The NIP7 protein is required for accurate pre-rRNA processing in human cells

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ABSTRACT

Eukaryotic ribosome biogenesis requires the function of a large number of trans-acting factors which interact transiently with the nascent pre-rRNA and dissociate as the ribosomal subunits proceed to maturation and export to the cytoplasm. Loss-of-function mutations in human trans-acting factors or ribosome components may lead to genetic syndromes. In a previous study, we have shown association between the SBDS (Shwachman–Bodian–Diamond syndrome) and NIP7 proteins and that downregulation of SBDS in HEK293 affects gene expression at the transcriptional and translational levels. In this study, we show that downregulation of NIP7 affects pre-rRNA processing, causing an imbalance of the 40S/60S subunit ratio. We also identified defects at the pre-rRNA processing level with a decrease of the 34S pre-rRNA concentration and an increase of the 26S and 21S pre-rRNA concentrations, indicating that processing at site 2 is particularly slower in NIP7-depleted cells and showing that NIP7 is required for maturation of the 18S rRNA. The NIP7 protein is restricted to the nuclear compartment and co-sediments with complexes with molecular masses in the range of 40S–80S, suggesting an association to nucleolar pre-ribosomal particles. Downregulation of NIP7 affects cell proliferation, consistently with an important role for NIP7 in rRNA biosynthesis in human cells.

INTRODUCTION

Biogenesis of eukaryotic ribosomes involves synthesis and assembly of four ribosomal RNAs (rRNA) with about 80 ribosomal proteins mediated by more than 170 trans-acting factors. A significant amount of the cell energy is devoted to the maturation of three of the four eukaryotic rRNA molecules (18S, 5.8S and 25/28S) which are generated from a single transcript containing the sequences of the mature rRNAs flanked by spacer sequences. During rRNA maturation, the spacer sequences are excised by a series of endo and exonucleolytic cleavages and, at specific positions defined by sequence complementarity to snoRNAs, bases or riboses are modified by methylation and uridines are converted to pseudouridines (1–4). The pre-rRNA processing pathway is best characterized in the Saccharomyces cerevisiae model system. In wild-type strains, pre-rRNA maturation follows a 5' to 3' processing hierarchy where the 5' ETS (external transcribed spacer sequence) is cleaved before processing in ITS1 (internal transcribed spacer sequence 1), which in its turn is cleaved before ITS2 (5–7).

Most of the yeast ribosome biogenesis factors have counterparts in higher eukaryotes, although not all the data obtained from the yeast system can be transferred to vertebrates. For instance, pre-rRNA maturation does not follow the 5' to 3' processing hierarchy in vertebrates. Instead, the order of processing depends on the species, cell type and physiological state. The mammalian pre-rRNA can be processed by simultaneous alternative pathways (8,9), following an initial cleavage that takes place at the site A' of the 47S pre-rRNA generating the 45S pre-rRNA. The three major pathways differ in the place of the first processing step in the 45S pre-rRNA. In pathway A, the first cleavage takes place at Site 1, in

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pathway B, at Site 2c and, in pathway C, at Site 4b (Figure 1). The pathway has continuously been reviewed and novel intermediates and a cleavage site in the 5'-ETS have recently been described for mouse and human cells (10,11).

Expression of genes encoding ribosome biogenesis factors (termed Ribi regulon) is tightly regulated, showing a coordinated response to nutrient supply, physiological conditions or genetic perturbations (12–15). Transcription of this group of genes requires concerted action of the three RNA polymerases which in animals is mediated by the transcription factor Myc (16–19). Synchronized expression of the Ribi regulon involves also feedback regulation by several ribosomal proteins (20–22). Accurate synthesis of ribosomes is critical to all cells. Mutations in S. cerevisiae genes required for ribosome biogenesis usually interfere with the order of pre-rRNA processing steps, causing accumulation of aberrant pre-rRNAs or fast degradation of pre-rRNA intermediates. Pre-rRNA processing defects can also lead to imbalance of the 40S/60S subunit ratio or affect subunit export to the cytoplasm. In humans, mutations causing loss of function in genes encoding structural proteins or ribosome biogenesis factors may lead to genetic syndromes. Currently, there are five known syndromes associated with mutations in such genes. A series of mutations in genes encoding both 40S subunit (23–25) and 60S subunit proteins (26–28) have been linked to the

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**Figure 1.** Schematic representation of the human pre-rRNA processing pathways. The 47S pre-rRNA is converted to the 45S pre-rRNA following the initial cleavages at sites A in the 5'-ETS and at site 6, at the 3'-end of the mature 28S rRNA. The 45S is processed by three alternative pathways. In pathway A, the first cleavage in 45S takes place at site 1, generating the 41S pre-rRNA whose 5'-end corresponds to the 5'-end of the mature 18S rRNA. The 41S pre-rRNA is subsequently processed at site 2c, separating the 21S pre-rRNA, from the 32S pre-rRNA. The 3'-end of the 21S pre-rRNA is processed to generate the 18S rRNA. The 32S pre-rRNA is cleaved at site 4b, generating the 12S pre-rRNA and the 28S rRNA. Finally, the 3'-end of the 12S pre-rRNA is processed further to generate the mature 5.8S rRNA. In pathway B, the first cleavage in 45S takes place at site 2c, generating the 34S and 32S pre-rRNAs. The 34S pre-rRNA is subsequently processed at site 1, generating the 21S pre-rRNA. The 3'-end of the 21S pre-rRNA is processed further to generate the 18S rRNA. The 32S pre-rRNA is processed as described for pathway A. In pathway C, the first cleavage in 45S takes place at site 4b, generating the 26S and the 28S rRNA. The 37S pre-rRNA is subsequently processed at site 1, generating the 26S pre-rRNA, which is subsequently cleaved at site 2 generating the 18S rRNA and the 17S pre-rRNA. This pre-rRNA is processed at site 3 generating the 12S pre-rRNA and the 3'-end of the 12S pre-rRNA is processed to generate the mature 5.8S rRNA. This representation of the pathways was based on the papers published by Bowman et al. (8) and Hadjiolova et al. (9). Open arrowheads indicate the subsequent cleavage sites. Dark arrowheads (P1–P7) underneath the 47S pre-rRNA indicate the positions of the oligonucleotide probes used in northern blot and the open arrows (PE1–PE3) indicate the oligonucleotide probes used in primer extensions analyses, respectively.
Diamond Blackfan Anemia (DBA). In addition, Dyskeratosis Congenita has been associated with mutations in the X-linked DKC1 gene (29), encoding dyskerin, the pseudouridine synthase component of box H/ACA snoRNP. Deficiency in dyskerin abrogates pseudouridylation of rRNA and affects translation initiation of mRNAs containing internal ribosomal entry sites (IRES) (30,31). Mutations in the RNA component of the RNase MRP have been linked to Cartilage–Hair hypoplasia (CHH) (32). Mutations in the gene encoding the protein treacle are linked to the Treacher Collins syndrome. Treacle interacts with RNA polymerase I and Nop56, a subunit of the box CD snoRNP, and is involved in rRNA methylation (33,34). The Shwachman-Bodian-Diamond syndrome (SDS) is associated to mutations in the SBDS gene (35). Studies using both yeast and human cells have shown that SBDS orthologs are involved in ribosome biosynthesis and function (36–38). Dysregulation of ribosome biosynthesis and translational capacity has also been associated to pathological conditions such as human breast cancer (39).

The S. cerevisiae NIP7 protein is required for pre-rRNA processing and ribosome biosynthesis (40). NIP7 orthologs are highly conserved, ranging from 160 to 180 amino-acid residues and sharing a two-domain architecture with the C-terminal region corresponding to the PUA domain (named after pseudouridine synthases and archaeosine-specific transglycosylases) with a predicted RNA-interaction activity (41) that in the case of the Pyrococcus abyssi and S. cerevisiae NIP7 orthologs was already confirmed (42). The human NIP7 interacts with Nop132 (43), the putative ortholog of the S. cerevisiae Nop8p that is also involved in ribosome biogenesis (44). Furthermore, the human NIP7 was found in complexes isolated by affinity-tagged purification of RPS19 (45), a component of the 40S subunit that plays an essential role in its synthesis (23), and of parvulin (Par14), a peptidyl-prolyl cis–trans isomerase (PPiase) required for pre-rRNA processing (46). In a previous work, we have detected the interaction of NIP7 with SBDS in the yeast two-hybrid system (47). In addition, an immobilized GST–SBDS fusion protein was able to pull-down NIP7 from HEK293 cell extracts, indicating that both proteins can be part of a multisubunit complex (47).

Although the evidence above points to a role for the human NIP7 protein in ribosome biosynthesis, so far there is no report on the functional analysis of this protein. In this work, we used RNA interference to downregulate NIP7 and investigate its function in human cell lineages. We show that downregulation of NIP7 affects cell proliferation and causes imbalance of the 40S/60S subunit ratio. We also identified defects at the pre-rRNA processing level with a decrease of the 34S pre-rRNA concentration and an increase of the 26S and 21S pre-rRNA concentrations, suggesting that processing at site 2 is particularly slower in NIP7-depleted cells. The results presented in this work suggest that NIP7 is required for accurate processing of the pre-rRNAs leading to 18S rRNA maturation and 40S subunit biogenesis. At least part of the pre-rRNA processing defects caused by NIP7 downregulation have been described for other situations in which ribosome biogenesis was impaired, such as treatment with leptomycin B, which inhibits exportin Crm1/Xpo1 and blocks ribosome subunit export from the nucleus, and for downregulation of 40S biogenesis factors (11). The NIP7 protein is restricted to the nuclear compartment and co-sediments with complexes with molecular masses in the range of 40S–80S suggesting an association to nucleolar pre-ribosomal particles. This work shows that NIP7 plays a critical role in pre-rRNA maturation in human cells.

**MATERIALS AND METHODS**

**Plasmid construction**

DNA cloning was performed using the Escherichia coli strain DH5α which was maintained in LB medium containing 50 mg ml⁻¹ of the required antibiotic used in transformant selection and manipulated according to standard techniques (48). The NIP7 540-bp coding sequence was isolated from pTL1-HSNIP7 (47) using the EcoRI/SalI restriction sites and inserted into the pET28a plasmid, generating pET28-HSNIP7. The NIP7 shRNA target was 21-residue oligonucleotide whose sequence was selected based on the Ambion siRNA Target Finder. The sequences of the oligonucleotides NIP7-shRNA-F and NIP7-shRNA-R used to generate the shRNA against the NIP7 mRNA and of the oligonucleotides scrambled shRNA-F and scrambled shRNA-R to generate the control scrambled shRNA, scRNA, is given in Table 1. The annealed oligonucleotides were cloned into pMaleficent (49) previously digested with Esp31/EcoRI, generating pMaleficent-shRNA-HSNIP7 and pMaleficent-scRNA-HSNIP7, respectively.

**Cell culture methods and RNA interference strategies**

HEK293 cells (ATCC number CRL-1573) were maintained in MEM (Minimum Essential Alfa Medium, Gibco-BRL) supplemented with 10% fetal bovine serum, 100 U.ml⁻¹ penicillin and 100 μg.ml⁻¹ streptomycin. The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. To generate permanent cells with downregulation of NIP7, we employed a mammalian transposon system designated pMaleficent/ pCMVHSB#17 (49). HEK293 transfections were performed by electroporation as described by Klan and Steinhiber (50). Following transfection, HEK293 cells were transferred into six-well plates and kept under genetin selection (700 μg.ml⁻¹). Proliferation rates of HEK293 derivative cells were determined using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetra-zolium bromide) assay previously described (47). Downregulation of NIP7 in HeLa cells was achieved by using transient transfections of siRNA. For each assay, 1.5 × 10⁵ cells (~60–80% confluence) were harvested in OPTI MEM (Invitrogen) and transfected with 5 or 10 nM siRNA oligonucleotides (NIP7-siRNA-F and NIP7-siRNA-R, Table 1) using 0.5 μl/ml lipofectamine RNAiMax Transfection Reagent (Invitrogen) following the
manufacturer’s instructions. Parallel control transfection assays were performed with scrambled ‘AllStars Negative Control siRNA’ (Qiagen); Cells were plated and cultured for 12 h in antibiotic free medium. Medium was changed and cells cultured for 36–96 h in complete fresh medium. For proliferation analysis of transfected HeLa and MCF10A cells, triplicates at a density of 1500 cells/well were plated and cultured for 4 h in antibiotic free medium. Medium was changed for 4 h at 37°C. Luminescent quantification was performed using a spectrophotometer (Cytofluor), excitation 590/25 nm; emission 530/25 nm. Background was measured using a spectrophotometer (Cytofluor), excitation 590/C14-C2. Luminescent quantification was performed on a FACSCalibur (BD Biosciences) equipped with a 488-nm argon laser. The fluorescence was measured using the FL2-Area and FL2-Width parameters. Data acquisition was performed using CellQuest software (BD Biosciences) and analysis using ModFit software (Verity Software). FACS analyses were performed with the permanent HEK293 derivative cells (SC, which express the scrambled shRNA and, CP4 cells that express the scrambled siRNA against the NIP7 mRNA) and with MCF10A cells transiently transfected with either a scrambled siRNA or a NIP7 siRNA 48 h after transfection.

### Analysis of NIP7 mRNA levels

NIP7 mRNA downregulation was confirmed by RT-qPCR using oligonucleotides complementary to NIP7 sequence (NIP7-shRNA-F and NIP7-shRNA-R). One microgram of total RNA was used for cDNA synthesis using the SuperScript II Reverse Transcriptase system (Invitrogen). The mRNA levels were quantified using the SuperScript II Reverse Transcriptase system (Invitrogen). The mRNA levels were quantified using the SuperScript II Reverse Transcriptase system (Invitrogen). The mRNA levels were quantified using the SuperScript II Reverse Transcriptase system (Invitrogen). The mRNA levels were quantified using the SuperScript II Reverse Transcriptase system (Invitrogen). The mRNA levels were quantified using the SuperScript II Reverse Transcriptase system (Invitrogen). The mRNA levels were quantified using the SuperScript II Reverse Transcriptase system (Invitrogen). The mRNA levels were quantified using the SuperScript II Reverse Transcriptase system (Invitrogen). 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mRNA levels were normalized to the amount of the HPRT and 36B4 mRNAs, respectively, which were determined using primers HPRT-F and HPRT-R or 36B4-F and 36B4-R, respectively. The sequences of all primers used in this work are given in Table 1. The NIP7 protein levels were determined by immunoblotting as described below.

**Sucrose gradient and cell fractionation**

Polysomes profiles were analyzed on sucrose gradients as previously described (51). The cells were cultivated up to 50% confluence. Following addition of 100 μg/ml-1 cycloheximide, 5 × 10⁷ cells were collected and lysed using 500 μl of polysome buffer (PB) containing 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 1% v/v Triton X-100 and 100 μg/ml-1 cycloheximide. Extracts were clarified by centrifugation at 20 000 g for 10 min at 4°C. Totals of 255 OD₂₆₀ units (300 μl) were loaded onto linear sucrose gradients (15–50%) prepared in PB. Polysomes were separated by centrifugation at 40 000 rpm for 4 h at 4°C using a Beckman SW41 rotor. Gradients were fractionated by monitoring absorbance at 254 nm. Protein precipitation and removal of sucrose for immunoblot analyses was performed as follows: 150 μl of each sucrose gradient fraction were mixed with 600 μl of methanol and subsequently mixed with 150 μl of chloroform; 450 μl of water were added to the mix and centrifuged at 20 000 × g for 5 min at 4°C. The aqueous layer was discarded and the pellet washed with 650 μl of methanol, followed by centrifugation as described above. The liquid was discarded and the pellet was taken up in protein sample buffer (48) and analyzed by immunoblotting. For isolation of nuclear and cytoplasmic extracts, 2 × 10⁷ cells were washed in ice cold PBS and harvested by centrifugation at 500 × g for 5 min at 4°C. Cells were lysed in 1 ml LSB (10 mM Tris–HCl pH 7.4, 320 mM sucrose, 2 mM MgCl₂, 3 mM CsCl₂, 0.4% NP-40, 1 mM DTT) containing a protease inhibitor cocktail (Roche) for 12 min on ice. Subsequently, the lysate was centrifuged at 800 g for 2 min at 4°C, the supernatant was collected and centrifuged at 20 000 × g for 10 min and the resulting supernatant was saved as the cytoplasmic extract. The pellet from the 800 × g centrifugation, containing the nuclei, was washed once with LSB and the nuclear proteins extracted in 300 μl HSB (250 mM NaCl, 50 mM Tris–HCl pH 8.0, 5 mM EDTA pH 8.0, 0.5% NP-40) containing a protease inhibitor cocktail (Roche) and 240 U of RNaseOUT (Invitrogen). The suspension was centrifuged at 20 000 × g for 10 min at 4°C and the supernatant saved as the nuclear fraction. Totals of 10 OD₂₆₀ units of nuclear extracts were fractionated on 15–50% sucrose gradients prepared in polysome buffer as described above. For immunoblotting, aliquots of sucrose gradient fractions were processed as described above.

**Immunoblot analysis**

Proteins were resolved by SDS–PAGE and transferred to PVDF membranes at 80 mA for 1 h in buffer containing 25 mM Tris–base, 200 mM glycine and 20% methanol. Subsequently, membranes were blocked for 2 h with TBST buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% low fat milk. The blots were incubated at room temperature for 2 h with primary antibodies and for 1 h with secondary antibodies. Polyclonal antisera for the human NIP7 protein were produced in rabbits and used at 1:5000 dilution. Rabbit polyclonal antibodies for RPS6, cytoskeletal actin and GAPDH (Bethyl Laboratories) were used at 1:300 and 1:2500 dilutions, respectively. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG (Calbiochem) and donkey anti-rabbit IgG (GE Healthcare) both at 1:5000 dilutions. The immunoblots were developed using the ECL western blotting analysis system (GE Healthcare).

**RNA analysis**

Total RNA from HEK293 cells, scRNA and shRNA-NIP7 clones were isolated by Trizol extraction (Invitrogen). RNAs were fractionated by electrophoresis on 1.2% (w/v) agarose/formaldehyde gels, followed by transfer to Hybond nylon membranes (GE Healthcare). Northern blot was performed using [³²P]-labeled oligonucleotide probes (P1–P7) complementary to specific regions of the 47S precursor RNA and submitted to autoradiography. The position of the probes were based on the human ribosomal DNA complete repeating unit, Genebank accession number U13369 (version U13369.1, GI:555853). The positions of the probes are as follows: P1 (5’ETS), complementary to nt 1401–1442; P2 (5’ETS), complementary to nt 1786–1825; P3 (ITS1), complementary to nt 6121–6160; P4 (5.8S), complementary to nt 6690–6729; P5 (ITS2), complementary to nt 7031–7070; P6 (ITS2), complementary to nt 7812–7838 and; P7 (28S), complementary to nt 7971–7997. Primer extension analysis was performed according to Gonzales et al. (52) using oligonucleotide probes PE1 (185), complementary to nt 3685–3714, PE2 (ITS1), complementary to nt 6299–6324 and PE3 (5.8S), complementary to nt 6655–6682 (Table 1). [³²P]-labeled-oligonucleotides MM21, MM30, MM36, MM44, MM54, MM64 and MM74 (Table 1) were used as molecular markers corresponding to 21, 30, 44, 54, 64 and 74 nt, respectively. For metabolic labeling of RNA, HEK293 cells cultured on 100-mm plates at ~70–80% confluence were pre-incubated for 30 min in phosphate-free DMEM (GIBCO) supplemented with 10% FCS. Subsequently, [³²P]-orthophosphate (15 μCi/ml) was added to the cultures that were incubated for 45 min and the medium was replaced by cold MEM (GIBCO) supplemented with 10% FCS. Cells were collected at time 0, 30, 60 and 120 min after changing the medium. Total RNA was extracted with Trizol (Invitrogen), and 10 μg were separated by electrophoresis on agarose/formaldehyde gels and revealed by autoradiography. RNA was extracted from sucrose gradient fractions as follows: 700 μl of each fraction were transferred to 1.5 ml tubes and 42 μl of a 10% SDS solution was added to obtain a final SDS percentage of 0.6. The suspension
was subjected to two sequential extractions, the first with 700 μl of phenol/chloroform/isoamyl alcohol (25:24:1) and the second with phenol/chloroform (1:1) and the aqueous phase was transferred to a new tube and the RNA precipitated with 700 μl of isopropanol for 1 h at -20°C. The RNA was sedimented by centrifugation (12,000 x g for 10 min at 4°C). The RNA pellet was washed with 75% ethanol, air dried and suspended in 10 μl of DEPC-treated water.

Electrophoretic mobility shift assays (EMSA)

For recombinant NIP7 protein production, we used plasmid pET28-HSNIP7, which was constructed by transferring the 540-bp NIP7 coding sequence from plasmid pTL1-HSNIP7 (47) to plasmid pET28a, using the EcoRI/SalI restriction sites. Expression of NIP7 was performed in E. coli BL21(DE3) cells transformed with vector pET28-HSNIP7 and incubated in LB medium containing kanamycin (50 μg/ml) at 37°C. At an OD600 of ~0.8, the culture was induced by adding 0.5 mM IPTG and incubating at 25°C for further 4 h. Cells were harvested by centrifugation, suspended in buffer containing 50 mM sodium phosphate pH 7.2, 100 mM NaCl, 10% glycerol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and treated with lysozyme (50 μg/ml) for 30 min on ice. Subsequently, the cells were disrupted by sonication and the histidine-tagged NIP7 purified by metal-chelating affinity chromatography, using a 20–200 mM imidazole gradient for elution. NIP7 was further purified on a heparin–sepharose column using the same buffer as above for binding and a 50 mM to 1 M KCl gradient for elution. For EMSA, 20 pmol of the RNA oligoribonucleotides poly-A(20), poly-U(20) and poly-AU(21), (5' UUA UUA UUU AUU UAU UUA UUA UUA 3'), were [32P]-labeled using 1 U T4 PNK and 20 μCi [γ-32P]-ATP. 0.4 pmol of [32P]-labeled oligoribonucleotide was incubated with the indicated concentrations of NIP7 in buffer A (20 mM Tris–Cl pH 8.0, 5 mM magnesium acetate, 150 mM potassium acetate, 0.2% v/v Triton X-100, 1 mM DTT, 1 mM PMSF) for 30 min at 37°C. Complexes were resolved on 8% polyacrylamide gels using TBE buffer pH 8.0 for electrophoresis and visualized by autoradiography. RNA competition assays were performed with 1, 5 and 10 pmol of either poly-A(20) or poly-AU(21) oligonucleotides.

RESULTS

Knockdown of NIP7

The strategy to generate HEK293 cells knockdown for NIP7 was based on the generation of stably-transfected cells expressing a shRNA targeting the NIP7 mRNA using pMaleficent as shRNA delivery vector (49). The efficiency of NIP7 downregulation was determined by analysis of the NIP7 mRNA and protein levels. Quantitative RT–PCR indicated that the reduction of NIP7 mRNA levels reached up to 80% of the parental and control cells (Figure 2A). Although the levels of the NIP7 mRNA are similar in clones CP4 and CP6, the reduction in NIP7 protein levels was more efficient in clone CP4 as observed by western blot analysis (Figure 2C). Downregulation of NIP7 in these clones was stable over the period of this study. CP4 cells showed significant reduction of the proliferation rate (Figure 2E).

RNA interference based on transient transfection of siRNA oligoribonucleotides was used to downregulate NIP7 also in MCF10A and HeLa cells. The NIP7 mRNA levels were reduced to levels <10% of control cells (Figure 2B). In siRNA-treated cells, NIP7 protein was below the detection level as determined by immunoblotting. A significant reduction in the proliferation rate of both cell lines was observed following transfection with the NIP7 siRNA (Figure 2F). The reduced proliferation rate prompted us to perform fluorescence-activated cell sorting (FACS) to investigate cell-cycle progression in these cells. In the case of the permanently transfected HEK293 derivative cells, CP4 show the highest number of cells in the G0–G1 phases and the lowest number in the S phase (Table 2). This effect on the cell cycle, despite of being mild, is consistent with the results observed for MCF10A cells. NIP7 downregulation in MCF10A resulted in an increase of cells in the G0–G1 phases with an equivalent reduction of the number of cells in the S phase (Table 2). This result shows that downregulation of NIP7 lead to accumulation of cells in the G1–S transition.

Analysis of polysomes in HEK293 cells depleted of NIP7

Defects in ribosome biogenesis can in some cases be detected by using sucrose density fractionation of polysomes especially when there is an imbalance in the 40S/60S subunit ratio. The CP4 clone, showing lower NIP7 levels, was chosen for further characterization. NIP7-depleted CP4 cells showed significant reduction of 40S ribosomal subunits (Figure 3C and D). This finding is consistent with the cell proliferation assay and suggests that the reduction in growth rate is due to defective 40S subunit biosynthesis.

Analysis of pre-rRNA processing intermediates in NIP7 deficient cells

Steady-state analysis of pre-rRNA processing was assessed by northern blotting using probes complementary to the transcribed spacer sequences and to the mature rRNAs. The boundaries of most pre-rRNAs intermediates relevant to this work have already been mapped (8–11). A set of northern blots was performed with probes P1, P2, complementary to the 5'S-ETS upstream and downstream of Site A0, respectively, and with probe P3 complementary to ITS1, upstream Site 2b (Figure 4). A second set of northern blots was performed with probes P4–P7 that are complementary to the 5.8S rRNA to ITS2 upstream and downstream Site 4b and to the 28S rRNA, respectively (Figure 5).

The precursors most affected are the 21S, 26S/A0-2c and 34S with the 21S and 26S/A0-2c pre-rRNAs showing increased levels and the 34S pre-rRNA showing reduced levels in NIP7 deficient cells (Figure 4). The 26S pre-rRNA running slightly faster than the mature 28S rRNA is detected with probes P2 and P3 but not by...
probe P1 and therefore extends from Sites A0 to 2c (Figure 4B, C and E). We concluded that this pre-rRNA corresponds to the 26S pre-rRNA described by Rouquette and co-workers (11) who defined its extension as from site A0 to a site in ITS1 downstream of nt 5687 and upstream of nt 6613, which must correspond to either Site 2b or 2c. Another precursor named 26S pre-rRNA, extending from Sites 1 to 4b, was described for pathway C (Figure 1, (8)). However, the possibility that this pre-rRNA is increased was excluded because it was not detected by probes P4 and P5 (Figure 5). Probes P1–P3 can detect both the 34S and

Table 2. Cell-cycle distribution of NIP7-depleted cells

| Cell type            | G0/G1 (%) | S (%) | G2 (%) |
|----------------------|-----------|-------|--------|
| HEK293               | 49        | 37    | 14     |
| HEK293/SC            | 43        | 42    | 15     |
| HEK293/CP4           | 51        | 33    | 16     |
| MCF10A/SC            | 47        | 35    | 18     |
| MCF10A/RNAi          | 73        | 13    | 14     |

Percentage numbers represent the average of three independent experiments.

Figure 2. Analysis of NIP7 knockdown. (A) NIP7 mRNA levels as determined by quantitative RT-qPCR in HEK293 derivative cells. HEK293, parental cells; SC, cells transfected with a scrambled shRNA; CP4 and CP6, cells transfected with shRNA against the NIP7 mRNA. NIP7 mRNA quantitation was performed using three different RNA extractions from clones CP4 and CP6. (B) NIP7 mRNA levels in transiently transfected MCF10A and HeLa cells. SC, control cells transfected with scrambled RNA; SiRNA, cells transfected with siRNA against NIP7. The histogram corresponds to one of the three independent transfections of MCF10A and HeLa cells using three replicates for each cell treatment. The amount of NIP7 mRNA in cells treated with the scrambled shRNA was considered as 100% to calculate its relative levels in the parental cells (HEK293) and in cells expressing the RNAi against the NIP7 mRNA (CP4) or in cells transfected with siRNA against the NIP7 mRNA. (C) Immunoblot showing the levels of the NIP7 protein in HEK293 derivative cells β-actin was used as an internal control. (D) Immunoblot using antiserum for the NIP7 protein in transiently transfected MCF10A and HeLa cells. (E) Proliferation rate of HEK293 derivative cells expressing the scrambled shRNA (SC) and the RNAi against the NIP7 mRNA (CP4) over a 7-day period. (F) Proliferation rate of transiently transfected MCF10A and HeLa cells. The graphs correspond to one of the two independent proliferation assays performed using three replicates for each cell treatment.
the 37S pre-rRNAs while probes P4 and P5 can detect both the 37S and the 32S pre-rRNAs (Figures 4 and 5). The 34S and 37S pre-rRNAs, comprising respectively \(6000\) and \(7520\) nt, may migrate with similar electrophoretic mobility in these gels. In the case of probes P1–P3 (Figure 4), a strong reduction of the signal is observed in this region of the northern blots, indicating that if any 37S pre-rRNA is generated in these cells its concentration is also decreased in NIP7-depleted cells.

A reduction in the concentration of the 32S pre-rRNA can be visually detected in the northern blots with probes P5, P6 and P7 in the NIP7 deficient cell line (Figure 5). The levels of the 41S pre-rRNA are slightly increased in NIP7 deficient cells (Figures 4C, E, 5A–C and F). This pre-rRNA, extending from sites 1 to 6, corresponds to the second largest band detected by probes P3–P6 (Figures 4C, E, 5A–C and F). The levels of the 47S and 45S pre-rRNAs, on the other hand, show a slight decrease in NIP7 deficient cells (Figures 4A–E and 5F).

Accumulation of the 26S/A0-2c pre-rRNA has been described in situations in which there is uncoupling of processing at Sites 1 and 2. Leptomycin B (LMB) inhibits exportin Crm1/Xpo1 and blocks ribosome subunit export from the nucleus. We have analyzed pre-rRNA processing of control cells (SC) and NIP7 shRNA cells (CP4) treated with LMB (Figure 4F).

Upon a 4 h LMB treatment, SC cells showed a sharp increase of the 26S/A0-2c pre-rRNA. In CP4 cells, that already contain increased levels of the 26S/A0-2c and 21S pre-rRNAs, LMB treatment has a small effect on the accumulation of the 26S/A0-2c pre-rRNA. Accumulation of the 26S/A0-2c is consistent with slower processing of sites 1 and 2 in NIP7-depleted cells. However, accumulation of the 21S pre-rRNA and of the 41S pre-rRNA indicates that processing at sites 2c/2b and 2 are even slower that at site 1, suggesting that NIP7 is particularly required for processing of the ITS1 sites.

Northern blot analysis with probe P3 was performed also with RNA samples from MCF10A cells (Supplementary Data), which detected accumulation of the 21S and 41S pre-rRNAs following downregulation of Nip7, indicating that Nip7 plays an important function in all cell types.

The 37S pre-rRNA (spanning from Sites A' to 4b) and the 17S pre-rRNA (spanning from Sites 2b to 4b) would be generated only if the pathway C proposed by Bowman and co-workers (8) is indeed taking place in HEK293 cells. Gel electrophoreses used in this work resolved the 28S rRNA (5035 nt) from the 26S/A0-2c pre-rRNA (~4642 nt). Therefore, it would be expected that the 37S pre-rRNA (~7520 nt) would also be separated from the 32S pre-rRNA (6337 bases from sites 3 to 6). However, probes 4 and 5 detected only one band in this region suggesting that HEK293 cells produce low levels of the 37S pre-rRNA. The 17S pre-rRNA could be detected by probes P3, P4 and P5 (Figures 4C, 5A and B) but only...
faint bands are detected in this region. The low levels of both 17S and 37S pre-rRNAs, together with the fact that the 17S immediate upstream precursor, namely the 26S pre-rRNA extending from sites 1 to 4b, was not detected by probes P4 and P5 (Figure 5A and B) are strong indications that pathway C is a minor pathway in HEK293 cells.

An additional band with mobility similar to the 5.8S rRNA is detected with probe P5 (‘Asterisk’ in Figure 5B). This band might correspond to a fragment comprising from site 4b to an upstream site in ITS2 but only if there would be a cryptic endonucleolytic cleavage site downstream of site 4, which can now be speculated, following the recent discovery of the endonucleolytic activity associated to the exosome, the main trans-acting factor responsible for maturation of the 5.8S rRNA 3’-end (53).

Analysis of the pre-rRNA processing kinetics was performed by metabolic labeling with [32P]-orthophosphate
By visual analysis of the autoradiograph, it is possible to observe the 21S pre-rRNA, although faint, in NIP7-depleted cells at the 2h time point. Quantitation of the bands and calculation of the ratio relative to the amount of the 47S pre-rRNA revealed that formation of the 34S pre-rRNA is reduced and the 21S pre-rRNA is increased in NIP7-depleted cells (Figure 6B), which is consistent with the steady-state analyses, showing a defect in processing of ITS1.
Primer extension analysis of pre-rRNA processing sites in NIP7 deficient cells

Primer extension analyses were performed to examine the 5'-end of rRNA products at the processing sites that appeared to be most affected according to the northern blot analyses (Figure 7). Primer extension with oligonucleotide PE1 complementary to a sequence in the 18S rRNA, 27 bases downstream of the predicted 5'-end of the mature 18S rRNA (Site 1), resulted in products with the expected size (57, 30 nt of the primer PE1 plus 27 nt of extension up to Site 1). Interestingly, however, two bands were observed for all samples, suggesting that the cleavage at site 1 may take place at two adjacent nucleotides. For the parental HEK293 cells and the control cells transfected with the scrambled shRNA, the two bands showed a 1:1 ratio (Figure 7A). NIP7-depleted cells showed different product ratios with a sharp reduction of the longer product (Figure 7A). These discrepancies are consistent with defective processing at site 1 in NIP7-depleted cells. Oligonucleotide PE2 complementary to a sequence in ITS1 (nt 6299–6324 in the pre-rRNA) downstream of probe P3 (complementary to ITS1 nt 6121–6160 in the pre-rRNA) was used to determine the efficiency of cleavage reactions at sites in ITS1. Extension of primer PE2 generated a product of 33 nt (26 nt of the primer and 7 nt of extension), which corresponds to the 5'-end of the pre-rRNAs generated by cleavage at site 2b (Figure 7B). A reduction in the primer extension product is observed in NIP7-depleted cells, corroborating the results of the northern hybridizations which show inhibition of cleavages in ITS1 as a consequence of the reduced levels of NIP7. Oligonucleotide PE3 is complementary to a sequence 32 bases downstream of site 3, the predicted 5'-end of the mature 5.8S rRNA (nt 6655–6682 in the pre-rRNA). Extension of primer PE3 generated two major products, one with 60 nt (28 nt of the primer plus 32 nt of extension), corresponding to the expected size of the product generated by cleavage at site 3 and another one, 8–10 nt longer, designated as 3L (Figure 7C). In eukaryotes, the 5'-end of the 5.8S rRNA has been described as heterogeneous (54–56) and this result suggests that mammals may have at least a second form of 5.8S rRNA showing extended 5'-end as observed for S. cerevisiae (57). Similarly to the extension of primers PE1 and PE2, knocking down NIP7 expression caused a reduction especially of the longer extension products at site 3. The primer extension results, therefore, confirm the observation described above that lowering NIP7 expression leads to defects in pre-rRNA processing, affecting more strongly processing of the 5'-ETS and ITS1 spacer sequences.

Extension of primer PE3 revealed also three additional products (Figure 7C) that end at positions U7, U14 and A17 of the mature 5.8S rRNA (shorter form). The 5.8S rRNA is known to be methylated at residues U14 and G77, so we can assume that the faint band at the U14 position is a primer extension stop due to methylation. There is no report in the literature about nucleotide or base modification at positions A17 and U7 of the mature 5.8S rRNA. Primer extension stops can be originated when the reverse transcriptase reaches 5'-ends, base/ribose modifications or highly stable secondary structures. The band at position A17 is barely detectable in control cells and increases in NIP7-depleted cells, suggesting that it results from NIP7 deficiency (Figure 7C). In this case it is possible to speculate that the stop was due to premature degradation of the 5.8S rRNA 5'-end in the context of NIP7 deficiency where the aberrant and misprocessed precursors might be directed for degradation. In case of stop at position U7, it is present in control and test cells and is therefore caused by something that is common to the three samples which could be any of the options mentioned above.
Figure 7. Analysis of primer extension products generated by reverse transcriptase. (A) Primer extension products using primer PE1 that is complementary to the 18S rRNA downstream site 1. (B) Primer extension products using primer PE2 that is complementary to ITS1 downstream site 2b. (C) Primer extension products using primer PE3 that is complementary to the 5.8S rRNA downstream site 3. U<sub>m14</sub> indicates the position of the 2-O-methyluridine at position 14 of the 5.8S rRNA. U7 and A17 indicate two unexpected primer extension stops. 1, 2b, 3 and 3L indicate the primer extension products extended to the respective processing sites. Arrowheads indicate the bands corresponding to the primers which were not extended: PE1, 30 nt; PE2, 26 nt and PE3, 28 nt, respectively. HEK293, parental cells; SC, cells transfected with scRNA; CP4, cells transfected with shRNA against the NIP7 mRNA. (D) Structure of the 47S pre-rRNA with the indication of the position of the oligonucleotides PE1, PE2 and PE3 used in the primer extension assays shown in A, B and C.
Human NIP7 associates to nuclear pre-ribosomal particles

Western blot analysis of the distribution of NIP7 in sucrose gradient fractions using the regular protocol to isolate polysomal extracts revealed that NIP7 co-sediments in the range of 60S-80S ribosomes (Figure 8B). This result is similar to the distribution of S. cerevisiae Nip7p in sucrose gradients (40). However, previous data indicated that human NIP7 is a nucleolar protein (43). Cell fractionation followed by immunoblot analysis can be used to determine protein subcellular localization with a reasonable accuracy that, in some cases, can complement immunofluorescence assays or localization based on fusion to fluorescent proteins. Therefore, cell fractionation experiments were performed and confirmed that NIP7 is restricted to the nuclear compartment (Figure 8C). In a second experiment the sucrose gradient fractionation was performed with nuclear extract. A regular sucrose gradient with whole-cell polysomal extract was performed in parallel so that the positions of the 40S and 60S ribosomal subunits, 80S ribosomes and polysomes of the whole-cell polysomal extracts could serve as reference for sedimentation of the nuclear pre-ribosomal complexes. The fractionation analysis of the nuclear extract on sucrose gradients revealed that NIP7 distribution is highly similar to the profile observed for its distribution in the gradient of the whole-cell extract (Figure 8, compare panels B and D).

![Graphical representation](image_url)

Figure 8. Analysis of NIP7 sedimentation on sucrose density gradients. (A) Polysome profile of HEK293 whole-cell polysomal extracts prepared according to Johannes and Sarnow (51). (B) Western blot analysis of NIP7 sedimentation on a sucrose density gradient of whole-cell polysomal extracts. 18S and 28S rRNAs and protein RPS6 are shown as references for ribosome and polysome sedimentation. GAPDH was used as reference for proteins not associated to ribosomes. (C) Western blots of HEK293 nuclear (Nuc) and cytoplasmic (Cyt) fractions showing NIP7 in the nuclear fraction. Lamin A/C and α-tubulin were used as controls for cell fractionation. (D) Western blot analysis of NIP7 following sucrose density gradient fractionation of a nuclear extract. The sucrose density gradients of whole-cell polysomal extract shown in B and nuclear extract shown in D were centrifuged and fractionated in parallel so that the positions of the 40S and 60S ribosomal subunits, 80S ribosomes and polysomes of the whole-cell polysomal extracts served as reference for sedimentation of the nuclear pre-ribosomal complexes. The profile of the nuclear extract is not shown because it is not informative of relevant peak fractions. RPS6 and Lamin A/C were used as references for 40S subunit and free protein sedimentation, respectively.
further suggesting that NIP7 co-sediments with pre-ribosomal particles. The control proteins used in these analyses showed the expected distribution. In the whole-cell extract gradient, RPS6 co-sediments in two major peaks, one between fractions 5 and 8, where the free 40S subunits and 80S ribosomes sediment, and between fractions 10 and 13, associated with polysomes (Figure 8B). In the gradient of nuclear extracts, on the other hand, most of RPS6, sediments in fractions 2 and 3, either as a soluble protein or as part of small complexes (Figure 8D). A smaller fraction of RPS6 sediments in fractions 5–9, indicating that it is associated to pre-ribosomal particles (Figure 8D). Lamin A/C was used as nuclear marker and behaves as a soluble protein in the sucrose gradient of the nuclear extract (Figure 8D). Consistently with the results shown above, NIP7 co-sediments with particles corresponding to pre-ribosomes in nuclear extracts gradients (Figure 8D). Combined, these results strongly indicate that NIP7 binds pre-ribosome particles in the nucleolus, thereby participating in the early pre-rRNA processing reactions.

**Human NIP7 interacts with poly-U and poly-AU RNAs in vitro**

The *S. cerevisiae* and *P. abyssi* Nip7 orthologs interact in vitro preferentially with poly-U homopolymers (42) and with poly-AU (J. S. Luz and C. C. Oliveira, personal communication). In order to determine whether recombinant human NIP7 also binds to RNA we initially tested its interaction with poly-U using electrophoretic mobility shift assays under native conditions. These assays showed that human NIP7 binds to polyuridine sequences although with low affinity (Figure 9A). Subsequent experiments showed that the human NIP7 protein binds to poly-AU with higher affinity than to poly-U (Figure 9B). In addition, competition assays were performed with increasing concentrations of unlabeled poly-A and poly-AU oligonucleotides. An excess of poly-A did not show effect on the NIP7–poly-AU interaction while the intensity of the band shift was reduced by increasing concentrations of unlabeled poly-AU oligonucleotides (Figure 9B and C), confirming the specificity of the NIP7–poly-AU interaction. The poly-AU oligonucleotid can form intra and intermolecular base-pair suggesting that NIP7 might have a higher affinity for structured RNAs.

**DISCUSSION**

Given the essential function of the *S. cerevisiae* NIP7 gene (40), the high conservation of NIP7 orthologs and the association to other ribosome synthesis proteins, we could predict that NIP7 would play an important function in human cells. We applied the RNA interference experimental approach to downregulate NIP7 expression and search for loss-of-function phenotypes. Downregulation of NIP7 in HEK293 cells was efficiently obtained using the transposon-based delivery system described by Heggestad et al. (49). Downregulation of NIP7 in HEK293, MCF10A and HeLa cells leads to reduction of cell proliferation rates, with cells accumulating in the G1 phase, indicating that NIP7 plays an important role in all human cells. Downregulation of NIP7 in HEK293 cells affected the levels of 40S ribosomal subunits and caused alterations in pre-rRNA processing which affected mainly the levels of the 34S, 26S and 21S pre-rRNA intermediates. Reduction of the 34S pre-rRNA indicates a slow processing defect at sites 2b and 2c. The increase of the 26S pre-rRNA concentration also correlates with slow processing of site 1 and of sites 2b/2c whereas the increase of the 21S pre-rRNA indicates slow processing at sites 2b/2c. Primer extension analysis of sites 1, 2b and 3 further supports the northern blot data. At least part of the pre-rRNA processing defects caused by NIP7 depletion have been described for other situations in which ribosome biogenesis was impaired, such as treatment with leptomycin B, which inhibits exportin Crml/Xpo1 and blocks ribosome subunit export from the nucleus, and for knockdown of 40S biogenesis factors (11). The data obtained in this study indicate that human NIP7 is required primarily for processing of the pre-rRNA intermediates leading to the synthesis of the 18S rRNA and 40S subunit. Taking into account the conditional depletion of yeast Nip7p leads to a deficit of 60S subunits and to accumulation of the 27S pre-rRNA (40), during the course of this work, we have tested whether the human and archaeal NIP7 orthologs could complement a yeast Δnip7 mutant strain (Supplementary Figure S1). This analysis showed that neither the human nor the archaeal NIP7 ortholog can complement a yeast Δnip7 strain. This finding indicates that although the three orthologs bind structured RNA, and the yeast and human NIP7 are involved in pre-rRNA processing, the function of yeast Nip7p is not fully conserved in human cells.

The interactions of human NIP7 with other proteins are consistent with its role in ribosome biosynthesis. The first interaction described was with Nop132 (43), the putative ortholog of the *S. cerevisiae* Nop8p (44). Tests using the yeast two hybrid system and GST pull-down assays detected association of NIP7 with SBDS, indicating that both proteins may be part of a multisubunit complex (47). Subsequently, human NIP7 was found in association with complexes isolated by affinity-tagged purification of the 40S subunit protein RPS19 (45). This protein was already shown to play an essential role in 40S subunit biosynthesis in human cells (23), consistently with the genetic findings that link mutations in the *RPS19* gene to the DBA (25). Orru’ and co-workers (45) described 159 proteins that co-purify with GST-RPS19. Although this complex is heterogeneous, it contains structural components of both the 40S and 60S subunits and a large set of proteins already known to function in ribosome synthesis. NIP7 is also found in complexes isolated by affinity-tagged purification of the parvulin (Par14), a peptidyl-prolyl cis–trans isomerase (PPIase) reported to function in pre-rRNA processing (46). These large complexes are found mostly in the nucleolus, although some components may be found throughout the whole nucleus, consistently with our data describing NIP7 in the nuclear extracts that sediment in sucrose gradients.
with molecular masses in the range of 40S–80S ribosomes. During the development of this work, it became clear that the cell extracts used in the sucrose gradient fractionations contained nuclear and nucleolar contaminants probably due to partial leakage of nuclear content during disruption of cells under the hypotonic buffer conditions used or due to partial disruption of nuclei by the 1% v/v Triton detergent that is added to the extraction buffer. Cell fractionation revealed NIP7 only in the nuclear fraction and sucrose gradient fractionation of nuclear extracts indicated that NIP7 co-sediments with high molecular complexes, consistently with its association to pre-ribosomal particles.

Depletion of *S. cerevisiae* Nip7p caused a general defect in pre-rRNA processing, with accumulation of normal (35S and 27S) and of the aberrant (23S) pre-rRNAs and a reduction in the concentration of the mature rRNAs. Despite the global defects on pre-rRNA processing, *S. cerevisiae* Nip7p depletion led to a deficit of 60S subunit (40) and was found in 60S complexes (58). In addition, *S. cerevisiae* Nip7p interacts with a group of proteins involved in ribosome synthesis, including the exosome subunit Rrp43p and the nucleolar proteins Nop8p, Nop53p and Sdo1p (37,44,59,60). These proteins have been implicated to 60S subunit synthesis, although it is important to point out that conditional depletion of the
exosome subunit Rrp43p also led to general defects in pre-rRNA processing resulting in deficit of 40S ribosomal subunits (60). This phenotype is highly unexpected, given that exosome components have previously been reported to be required for excision of the ITS2 segment during maturation of the 3′-end of the 5.8S rRNA, a component of the 60S subunit (61). Further analysis of Rrp43p-depleted cells (60) and of temperature sensitive mutant strains (62) confirmed that deficiency of Rrp43p causes global defects in pre-rRNA processing, making it difficult to distinguish the primary from the secondary defects based only on data obtained from conditional mutants.

Despite the discrepancies between the defects observed in yeast and human NIP7 deficient cells, both human and yeast NIP7 proteins share the ability to bind poly-U in vitro, although human NIP7 has shown higher affinity for a poly-AU oligoribonucleotide. NIP7 cognate RNA target sequences have not been identified yet and its preference for binding to poly-U and poly-AU suggests that it might interact with uridine-rich sequences of the pre-rRNA similarly to Rrp5p, which was described to interact with a uridine-rich sequence in the ITS1 of the S. cerevisiae pre-rRNA (63). NIP7 orthologs share a two-domain architecture with the C-terminal PUA domain mediating interaction with RNA (42). This domain organization, suggests that NIP7 is an adaptor protein with the C-terminal domain interacting with RNA targets and the N-terminal domain mediating interaction with protein targets.

Saccharomyces cerevisiae NIP7 is part of the Ribi regulon (13,15) and its transcription levels were also shown to correlate with other Ribi regulon genes in response to stress caused by the alkylating agent methyl methanesulfonate (64). In addition, a study based on the analysis of gene expression in response to abrupt changes of environmental conditions has associated S. cerevisiae NIP7 to the early repressed ribosomal genes (65). As for the human NIP7 gene, its core promoter contains the 5′-C ACGTG-3′ sequence, also known as E(CG) sequence (66), that is recognized by the Myc:Max heterodimer, indicating that NIP7 is part of the human Ribi regulon proposed to be under control of the Myc transcription factor (16–18).

In conclusion, the data presented in this study show that human NIP7 plays an important role for cell proliferation and implicate NIP7 primarily in the processing of pre-rRNA intermediates leading to maturation of the 18S rRNA and 40S ribosomal subunit biosynthesis. The pre-rRNA processing defects clearly indicate that NIP7 plays a critical role in pre-rRNA processing in human cells. Association of NIP7 to RPS19 complexes (45) helps to explain the pre-rRNA processing defects of the 18S rRNA pathway observed in this work for NIP7-depleted cells. We also show evidence that the human NIP7 protein is restricted to the nuclear compartment and that its sedimentation pattern in sucrose gradient fractionation indicates that it is associated to nuclear pre-ribosomal complexes.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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