle by Northern blotting and detected 18S-E pre-rRNA, the last precursor to mature 18S rRNA (Fig. 1 D). In summary, we could isolate and characterize a late human 40S subunit precursor, which closely resembles its counterpart in yeast described by Schafer et al. (2003).

hRio2 directly binds CRM1 in an hRanGTP-dependent manner

Previous work in both yeast and human cells showed that Rio2 localizes to the cytoplasm at steady state and accumulates in the nucleus upon inhibition of the Xpo1p/CRM1 export pathway (Schafer et al., 2003; Vanrobays et al., 2003; Rouquette et al., 2005; Bonazzi et al., 2007). To look into the kinetics of this process, we analyzed the localization of endogenous hRio2 at several time points after the incubation of cells in the presence of the CRM1 inhibitor leptomycin B (LMB; Kudo et al., 1999). After only 30 min of LMB treatment, the majority of cells displayed hRio2 localization throughout the nucleus (Fig. 2 A). This rapid effect suggests that hRio2 may be a direct export substrate of CRM1. Interestingly, hRio2 was excluded from nucleoli at later time points, indicating a second phase of hRio2 relocalization. We further analyzed the cells for the localization of hEnp1. Even though hEnp1 is a nucleolar protein at steady

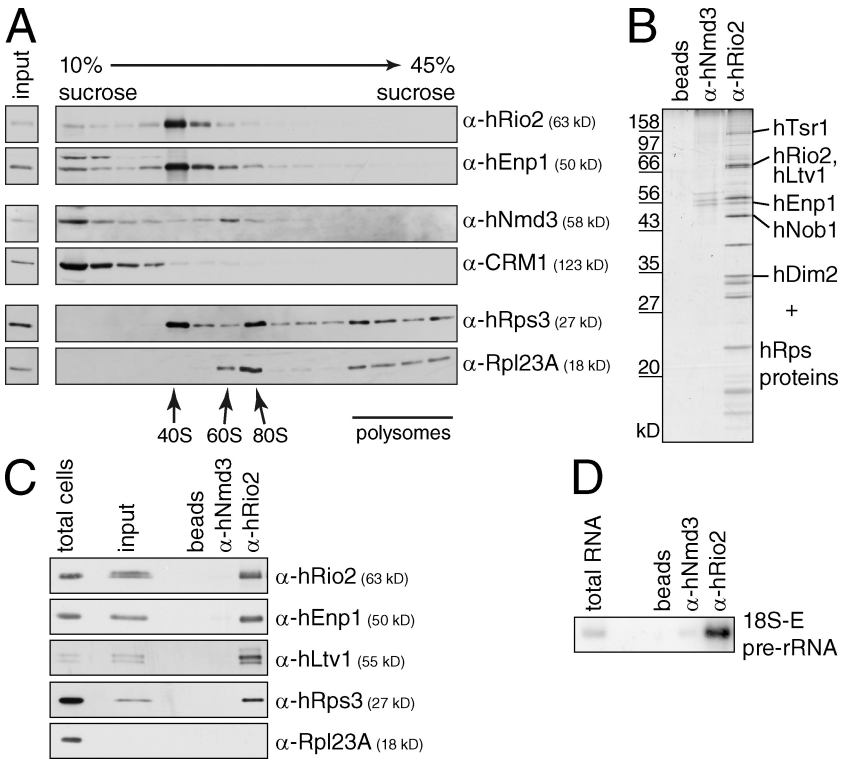


Figure 1. **hRio2 is a component of late 40S precursors in human cells.** (A) hRio2 and hEnp1 cosediment with pre-40S or 40S subunits. Cell extract was centrifuged on a 10–45% sucrose gradient. Fractions were analyzed by Western blotting. (B) hRio2 coprecipitates a pre-40S particle. hRio2 was immunoprecipitated from pooled 40S-containing gradient fractions. Proteins coprecipitated with hRio2 were analyzed by silver staining and identified by MS. (C) Western blot analysis confirms the presence of hRio2, hEnp1, hLtv1, and hRps3 in the hRio2-associated particle. (D) hRio2-associated particles contain 18S-E pre-rRNA. rRNA was isolated from the hRio2 immunoprecipitate and analyzed by Northern blotting.

state, its localization is also influenced by CRM1 inactivation because LMB treatment led to an increase in nucleoplasmic hEnp1 staining (Fig. 2 A). Relocalization of hEnp1 occurred with similar kinetics as the nucleolar exclusion of hRio2 and might reflect the nucleoplasmic accumulation of hRio2- and hEnp1-containing 40S precursors in CRM1-inhibited cells. In line with this interpretation, analysis of extracts from LMB-treated cells on sucrose gradients revealed that both hRio2 and hEnp1 are associated with 40S-sized particles after CRM1 inhibition (Fig. S1, A–C).

Because the LMB-induced relocalization of hRio2 occurs rapidly, nuclear export of hRio2 may directly be mediated by CRM1. Using a series of EGFP-tagged hRio2 constructs (not depicted), we could map a putative binding site for CRM1 in hRio2 to a region around aa 400 of its primary sequence, which contains a sequence motif that does not fully match but closely resembles the consensus for Leu-rich NESs recognized by CRM1 (Fig. 2 B; for review see Kutay and Güttinger, 2005). Indeed, mutation of Leu400 and Ile403 to Ala (NESmut) or deletion of aa 399–408 (NESΔ10) relocalized hRio2 to the nucleus (Fig. 2 C), indicating that this sequence motif is a functional NES. Sequence alignment reveals that the NES in the C-terminal domain of hRio2 is well conserved in vertebrates (Fig. 2 B). The C-terminal domains of Rio2 sequences from lower eukaryotes like yeast are mostly much shorter than in human Rio2 and generally do not align well (Vanrobays et al., 2003; unpublished data). Nevertheless, a similar NES-like sequence can also be found, for instance, in yeast Rio2p (Fig. 2 B). To test whether the NES of hRio2 can mediate nuclear export of a reporter, we fused aa 388–410 of hRio2 to the C terminus of EGFP. Although EGFP displayed a predominantly nuclear localization, EGFP-NES(Rio2) localized to the cytoplasm (Fig. 2 D). Nuclear export of EGFP-NES(Rio2) was CRM1 dependent, as it relocalized to the nucleus upon LMB treatment. Similar results were obtained using the NES of hNmd3 as a positive control. Thus, the NES identified in hRio2 can mediate nuclear export in vivo.

To confirm that hRio2 is exported by CRM1, we performed in vitro binding assays using recombinant zz-hRio2 and CRM1 in the absence and presence of RanGTP, which increases the affinity of exportins for their substrates (Fornerod et al., 1997; Kutay et al., 1997). Indeed, binding of CRM1 to zz-hRio2 was observed in the presence but not in the absence of RanGTP (Fig. 2 E). In contrast, CRM1 did not associate with either zz-hEnp1 or the zz tag alone. Recombinant zz-hRio2(NESmut) and zz-hRio2(NESΔ10) could be purified in similar purity and amounts as zz-hRio2(wild type [WT]) and displayed similar in vitro kinase activity (Fig. 2 F), indicating that the mutations did not grossly alter hRio2 structure. However, both zz-hRio2(NESmut) and zz-hRio2(NESΔ10) displayed strongly reduced CRM1 binding (Fig. 2 E). Together, these results show that hRio2 directly binds CRM1 via an NES in its C-terminal domain and is an export substrate of CRM1. Because hRio2 is a component of late pre-40S particles (Fig. 1), it is a candidate adapter protein for CRM1 in pre-40S export.

hRio2 contributes to the efficient nuclear export of 40S subunit precursors

To study pre-40S export in human cells, we developed a novel reporter system based on a HeLaK cell line, which allows for the tetracycline (tet)-dependent induction of a YFP-tagged ribosomal protein of the 40S subunit, i.e., hRps2-YFP. Only low levels of hRps2-YFP were detected in these cells in the absence of tet induction (Fig. 3). However, tet addition led to a strong

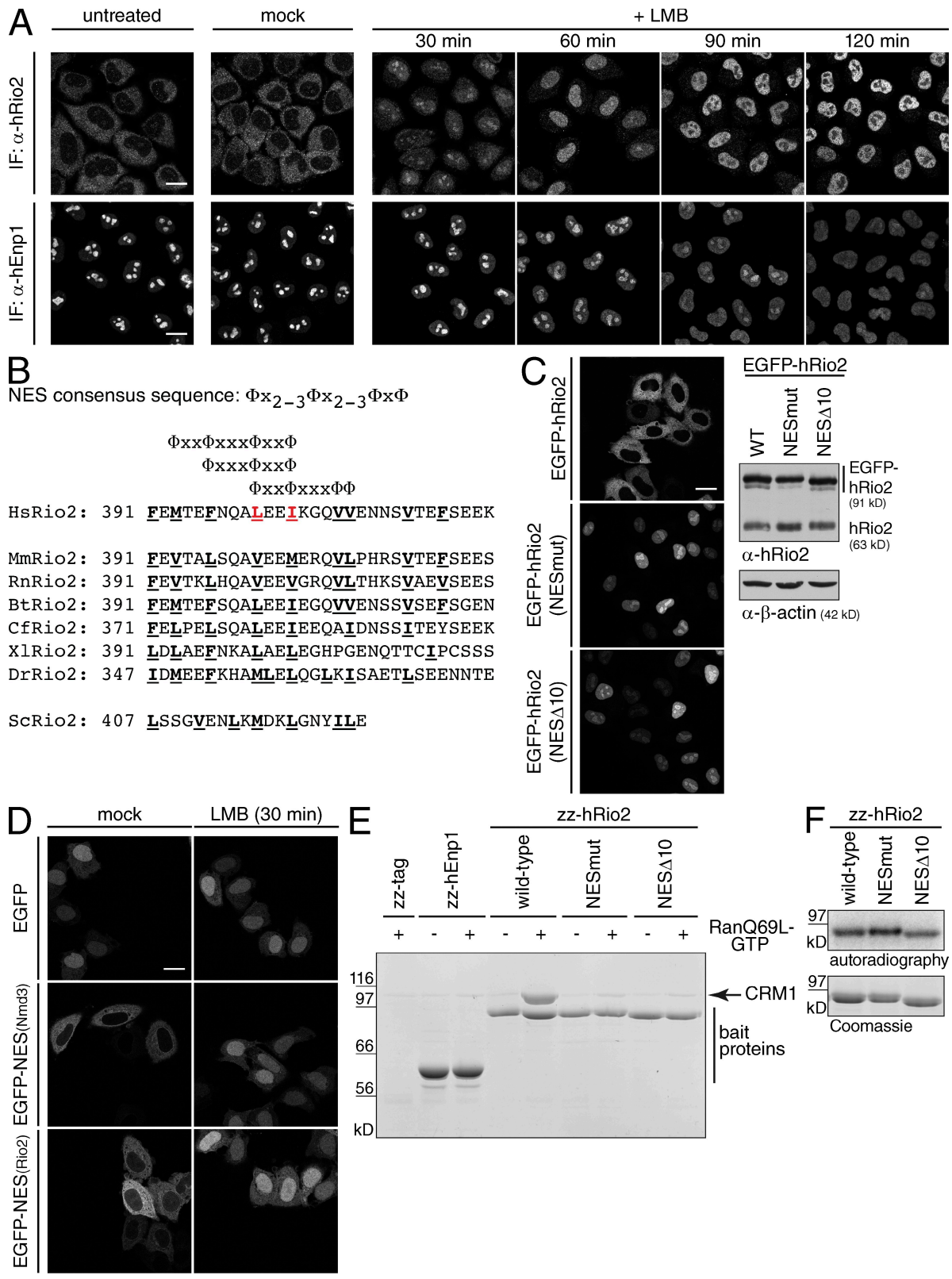


Figure 2. **hRio2 is exported from the nucleus in a CRM1-dependent manner.** (A) hRio2 accumulates in the nucleoplasm upon CRM1 inhibition. HeLaK cells were treated with 20 nM LMB or solvent (ethanol) for the times indicated, followed by fixation and immunofluorescence (IF) analysis for hRio2 or hEnp1. (B) Possible alignments of the NES consensus (for review see Kutay and Güttinger, 2005) to aa 391–420 of hRio2 and selected Rio2 homologues. Rio2 sequences from *Homo sapiens* (Hs), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Bos taurus* (Bt), *Canis familiaris* (Cf), *Xenopus laevis* (Xl), and *Danio rerio* (Dr) were aligned using ClustalW. Φ, large, hydrophobic amino acid (underlined and bold in Rio2 sequences); x, any amino acid. Leu400 and Ile403, the two residues mutated in hRio2(NESmut), are depicted in red. (C) Mutation of the NES of hRio2 leads to its nuclear accumulation. HeLaK cells were transiently transfected with EGFP-hRio2, EGFP-hRio2(NESmut) or EGFP-hRio2(NESΔ10). 24 h after transfection, cells were analyzed by fluorescence microscopy and Western blotting for the localization and expression of hRio2 constructs, respectively. (D) The NES of hRio2 mediates CRM1-dependent nuclear export of a

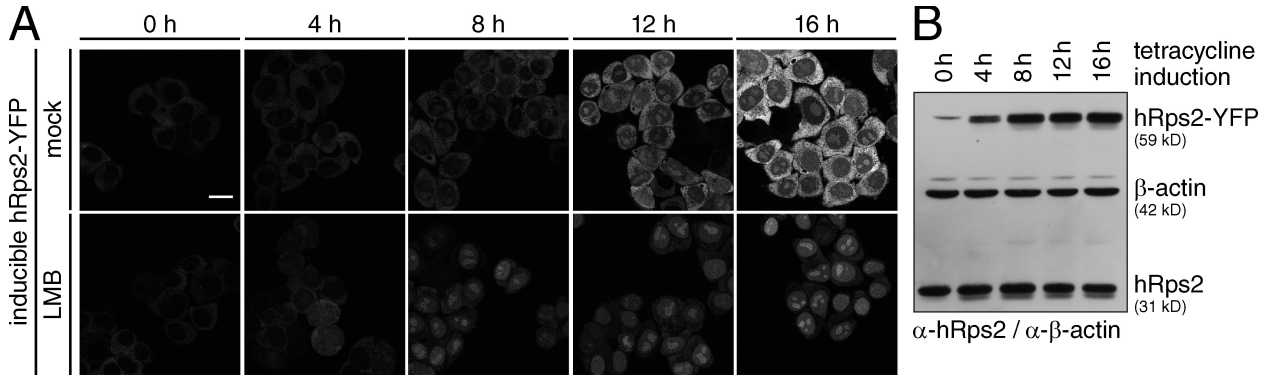


Figure 3. **Inducible hRps2-YFP reporter cells to study 40S subunit biogenesis.** (A) Inducible hRps2-YFP reporter cells show nuclear accumulation of hRps2-YFP upon CRM1 inhibition. hRps2-YFP expression in reporter cells was induced by the addition of tet followed by treatment with 20 nM LMB or solvent (ethanol) for the times indicated. (B) Western blot analysis of A. Bar, 20 μm.

increase in hRps2-YFP signal, and the reporter localized predominantly to the cytoplasm. To determine whether hRps2-YFP becomes incorporated into translating ribosomes, we performed sucrose gradient analysis. The majority of hRps2-YFP was associated with 40S, 80S, and polysome fractions, indicating that it was indeed faithfully incorporated into functional 40S subunits (Fig. S1 D). A small pool of free hRps2-YFP was also observed, caused by the induction of newly synthesized, not yet incorporated protein upon tet addition (Fig. S1 E) and perhaps also partially by the increased total amount of hRps2 in these cells, endogenous and YFP tagged. Upon inhibition of pre-40S export by LMB in the reporter cell line, a nuclear accumulation of hRps2-YFP was observed (Fig. 3 A), demonstrating that our reporter allows for a sensitive readout of defects in 40S biogenesis and export and provides a sensitive assay to study the fate of newly assembled 40S subunits.

Using these reporter cells, we performed RNAi experiments to determine the effects of hRio2 depletion on pre-40S export. Control experiments targeting CRM1 and hNmd3 showed the expected results, namely a nuclear accumulation of hRps2-YFP in CRM1-depleted cells and no effect on hRps2-YFP localization after knockdown of the pre-60S export adapter hNmd3 (Fig. 4 A). Surprisingly, hRio2 depletion, which was efficient (Fig. 4 A and Fig. S2 A), led to a mixed phenotype: ∼25% of cells displayed nuclear accumulation of the reporter protein, whereas in the others, hRps2-YFP was predominantly cytoplasmic or showed both nuclear and cytoplasmic staining. Moreover, we performed sucrose gradient analyses from extracts of hRio2-depleted as well as LMB-treated reporter cells and did not observe a significant change in the amount of hRps2-YFP detected in the free protein fractions, suggesting that the observed nuclear accumulation of hRps2-YFP in these cells is not caused by an assembly defect but by reduced pre-40S export (unpublished data). The pre-40S export defect upon hRio2 depletion was clearly not as prominent as after knockdown of CRM1, and not all hRio2-depleted cells showed a defect. Therefore, hRio2 appears not to be absolutely required for 40S export, but hRio2 knockdown may reduce pre-40S export efficiency. In line with this argument, hRps2-YFP accumulation in the nucleus of hRio2-depleted cells progressively disappeared when cells were released from tet treatment, demonstrating that export still takes place in hRio2-depleted cells, albeit at a slower rate (Fig. S2 B).

We further analyzed hRio2-depleted reporter cells for the localization of another pre-40S component, hNob1, and observed that hNob1 was strongly accumulated in the nucleoplasm of cells displaying predominantly nuclear hRps2-YFP localization (Fig. 4 A). Thus, nuclear hNob1 localization appears to reflect pre-40S accumulation, supporting the idea that hRio2 depletion affects pre-40S export. Furthermore, several other 40S-associated trans-acting factors, namely hLtv1, hEnp1, and hDim2, also displayed nuclear accumulation in some but not all hRio2-depleted cells (Fig. S3 A).

Similar results were obtained using an hRps3A-YFP reporter cell line (unpublished data), indicating that the observed pre-40S export defects are not specific to hRps2-YFP–expressing cells. Moreover, we investigated the effects of hRio2 depletion in a HeLa Yale (HeLaY) cell line that does not express a reporter protein using the localization of the trans-acting factors hNob1 (Fig. 4 A), hLtv1, hEnp1, and hDim2 (Fig. S3 B) as readouts for pre-40S maturation. Also, in HeLaY cells, RNAi against hRio2 reproducibly led to a mixed phenotype: for hNob1, ∼10% of cells displayed nuclear accumulation; the other cells showed little or no alteration of hNob1 localization (Fig. 4 A). In summary, we conclude that hRio2 contributes to pre-40S export because nuclear accumulation of 40S precursors can be observed in hRps2-YFP and hRps3A-YFP reporter cells as well as in HeLaY cells after

reporter. HeLaK cells were transiently transfected with EGFP, EGFP-NES(Nmd3), or EGFP-NES(Rio2). 24 h after transfection, cells were treated with 20 nM LMB or solvent (ethanol) for 30 min, followed by fixation and fluorescence microscopy. (E) hRio2 directly binds CRM1 in the presence of RanGTP, dependent on its NES. Recombinant zz-hRio2 WT, NESmut, and NESΔ10 were immobilized on IgG Sepharose and incubated with CRM1 in the presence or absence of hRanQ69L-GTP. Bound proteins were analyzed by Coomassie staining. (F) hRio2 WT, NESmut, and NESΔ10 display autophosphorylation activity in vitro. zz-tagged hRio2 constructs were tested for autophosphorylation activity as described in Materials and methods. Bars, 20 μm.

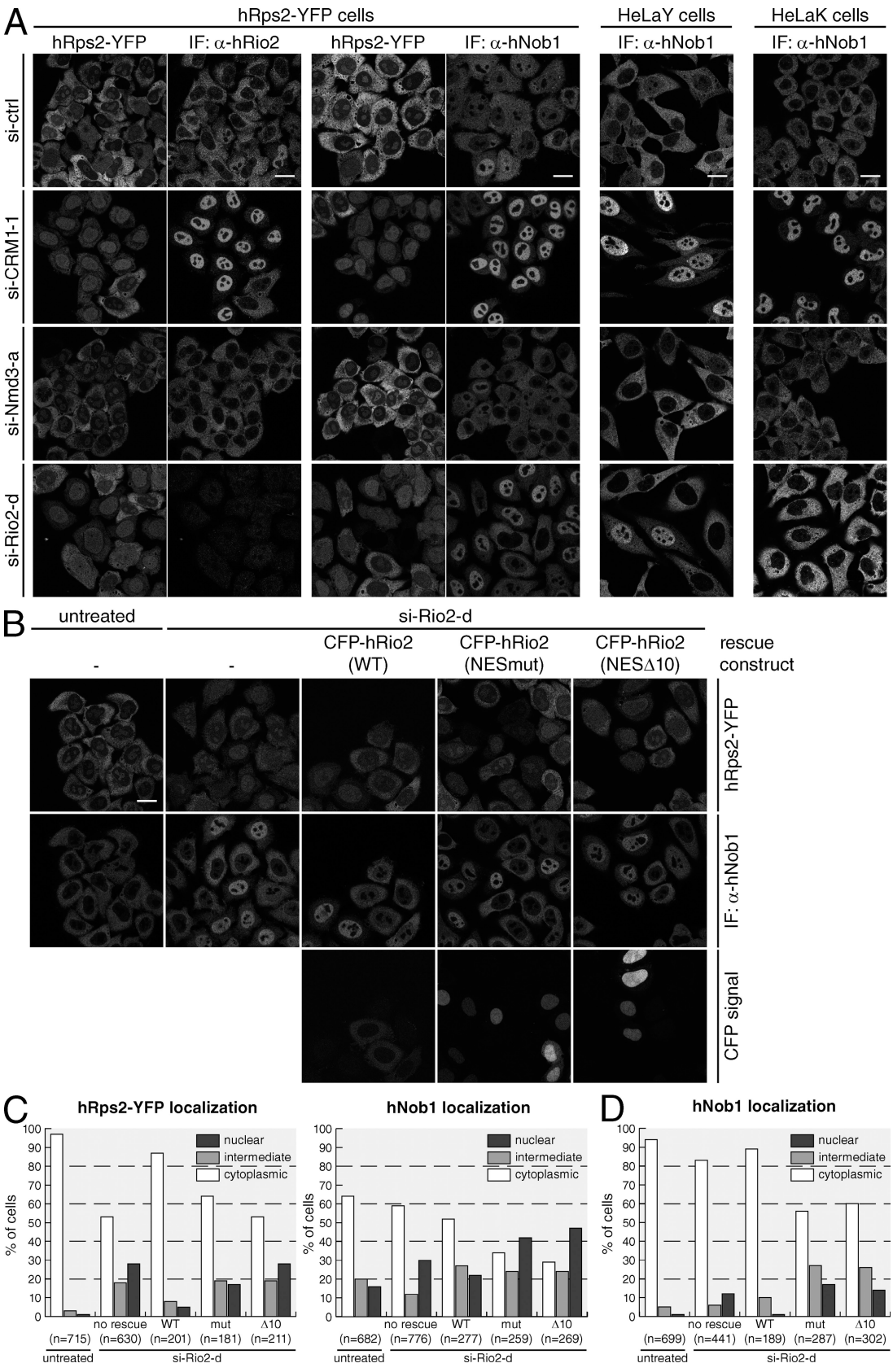


Figure 4. **hRio2 is not absolutely required for pre-40S export but contributes to 40S export efficiency.** (A) hRio2 depletion causes pre-40S export defects in hRps2-YFP reporter cells and HeLaY cells but not in HeLaK cells. Cells were transfected with the siRNAs (si) indicated. In reporter cells, hRps2-YFP expression was induced 35 h after transfection. 48 h after transfection, cells were fixed, followed by immunofluorescence (IF) analysis for hRio2 or hNob1. Note that ~25–30% of reporter cells and 10–15% of HeLaY cells displayed nuclear accumulation of hRps2-YFP or hNob1. ctrl, control. (B) Pre-40S export defects upon hRio2 depletion can be rescued by overexpression of CFP-tagged hRio2(WT) but not hRio2(NESmut) or hRio2(NESΔ10). RNAi experiments were

hRio2 knockdown. Notably, there is no absolute requirement for hRio2 in pre-40S export, which may be explained by the presence of several redundant adapters for CRM1 in 40S precursors (see Discussion).

To ensure that the pre-40S export defects were indeed caused by hRio2 depletion, rescue experiments were performed using EGFP- or CFP-tagged hRio2, which allows for detection of cells in which the rescue construct was expressed. In addition to WT hRio2, we also used the NES mutant and deletion variants of hRio2. As expected, overexpression of CFP-hRio2(WT) rescued the mislocalization of hRps2-YFP in hRio2-depleted hRps2-YFP reporter cells (Fig. 4, B and C). In contrast, nuclear accumulation of hRps2-YFP was only partially rescued by CFP-hRio2(NESmut) and not rescued by CFP-hRio2(NESΔ10), suggesting that the CRM1-binding site of hRio2 indeed contributes to pre-40S export.

When analyzing hNob1 localization in these rescue experiments (Fig. 4, B and C), CFP-hRio2(WT) partially relieved nuclear accumulation of hNob1, whereas CFP-hRio2(NESmut) and CFP-hRio2(NESΔ10) did not rescue hNob1 localization but rather increased the number of cells displaying nuclear hNob1 compared with hRio2-depleted cells. The same rescue experiments were also performed in HeLaY cells and analyzed by hNob1 immunostaining (Fig. 4 D). hNob1 mislocalization caused by hRio2 knockdown was almost completely rescued by EGFP-hRio2. In contrast, EGFP-hRio2(NESmut) or EGFP-hRio2(NESΔ10) did not rescue but instead led to a further increase in nuclear accumulation of hNob1 compared with hRio2-depleted cells. Collectively, our data in both HeLaY and hRps2-YFP cells support a role for hRio2 in pre-40S export that is dependent on its NES. Therefore, hRio2 might serve as an adapter protein for CRM1 in 40S subunit export (see Discussion).

Cytoplasmic maturation of pre-40S particles is stalled in the absence of hRio2

One interesting observation consistent in all hRio2 knockdown experiments in the different cell lines was an obvious cytoplasmic accumulation of 40S trans-acting factors in many cells (Fig. 4 and Fig. S3). This observation was particularly striking in HeLaK cells, in which we did not even observe nucleoplasmic accumulation of these proteins. Thus, HeLaK cells turned out to be a suitable model to further study these effects independently of nuclear export defects. Indeed, immunofluorescence analysis of hRio2-depleted HeLaK cells revealed that ∼90% of cells displayed a partial cytoplasmic accumulation of hEnp1 and hDim2, which are restricted to the nucleus in control cells (Fig. 5 A). Moreover, both hNob1 and hLtv1 appeared to be more strongly cytoplasmic after hRio2 knockdown (Fig. 5 A). Together, these results suggest that in the absence of hRio2, these four trans-acting factors are found on cytoplasmic 40S precursors that are stalled in their maturation.

The yeast homologues of hNob1 and hLtv1 have been described as proteins whose cytoplasmic localization depends on CRM1 (Schafer et al., 2003; Seiser et al., 2006), and our results show that the same applies in human cells because both proteins accumulated in the nucleus of LMB-treated HeLaK cells (Fig. 5 C). We tested whether nucleocytoplasmic shuttling of these factors would be affected after hRio2 knockdown by combining RNAi and LMB treatment. Interestingly, the shuttling kinetics of hNob1 and hLtv1 are slowed down in the absence of hRio2 because both factors displayed more cytoplasmic staining after 1 or 2 h of CRM1 inhibition compared with control cells (Fig. 5 C). This finding is in line with the idea that these trans-acting factors are associated with cytoplasmic 40S precursors after depletion of hRio2. To directly address this point, we analyzed extracts from control and hRio2-depleted cells on sucrose gradients. As shown in Fig. 5 D, hDim2 and hEnp1 were more efficiently extracted from hRio2-depleted cells, which is consistent with their partial relocalization to the cytoplasm. Importantly, hNob1, hLtv1, hEnp1, and hDim2 all cosedimented with pre-40S or 40S subunits in both conditions. Therefore, in cells lacking hRio2, all of the 40S-associated factors tested are bound to 40S precursors stalled in their maturation in the cytoplasm.

hRio2 kinase activity is required for the release of hDim2, hLtv1, and hNob1 but not hEnp1 from cytoplasmic 40S precursors

To determine whether the kinase activity of hRio2 is required for its function in cytoplasmic 40S maturation, we generated a kinase-dead (KD) variant of hRio2. Based on a sequence alignment of the kinase domain of Rio2 from several species with protein kinase A (unpublished data), we identified Lys123 and Asp246 as two conserved residues in the kinase domain of hRio2 that are likely essential for its kinase activity. Indeed, mutation of these residues to Ala led to loss of autophosphorylation activity, and thus, hRio2(K123A,D246A) represents a KD variant, hRio2(KD) (Fig. 6 A). We then used EGFP-hRio2(WT) or EGFP-hRio2(KD) in rescue experiments and analyzed cells for the localization of trans-acting factors. The aberrant partial relocalization of hEnp1 to the cytoplasm upon hRio2 depletion could be rescued by overexpression of either WT or KD EGFP-hRio2 (Fig. 6, B–D). In contrast, only EGFP-hRio2(WT) but not EGFP-hRio2(KD) was able to restore hDim2 localization to nucleoli after hRio2 knockdown (Fig. 6, B–D). For the cytoplasmic proteins hLtv1 and hNob1, rescue experiments were combined with LMB treatment to check whether or not their shuttling behavior could be rescued. EGFP-hRio2(WT) indeed almost fully restored rapid shuttling of both factors (Fig. 6, E and F). However, EGFP-tagged KD hRio2 not only failed to rescue the fast LMB-dependent relocalization of hLtv1 and hNob1 but even exacerbated the retention of both proteins in the

performed using hRps2-YFP reporter cells as in A, but, in addition, cells were transfected with CFP-tagged hRio2 constructs after 24 h of RNAi. (C) Quantification of the experiment shown in B. Cells displaying predominantly nuclear, intermediate, or predominantly cytoplasmic hRps2-YFP or hNob1 localization were counted. For rescues, hRps2-YFP or hNob1 localization was analyzed for transfected cells only. n, number of cells counted (from one representative experiment). (D) Nuclear accumulation of hNob1 in the nucleus of HeLaY cells after hRio2 depletion is rescued by overexpression of EGFP-tagged hRio2 but not hRio2(NESmut) or hRio2(NESΔ10). Rescue experiments using HeLaY cells were performed as in B and analyzed as in C. Bars, 20 µm.

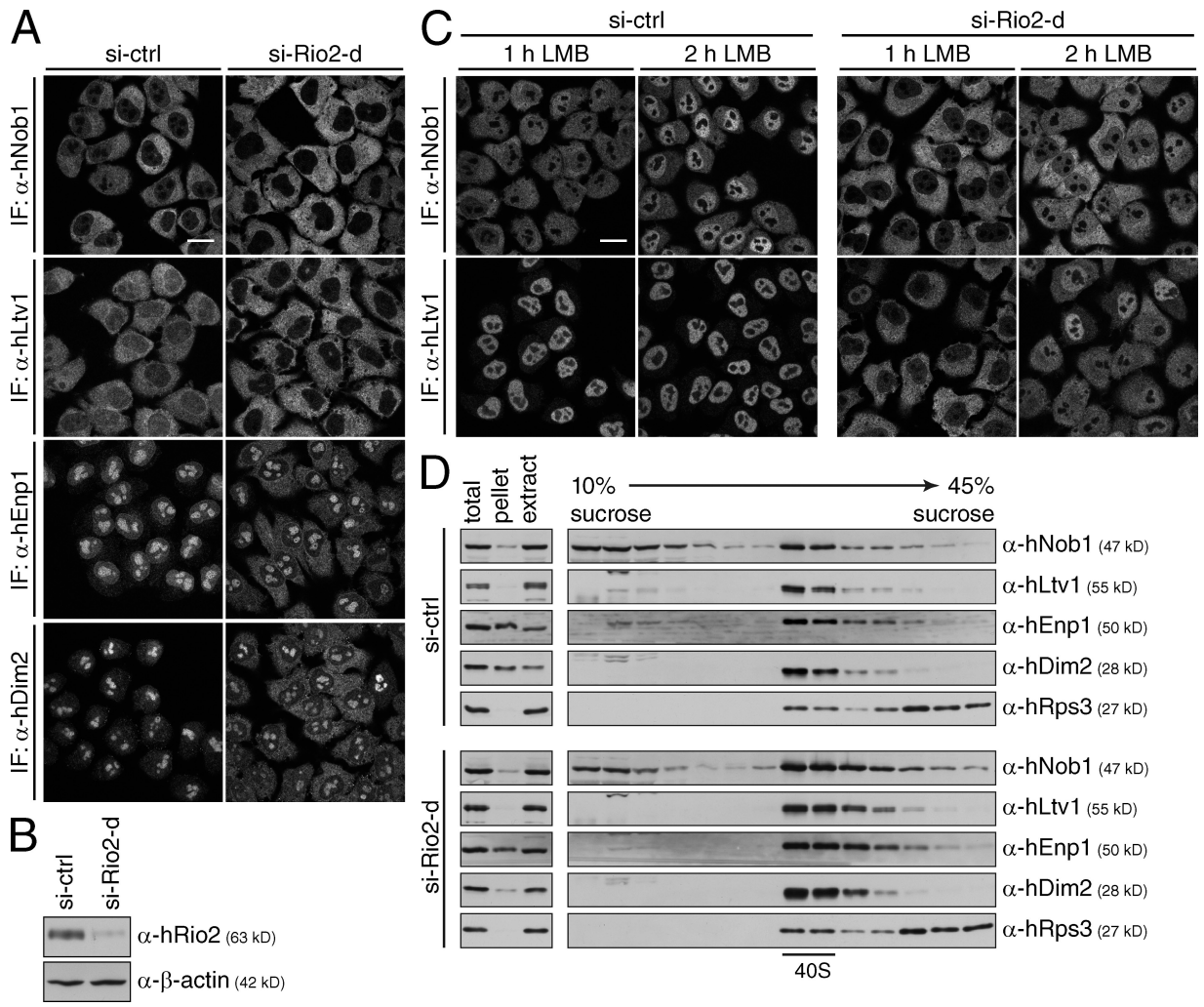
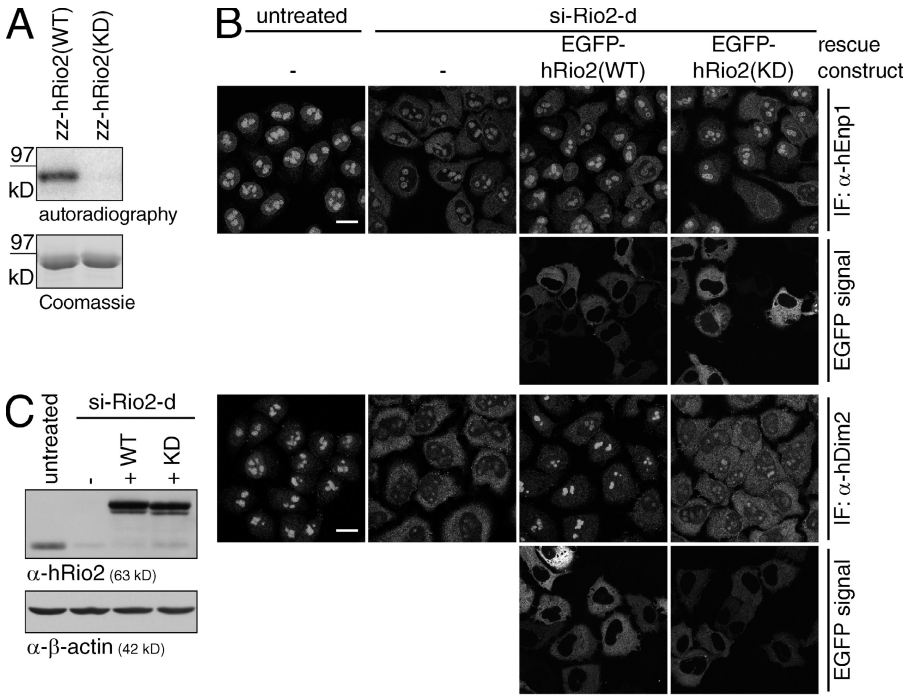


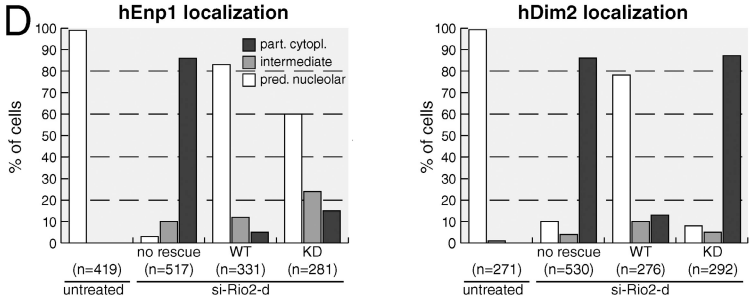
Figure 5. **In the absence of hRio2, the trans-acting factors hNob1, hLtv1, hEnp1, and hDim2 accumulate on cytoplasmic 40S precursors that are stalled in their maturation.** (A) hNob1, hLtv1, hEnp1, and hDim2 accumulate in the cytoplasm of hRio2-depleted cells. RNAi experiments in HeLaK cells were performed as described in Fig. 4 A, followed by immunofluorescence (IF) analysis for hNob1, hLtv1, hEnp1, and hDim2. (B) Western blot analysis of the RNAi experiment in A. (C) hNob1 and hLtv1 display slower shuttling kinetics in hRio2-depleted cells. 48 h after transfection, cells from the RNAi experiment in A were treated with 20 nM LMB for 1 or 2 h, followed by fixation and immunofluorescence analysis for hNob1 and hLtv1. (D) hNob1, hLtv1, hEnp1, and hDim2 are associated with 40S precursors in hRio2-depleted cells. Cell extracts from control and hRio2-depleted cells were centrifuged on a 10–45% sucrose gradient. Samples of total cells, cell extract, and pellet obtained during extract preparation as well as gradient fractions were analyzed by Western blotting. ctrl, control; si, siRNA. Bars, 20 µm.

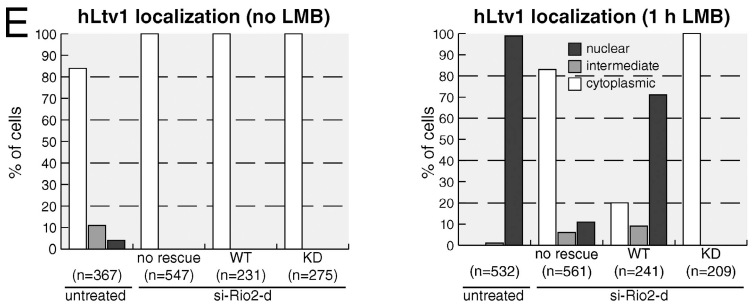
cytoplasm compared with hRio2-depleted cells. In summary, the kinase activity of hRio2 appears to be required for final 40S maturation in the cytoplasm to occur and for the release of hDim2, hLtv1, and hNob1 from 40S precursors for the next round of 40S biogenesis. Surprisingly, hEnp1 recycling only required the physical presence of hRio2 but not its kinase activity, suggesting that hEnp1 is released from 40S precursors at an earlier cytoplasmic maturation step for which the presence of inactive hRio2 is sufficient.

hRio2 kinase activity is required for pre-rRNA processing

Cytoplasmic 40S maturation includes a pre-rRNA cleavage step to produce 18S rRNA from 18S-E pre-rRNA in human cells and from 20S pre-rRNA in yeast, and a role for hRio2/Rio2p in this process has previously been described (Geerlings et al., 2003; Vanrobays et al., 2003; Rouquette et al., 2005). Indeed, using Northern blotting and FISH, we could confirm that hRio2 depletion leads to the accumulation of 18S-E pre-rRNA in the cytoplasm (Fig. 7). To test whether hRio2 kinase activity is required for 18S-E pre-rRNA processing, we performed rescue experiments followed by FISH analysis. EGFP-hRio2(WT) was able to restore pre-rRNA cleavage, as the cytoplasmic accumulation of 18S-E pre-rRNA was no longer detected in EGFP-hRio2–expressing cells (Fig. 7, A and B). In contrast, cells transfected with EGFP-hRio2(KD) still displayed a strong cytoplasmic FISH signal, indicating that 18S-E pre-rRNA processing did not occur in these cells. This was supported by Northern blot analysis, which revealed that EGFP-hRio2(WT) but not EGFP-hRio2(KD) overexpression reduced the RNAi-induced 18S-E pre-rRNA accumulation by ∼35% in the whole cell population, as expected based on the 39% transfection efficiency obtained for this construct (Fig. 7, C and D). Thus, although the physical presence of hRio2 is sufficient for the







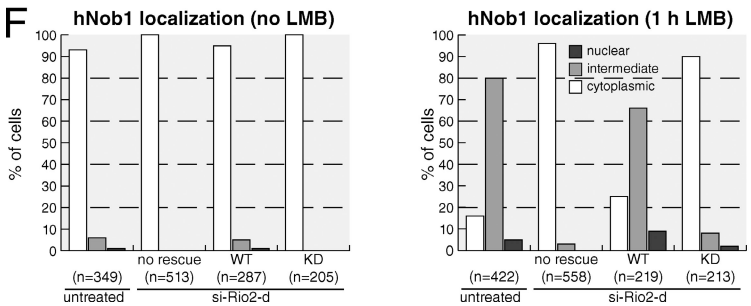


Figure 6. **hRio2 kinase activity is required for the recycling of hDim2, hLtv1, and hNob1 but not hEnp1 from cytoplasmic 40S precursors.** (A) Mutation of Lys123 and Asp246 of hRio2 to Ala results in a KD hRio2 mutant. zz-tagged hRio2(WT) and hRio2(K123A,D246A) (hRio2[KD]) were tested for autophosphorylation activity as described in Materials and methods. (B) In hRio2-depleted cells, KD hRio2 rescues the relocalization to the cytoplasm of hEnp1 but not hDim2. Rescue experiments in HeLaK cells were performed as described in Fig. 4 B using EGFP-hRio2(WT) and EGFP-hRio2(KD) as rescue constructs. (C) Western blot analysis of A revealing efficient hRio2 knockdown by RNAi and similar expression levels of the rescue constructs. (D) Quantification of the experiment shown in A. Cells displaying predominantly nucleolar, intermediate, or partially cytoplasmic hEnp1 or hDim2 localization were counted. For rescues, hEnp1 and hDim2 localization was analyzed for transfected cells only. (E and F) Slower shuttling kinetics of hLtv1 and hNob1 after hRio2 depletion are rescued by WT but not KD hRio2. Rescue experiments were performed as described in B and combined with treatment with 20 nM LMB for 1 h as described in Fig. 5 C. Cells were analyzed by immunostaining for hLtv1 (E) and hNob1 (F) and counted as in Fig. 4 C. For rescues, only transfected cells were counted. n, number of cells counted (from one representative experiment); si, siRNA. Bars, 20 μm.

release of hEnp1 from cytoplasmic 40S precursors, hRio2 kinase activity is necessary for the recycling of hDim2, hLtv1, and hNob1 as well as 18S-E pre-rRNA cleavage. Therefore, cytoplasmic 40S maturation consists of at least two distinct steps, both of which depend on hRio2.

Discussion

Characterization of a late 40S precursor particle from human cells

Although ribosomal precursor particles have been extensively characterized in yeast, little is known about their composition in human cells. Previously, several potential preribosomal particles were purified by immunoprecipitation of epitope-tagged factors (Yanagida et al., 2001; Fujiyama et al., 2002; Hayano et al., 2003). In contrast, we made use of an hRio2-specific antibody, thereby targeting the endogenous protein rather than an overexpressed factor that may show nonspecific interactions. Furthermore, by precipitating hRio2 from pooled 40S fractions rather than nonfractionated extract, we could strongly reduce background binding (unpublished data). Using this approach, we isolated an hRio2-associated particle composed of ribosomal proteins of the small subunit, a series of trans-acting factors, and 18S-E pre-rRNA, the last precursor of mature 18S rRNA, indicating that a late 40S precursor was purified.

The isolated human pre-40S particle contains homologues to most trans-acting factors found in late 40S precursors in yeast (Schafer et al., 2003), namely hTsr1, hLtv1, hRio2, hEnp1, hNob1, and hDim2. In addition, trans-acting factors identified on yeast late 40S preribosomes included the 18S rRNA dimethylase Dim1p and the CK1 (casein kinase 1) isoform Hrr25p. Homologues to these two factors were not present in our MS analysis. However, low levels of hDim1 can be detected in the hRio2 immunoprecipitate by Western blotting (unpublished data). Possibly, hDim1 is only weakly associated with hRio2-containing 40S precursors in human cells. It also remains to be seen whether a CK1 isoform is associated with these particles but escaped MS detection. Clearly though, most if not all pre-40S components found in yeast are also present in human 40S precursors. Conversely, we did not detect 40S-associated trans-acting factors that are specific to human cells and absent in yeast pre-40S particles. Thus, the composition of late 40S preribosomes seems to be highly conserved from yeast to human.

A Leu-rich NES in hRio2 contributes to efficient pre-40S export

hRio2 localizes to the cytoplasm at steady state but rapidly accumulates in the nucleus upon inhibition of CRM1 using LMB (Rouquette et al., 2005; Bonazzi et al., 2007; this study). We could show that hRio2 contains a functional NES located in its C-terminal domain and that hRio2 directly interacts with CRM1 via this NES. As a component of 40S precursors that directly binds CRM1, hRio2 could therefore serve as an adapter for CRM1 in pre-40S export.

To study nuclear export of 40S subunits, we have developed a new experimental approach that allows for a very sensitive readout for defects in 40S biogenesis and export. Using a tet-inducible Rps2-YFP reporter, we are able to specifically detect preribosomes that are newly assembled during the time of tet induction. In comparison with our previous assays relying on rRNA localization by FISH or on noninducible Rps (ribosomal protein of the small subunit) reporters (Thomas and Kutay, 2003), much clearer effects of CRM1 inhibition on 40S export can be observed (Fig. 3).

In this reporter cell line, depletion of hRio2 by RNAi caused nuclear accumulation of hRps2-YFP in ∼25% of cells, indicating a role for hRio2 in pre-40S export. Moreover, in support of the conclusion that the efficiency of pre-40S export is reduced in the absence of hRio2, several trans-acting factors like hNob1 accumulated to some degree in the nucleus both in hRps2-YFP reporter cells and HeLaY cells. hNob1 is a pre-40S component, which is predominantly cytoplasmic at steady state and redistributes to the nucleus upon inhibition of 40S export (Fig. 4). As hNob1 is a component of the 40S biogenesis machinery but not part of the end product, the translating 40S subunit, its localization allows for a kinetic readout on how 40S maturation progresses. Using a series of rescue constructs, we could show that the role of hRio2 in pre-40S export depends on its CRM1-binding site. Upon mutation or deletion of its NES, hRio2 lost the ability to rescue the pre-40S export defects observed in hRio2-depleted hRps2-YFP and HeLaY cells. These NES-deficient rescue mutants of hRio2 even increased the nuclear accumulation of hNob1.

Analogous to the role of hNmd3 in 60S export, the simplest model for the involvement of hRio2, and particularly its NES, in nuclear export of 40S precursors is that hRio2 contributes to efficient pre-40S export as an adapter protein for CRM1. Yet, we have not been able to conclusively demonstrate CRM1 recruitment to 40S precursors via hRio2 (unpublished data). Interestingly, when blocking pre-40S export by CRM1-independent means, namely by RNAi-mediated depletion of hRps15 (Rouquette et al., 2005), hRio2 showed only a slight nuclear accumulation, whereas the majority of the protein remained cytoplasmic (Fig. S4), which is similar to results in yeast (Leger-Silvestre et al., 2004). This might be interpreted such that either hRio2 does not enter the nucleus to participate in the translocation of 40S precursors through nuclear pore complexes or it joins the particle after association of hRps15. Our observation of hRio2 being associated with 40S precursors in LMB-treated cells (Fig. S1, A–C) argues for a nuclear function for hRio2 and supports the conclusion that hRio2 directly acts in pre-40S export. One model reconciling these data would predict that hRio2 association with pre-40S particles is hRps15 dependent and represents one of the very last steps of nuclear 40S maturation, followed by 40S export. In the absence of hRps15, hRio2 would remain a free protein that can directly bind CRM1 and rapidly be exported to the cytoplasm.

Surprisingly, although depletion of hRio2 caused nuclear accumulation of hRps2-YFP as well as several trans-acting factors in our reporter cells, no such defects were observed in HeLaK cells, from which the reporter system was developed over several steps of clonal selection. However, upon RNAi against hRio2 in the cell line bearing the flippase recognition target (FRT) integration site and expressing the tet repressor

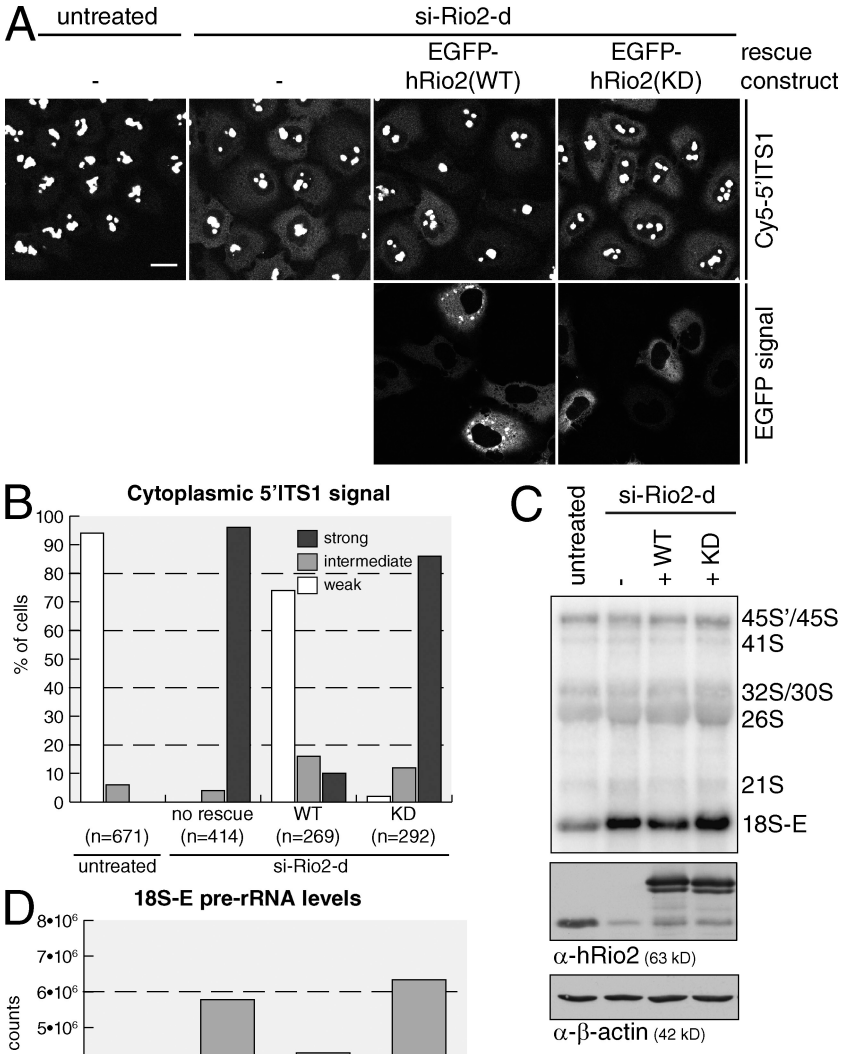


Figure 7. **hRio2 kinase activity is required for cytoplasmic 18S-E pre-rRNA processing.** (A) Cytoplasmic accumulation of 18S-E pre-rRNA after hRio2 knockdown is rescued by WT but not KD hRio2. HeLaK cells were transfected with Rio2-d siRNA (si-Rio2-d). Rescue constructs were transfected after 48 h of RNAi. Cells were fixed after 72 h of RNAi, followed by FISH analysis. FISH pictures were processed in parallel by enhancing levels, followed by setting γ correction to 0.75. Note that the 5′ ITS1 probe shows the localization of all 18S rRNA precursors, but only 18S-E pre-rRNA is detected in the cytoplasm (Rouquette et al., 2005). (B) Quantification of the experiment shown in A. Cells displaying weak, intermediate, or strong cytoplasmic 5′ ITS1 staining were counted. For rescues, only transfected cells were counted. n, number of cells counted (from one representative experiment). (C) 18S-E pre-rRNA accumulation upon hRio2 depletion is rescued by WT but not KD hRio2. Rescue experiments were performed as in A, followed by Northern blot analysis. Western blotting revealed efficient hRio2 knockdown by RNAi and similar expression levels of the rescue constructs. Note that transfection efficiencies were 39% for EGFP-hRio2(WT) and 41% for EGFP-hRio2(KD). (D) 18S-E pre-rRNA levels from the experiment in C were quantified using IQMac (GE Healthcare). Bar, 20 μm.

(TetR), but not yet stably transfected with hRps2-YFP, nuclear accumulation of hNob1 could also be observed (Fig. S2 C). These results indicate that the pre-40S export defects observed in the reporter cells do not represent an artifact caused by hRps2-YFP overexpression. Importantly, pre-40S export defects after knockdown of hRio2 were also found in HeLaY cells.

Clearly though, based on our data as well as published work on hRio2 (Rouquette et al., 2005), there is no absolute requirement for hRio2 in 40S export. One possible explanation for this observation is that 40S precursors may contain several CRM1-binding proteins. Indeed, two other pre-40S components have been suggested to act as adapter proteins for Xpo1p in yeast, namely Dim2p and the nonessential protein Ltv1p (Seiser et al., 2006; Vanrobays et al., 2008). However, evidence for a direct binding of Xpo1p to Ltv1p or Dim2p is lacking. Moreover, as for hRio2, no conclusive evidence has been provided in these previous studies to show that either of these proteins recruits Xpo1p to 40S precursors. Yet, it seems possible that recruitment of CRM1 to export-competent pre-40S particles is a redundant process relying on several CRM1-binding adapter proteins. Indeed, considering that pre-60S export involves at least three export receptors in yeast, it is not surprising that pre-40S export may require more than one export adapter to recruit multiple copies of CRM1.

Therefore, we have also investigated the potential of hLtv1 and hDim2 to serve as CRM1 adapter proteins to the small subunit. hLtv1 directly binds CRM1 via a C-terminal Leu-rich NES (unpublished data). However, cells depleted for both hRio2 and hLtv1 do not display an export defect stronger than that caused by hRio2 depletion alone, and overexpression of an NES-deficient mutant of hLtv1 in human cells does not cause a pre-40S export defect (unpublished data). Collectively, our experiments so far

failed to provide positive evidence for a contribution of hLtv1 to 40S export. Recently, a conserved NES in yeast Dim2p has been implicated in 40S biogenesis and export (Vanrobays et al., 2008). Notably, we can indeed show that hDim2 directly binds CRM1 (Fig. S5); however, mutation of the CRM1-binding site proposed in hDim2 (Vanrobays et al., 2008) did not abolish in vitro CRM1 binding of hDim2 (Fig. S5). For these reasons, the elucidation of the potential roles of hDim2 and hLtv1 in pre-40S export remains a challenging subject for future studies.

hRio2 is required for distinct steps of cytoplasmic 40S maturation

In contrast to the nonessential role of hRio2 in pre-40S export, cytoplasmic 40S maturation strongly depends on hRio2. Our RNAi analysis revealed that hRio2 is necessary for the recycling of four 40S-associated trans-acting factors. Furthermore, by performing rescue experiments with kinase-active and KD hRio2, we could separate two events in cytoplasmic 40S biogenesis.

In a first step, which depends on the physical presence of hRio2 but not on its kinase activity, hEnp1 is released from 40S precursors and reimported into the nucleus. Several models can be put up to explain the requirement for hRio2 in this process. For example, a so far unknown hEnp1 recycling factor may depend on hRio2 to be activated or to associate with 40S precursors. Alternatively, the absence of hRio2 may alter the structure of pre-40S particles such that hEnp1 release is no longer possible. As the kinase activity of hRio2 is not required for this recycling step, it appears that phosphorylation of hEnp1 by hRio2 is not involved in hEnp1 release from the subunit. Interestingly, phosphorylation of yeast Enp1p is known to promote its dissociation from 40S precursors in the course of the formation of the 40S "beak" structure (Schafer et al., 2006). In agreement with our results, this previous study demonstrated a requirement for the casein kinase isoform Hrr25p but not Rio2p in Enp1p phosphorylation. However, the recycling of yeast 40S trans-acting factors from the cytoplasm to the nucleus has not yet been addressed, and it remains to be seen whether Hrr25p-mediated phosphorylation of Enp1p contributes to its recycling to the nucleus. Our results identify an important, kinase-independent role for hRio2 in hEnp1 release from 40S precursors.

In contrast to its function in hEnp1 recycling, hRio2 kinase activity is essential for a second phase of cytoplasmic 40S maturation, in which cleavage of 18S-E pre-rRNA takes place and hDim2, hLtv1, and hNob1 are released from maturing 40S preribosomes. It is still unclear how the kinase activity of hRio2 relates to its function in pre-rRNA processing. hRio2 may phosphorylate and thereby activate the endonuclease itself or a cofactor of the endonuclease. Alternatively, hRio2 kinase activity could be required for a conformational change in the 40S precursor that is a prerequisite for endonuclease activity, or for the removal of an inhibitory factor. Indeed, it is tempting to speculate that hRio2 triggers the release of hDim2, hLtv1, and/or hNob1 by phosphorylating one or several of these factors, which, in turn, allows pre-rRNA processing to occur. Unraveling the molecular mechanism of hRio2 function will likely require knowledge of the substrate of hRio2 kinase activity, but despite our attempts to identify hRio2 phosphorylation targets, they remain elusive to date.

In summary, we could show that late maturation of 40S preribosomes depends on hRio2 at several distinct steps. hRio2 supports pre-40S export efficiency and may act as an adapter protein for CRM1. After nuclear export, hRio2 but not its kinase activity is required for release of hEnp1 from 40S precursors. Then, phosphorylation of an unknown target by hRio2 leads to the recycling of hDim2, hLtv1, and hNob1 and to 18S-E pre-rRNA processing. Therefore, cytoplasmic 40S biogenesis consists of at least two steps, both of which depend on hRio2. Further analysis of other trans-acting factors involved in this process may lead to the identification of additional maturation events, which collectively allow 40S subunits to reach translation competence.

Materials and methods

Cell lines and reagents

HeLaY and HeLaK cells were gifts from A. Helenius and D. Gerlich (ETH Zurich, Zurich, Switzerland). HeLaK and HeLaY cells differ in their morphology and growth behavior, with HeLaK cells growing faster and patchier than HeLaY. The tet-inducible hRps2-YFP reporter cell line was established based on a HeLaK cell line bearing H2B-mRed and an FRT integration site (gift from D. Gerlich and P. Meraldi [ETH Zurich]) by first stably integrating an HA-tagged TetR using a pIRESpuro plasmid (gift from A. Hayer [ETH Zurich] and A. Helenius), resulting in the HeLaK FRT TetR cell line. Then, hRps2-YFP (cloned into the HindIII and NotI sites of pcDNA/FRT/TO; Invitrogen) was integrated into the FRT site by cotransfection with pOG44 (Invitrogen). LMB was purchased from LC Laboratories.

Antibodies

Antibodies were raised against purified recombinant His-tagged proteins and affinity purified using the respective antigens coupled to SulfoLink (Thermo Fisher Scientific). Peptide-specific antibodies against a C-terminal peptide of hNmd3 (C-DLHISQDATGEEGASMLT) and a C-terminal peptide of hLtv1 (C-RQEKELLNLKKNVEGLKL) were also raised in rabbits and purified as described above. Peptide-specific α-hLtv1 antibody was used for Western blotting, and anti–recombinant hLtv1 was used for immunofluorescence. α-CRM1 (Lund et al., 2004) and α-Rpl23A (Pool et al., 2002; Thomas and Kutay, 2003) antibodies have been previously described. Anti–β-actin was purchased from Sigma-Aldrich, anti-hRpl10 was purchased from Santa Cruz Biotechnology, Inc., and secondary antibodies for immunofluorescence were purchased from Invitrogen.

Molecular cloning, protein expression, and purification

Coding regions of hDim2, hEnp1, hLtv1, hNob1, hRio2, hRps2, and hRps3 were amplified by PCR from HeLaY cell cDNA. For expression in *Escherichia coli*, fragments were cloned into pQE (QIAGEN) or pET (EMD) derivatives. For transient transfection in HeLa cells, hRio2 was cloned into the XhoI–BamHI sites of pEGFP-C1 (Clontech Laboratories, Inc.) or an analogous pCFP vector. Note that three individual cDNA clones of hRio2 contained the same three nucleotide changes, leading to one amino acid change compared with the GenBank/EMBL/DDBJ database sequence (NM_018343; from Arg to Gly at position 349).

Site-directed mutagenesis of hRio2 (L400A and I403A [NESmut] or K123A and D246A [KD hRio2]) was performed using the QuikChange kit (Agilent Technologies). Deletion of the NES of hRio2 (NESΔ10 construct) was performed using PCR fragments amplified from the full-length hRio2 construct. aa 309–408 of hRio2 were replaced by a Gly-Thr sequence based on a KpnI site used for cloning. Coding sequences for aa 388–410 of hRio2 and 475–493 of hNmd3 were inserted into the XhoI–BamHI sites of pEGFP-C1.

zz-hRio2, zz-hRio2 mutants, and zz-hEnp1 were expressed in BLR(pRep4) cells at 25°C. Cells were harvested by centrifugation and lysed by sonication in 50 mM Tris/HCl, pH 7.6, 700 mM NaCl, 5 mM $MgCl_2$, and 5% (vol/vol) glycerol. The lysate was ultracentrifuged and passed over nickel–nitrilotriacetic acid agarose (QIAGEN), and the protein was eluted with 400 mM imidazole in lysis buffer. Purified proteins were rebuffered to 50 mM Tris/HCl, pH 7.6, 200 mM NaCl, and 5 mM $MgCl_2$. Recombinant hRanQ69L-GTP and CRM1 were prepared as previously described (Muhlhausser et al., 2001).

Transient transfections and RNAis

Transient transfections were performed using FuGENE reagent (Roche). After 24 h, cells were fixed in 4% PFA in PBS, washed, and mounted for confocal microscopy. For RNAi experiments, the following siRNA oligonucleotides (Microsynth) were used (sense sequence): si-CRM1-1 (5′-UGUGGUGAAUUGCUUAUAC-3′) and si-Nmd3-a (5′-GAAUGGUGCUAUCCUUCAA-3′) at 35 nM concentration and si-Rio2-d (5′-GGAUCUUGGAUAUGUUUAA-3′) at 17.5 nM. As a negative control, AllStars (QIAGEN) was used at 35 nM. Note that si-Rio2-d targets the 3′ untranslated region of hRio2 mRNA, allowing rescue experiments with WT hRio2. siRNAs were transfected using Oligofectamine (Invitrogen). After 48 h of RNAi, cells were fixed for immunofluorescence analysis or harvested for Western blotting, extract preparation, or RNA isolation. In rescue experiments, cells were transfected with rescue constructs 24 h before harvest/fixation. In hRps2-YFP cells, reporter protein expression was induced with 0.125 µg/ml tet for 13 h before harvest/fixation.

Immunofluorescence analysis

Cells were fixed in 4% PFA and permeabilized in 0.1% Triton X-100 and 0.02% SDS in PBS for 5 min. After blocking with 10% goat serum in 2% BSA/PBS for 30 min, primary antibodies diluted in blocking solution were added for 1 h. Cells were washed three times for 4 min in 2% BSA/PBS and incubated with secondary antibody (Alexa Fluor 488– or Alexa Fluor 633–labeled goat anti–rabbit antibody) diluted in blocking solution for 30 min. Cells were again washed three times, incubated briefly in 0.1% Triton X-100 and 0.02% SDS in PBS, and fixed with 4% PFA. After washing with PBS, coverslips were mounted in VectaShield (Vector Laboratories) for confocal microscopy.

Confocal microscopy

Images from fixed transfected or immunostained cells were taken at RT with a confocal scanning system (SP2; Leica) using a 63× NA 1.4 differential interference contrast oil HCX Plan-Apochromat objective and the confocal software (Leica). Excitation at 405 nm was used for CFP fluorescence, 488 nm for EGFP, YFP, or Alexa Fluor 488, and 633 nm for Alexa Fluor 633. γ correction for FISH pictures was performed using Photoshop (Adobe).

CRM1-binding assay

200 pmol of purified bait proteins was immobilized on IgG Sepharose (GE Healthcare) in 50 mM Tris/HCl, pH 7.6, 175 mM KCH_3CO_2, 2 mM $MgCl_2$, and 0.001% (vol/vol) Triton X-100. Beads were incubated with 1 µM CRM1 and 0.16 mg/ml BSA in a volume of 100 µl in the presence or absence of 3 µM hRanQ69L-GTP. Binding reactions were incubated on ice for 2 h. After washing three times, beads were transferred to spin columns (Mobicol; Life Systems Design), washed once with 50 mM Tris/HCl, pH 7.6, and eluted in SDS-PAGE sample buffer.

Kinase assays

10-µl reactions containing 1 µg of purified protein and 1 µCi γ-[^{32}P]ATP in 50 mM Tris/HCl, pH 7.6, 200 mM NaCl, and 5 mM $MgCl_2$ were incubated at 30°C for 30 min. Reactions were stopped by the addition of 30 µl of SDS sample buffer and analyzed by SDS-PAGE, and the gels were dried and analyzed by phosphoimaging.

Extract preparation

For extract preparation, cells were incubated for 10 min with 100 µg/ml cycloheximide (Sigma-Aldrich), washed once with PBS, detached with PBS containing 0.5 mM EDTA and 100 µg/ml cycloheximide, and centrifuged (2000 *g* for 5 min at 4°C). Cells were washed with 10 mM Tris/HCl, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, and 50 µg/ml cycloheximide, centrifuged again, and resuspended in the same buffer containing 1 mM EGTA, 1 mM DTT, 0.05% Triton X-100, and protease inhibitors. After incubation on ice for 20 min, lysis was performed by passing cells through a 27-gauge needle. After centrifugation (1000 *g* for 5 min at 4°C), the supernatant was used as cell extract. For Fig. S1 (A–C), the extraction of cells was performed with 50 mM Tris/HCl, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40, 50 µg/ml cycloheximide, and protease inhibitors.

Sucrose gradient analysis

For analysis of RNAi experiments, cell extracts (400 µg of total protein) were loaded onto linear 10–45% sucrose gradients in 50 mM Hepes/KOH, pH 7.5, 100 mM KCl, and 3 mM $MgCl_2$ and centrifuged for 2 h at 55,000 rpm at 4°C in a TLS55 rotor (Beckman Coulter). 160-µl fractions were collected, precipitated with TCA, and analyzed by Western blotting. To analyze the association of hRio2 and hEnp1 with ribosomal particles, cell extract (1 mg total protein) was loaded on 10–45% sucrose gradients and centrifuged for 3 h at 40,000 rpm at 4°C in an SW41 rotor (Beckman Coulter). Fractions were collected and precipitated with TCA for Western blotting. To prepare pooled 40S fractions for immunoprecipitation, HeLa cell low salt extract (Kutay et al., 1998) was fractionated on 10–45% sucrose gradients by centrifugation for 13 h and 50 min at 18,600 rpm at 4°C in an SW32 rotor (Beckman Coulter). 40S-containing fractions were pooled.

Immunoprecipitation

hRio2- and hNmd3-specific antibodies coupled to a 9:1 mixture of protein A/protein G–Sepharose beads (GE Healthcare) were incubated with pooled 40S fractions in 50 mM Tris/HCl, pH 7.6, 225 mM KCH_3CO_2, 2 mM $MgCl_2$, and 0.001% (vol/vol) Triton X-100. After washing, beads were eluted with 0.2 M Gly/HCl, pH 2.2. Eluates were precipitated with TCA and analyzed by SDS-PAGE. Bands were cut from the gel, digested with trypsin, and analyzed by MS at the Functional Genomics Center Zurich.

Northern blotting

RNA was isolated from HeLaK cells using the RNeasy Mini kit (QIAGEN). 2 µg RNA was separated on a 1% agarose gel in 30 mM triethanolamine, 30 mM tricine, and 1.25% formaldehyde, transferred to a Hybond-N+ membrane (GE Healthcare) in 0.5× TBE buffer (45 mM Tris base, 44 mM boric acid, and 1 mM EDTA) by electrotransfer, and UV cross-linked to the membrane. Equal loading of the samples was analyzed by ethidium bromide staining. The membrane was prehybridized in 0.5 M Na phosphate, pH 7.2, 1 mM EDTA, 7% SDS, and 1% BSA for 30 min and hybridized with a radiolabeled 5′ ITS1 probe (5′-CCTCGCCCTCCGGGCTCCGTTAATGATC-3′) in the same buffer at 45°C overnight. After rinsing twice with 2× SSC/0.1% SDS and washing once for 10 min with 1× SSC/0.1% SDS, the membrane was exposed for phosphoimaging.

FISH

FISH was performed as described previously (Rouquette et al., 2005) using a 5′ Cy5–labeled, 5′ ITS1–specific probe (5′-CCTCGCCCTCCGGGCTCCGTTAATGATC-3′; Microsynth).

Online supplemental material

Fig. S1 shows sucrose gradient analyses to reveal pre-40S association of hRio2 and hEnp1 in LMB-treated cells as well as incorporation of hRps2-YFP into ribosomes in the reporter cell line. Fig. S2 shows Western blot analysis for the experiments of Fig. 4 A and a tet release experiment in the reporter cell line as well as the analysis of pre-40S export defects upon hRio2 depletion in HeLaK FRT TetR cells. Fig. S3 demonstrates that hRio2-depleted hRps2-YFP and HeLaY cells display recycling defects. Fig. S4 shows the effect of hRps15-depletion on hRio2 localization in HeLaK cells. Fig. S5 demonstrates that mutation of Ile153A, Leu155, and Ile158 of hDim2 to Ala does not abolish its CRM1-binding activity. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904048/DC1.

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