

The human Obg protein GTPBP10 is involved in mitoribosomal biogenesis

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Received May 17, 2018; Revised July 19, 2018; Editorial Decision July 20, 2018; Accepted July 23, 2018

ABSTRACT

The human mitochondrial translation apparatus, which synthesizes the core subunits of the oxidative phosphorylation system, is of central interest as mutations in several genes encoding for mitoribosomal proteins or translation factors cause severe human diseases. Little is known, how this complex machinery assembles from nuclear-encoded protein components and mitochondrial-encoded RNAs, and which ancillary factors are required to form a functional mitoribosome. We have characterized the human Obg protein GTPBP10, which associates specifically with the mitoribosomal large subunit at a late maturation state. Defining its interactome, we have shown that GTPBP10 is in a complex with other mtLSU biogenesis factors including mitochondrial RNA granule components, the 16S rRNA module and late mtLSU assembly factors such as MALSU1, SMCR7L, MTERF4 and NSUN4. GTPBP10 deficiency leads to a drastic reduction in 55S monosome formation resulting in defective mtDNA-expression and in a decrease in cell growth. Our results suggest that GTPBP10 is a ribosome biogenesis factor of the mtLSU required for late stages of maturation.

INTRODUCTION

Mammalian mitochondrial ribosomes (mitoribosomes) are essential as they synthesize the mitochondrial DNA (mtDNA)-encoded core subunits of the oxidative phosphorylation (OXPHOS) system. The significance of this machinery is demonstrated by a growing number of patients with severe mitochondrial diseases associated with mutations in genes encoding for mitoribosomal proteins (MRPs) (1). Although mitoribosomes derived from a bacterial ancestor, there are substantial differences in structure and

composition comparing the 55S human mitoribosome and the 70S bacterial ribosome. The 70S particle, composed of a 30S small ribosomal subunit (SSU) and a 50S large ribosomal subunit (LSU), shows a very compact structure with approximately 70% rRNA and 30% proteins. The 55S mitoribosome, which consists of a 28S mitochondrial SSU (mtSSU) and a 39S mitochondrial LSU (mtLSU), exhibits a more porous structure with a reverse rRNA:protein ratio (2). During evolution the human mitoribosome acquired additional proteins and N- and C-terminal extension to the pre-existing ones leading to ~50% of the MRPs being unique to the 55S without any homologue in bacteria. Additionally, the 55S is of dual genetic origin, meaning the rRNA (12S and 16S rRNA) and the tRNA of the central protuberance are encoded by the mtDNA, whereas all the 82 MRPs are encoded by the nucleus suggesting a complex assembly pathway with the requirement of coordinated communication between different cellular compartments. In the last decades the bacterial ribosome was extensively studied in its function and assembly *in vitro* and *in vivo* (3). Having a common ancestor, similarities in the assembly pathway comparing the 70S and the 55S particles are expected. However, due to the aforementioned differences in structure and composition, significant differences are highly likely to be present for the assembly of this complex machinery. For the *in vivo* assembly of the 70S ribosome several ancillary factors are required including RNA helicases, RNA modifying enzymes, chaperones and GTPases (3). The GTPases comprise the highly conserved Obg family, whose members are present in bacteria, but also in eukaryotes. It has been demonstrated in various bacterial species that Obg proteins associate with the LSU (4–8), however, the molecular function of Obg proteins in ribosome biogenesis is still elusive. Feng *et al.* have suggested that the *E. coli* Obg protein (ObgE) acts as an anti-association factor to prevent the formation of the 70S ribosome. Thus, ObgE might function as a quality control factor in late assembly stages of the LSU in bacteria (9). In human, there are

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two homologues of ObgE, namely GTPBP5 (OBGH1) and GTPBP10 (OBGH2) (10). Both exhibit GTPase activity *in vitro* and complemented ObgE deleted *E. coli* strains suggesting conserved function of Obg proteins throughout evolution. However, while GTPBP5 was localized to mitochondria, associating with the mtLSU, GTPBP10 was proposed to be present in the nucleolus suggesting different functions of the human Obg proteins (10,11).

Here, we show that GTPBP10 is a mitochondrial protein, peripherally associated with the inner mitochondrial membrane. GTPBP10 associates with the mtLSU at a late maturation state, but not with the mtSSU or with the assembled 55S monosome. The loss of GTPBP10 leads to a significant decrease in monosome formation associated with reduced steady state levels of selected MRPs as well as of 16S rRNA leading to ablated mtDNA gene expression. In addition, we show that both the N-terminal Obg domain, and also the GTPase domain of GTPBP10 are required for mtLSU binding. In contrast to previous observations, our data suggest that both human Obg homologues and not only GTPBP5 localize to mitochondria (10) and that GTPBP10 is required for mtLSU biogenesis at a late assembly stage.

MATERIALS AND METHODS

Cell culture

The cultivation media and chemicals were purchased by Sigma or GIBCO unless specified otherwise. Human embryonic kidney cell lines (HEK293-Flp-In T-Rex; HEK293T), 143B wild-type, and 143B Rho⁰ cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM Sodium pyruvate, 50 µg/ml uridine, 100 units/ml Penicillin and 100 µg/ml Streptomycin at 37°C under 5% CO₂ humidified atmosphere. Cells were systematically tested for the presence of Mycoplasma. For testing cell growth 7.5 × 10⁴ cells were seeded into 6-well plates and cell number was monitored after 1, 2 and 3 days using a Neubauer chamber.

Stable inducible HEK293T cell lines expressing C-terminal FLAG tagged mS40 and GTPBP10 wild type (WT) or mutated variants of GTPBP10 were generated as described previously (12,13). Briefly, HEK293T cells were simultaneously transfected with pOG44 and pcDNA5/FRT/TO plasmids containing the respective FLAG construct using GeneJuice (Novagen) or Superfect (Qiagen) as transfection reagent according to manufacturer's instructions. Selection of clones with FLAG construct insertion was started on the second day after transfection using Hygromycin B (100 µg/ml) and Blasticidin S (5 µg/ml). After approximately two weeks, single clones were isolated, induced with tetracycline and analysed for expression by Western blotting.

Gtpbp10^{64R65K} HEK293T cell line was generated applying the CRISPR/Cas9 technology as previously described (13,14). HEK293T cells were transfected with pX330-gRNA-GTPBP10 plasmid containing guide RNA sequence targeting *Gtpbp10* gene (CCAGCCACAAACCGTTTCCG AGG) and with the pEGFP-N1 plasmid for GFP-mediated single cell sorting. Resulting clones were subjected to pri-

mary screening by immunoblotting and the mutation in the GTPBP10 coding sequence was analysed by sequencing.

Cell lysates, mitochondria isolation from cultured cells and mitoplasts preparation

Cells were lysed in NP-40 containing lysis buffer (50 mM Tris/HCl pH 7.4, 130 mM NaCl, 2 mM MgCl₂, 1% NP-40, 1 mM PMSF and 1× Protease inhibitor cocktail (Roche)), vortexed for 30 s, centrifuged for 2 min at 560 g and supernatant saved for further analyses.

Mitochondria were isolated as described previously (15). Briefly, cells were resuspended in homogenization buffer (300 mM Trehalose, 10 mM KCl, 10 mM HEPES pH 7.4) with addition of 1 mM PMSF and 0.2% BSA and potted with Homogen^{plus} Homogenizer (Schuett-Biotec, Germany). Suspension was subjected to differential centrifugation and isolated mitochondria were pelleted at 11 000 g for 10 min, washed with homogenization buffer and subjected to further analyses. For mitoplasts preparation, mitochondria were resuspended in homogenization buffer containing 0.1% Digitonin, incubated on ice for 30 min and treated with proteinase K for 15 min. After blocking Proteinase K with 2 mM PMSF, resulting mitoplasts were washed five times prior further analyses.

Protein localization assays

Proteinase K assay and Carbonate extraction of membrane proteins were executed as previously described (16).

Immunodetection of proteins

Primary antibodies used in this study were: rabbit anti-GTPBP10 (Novusbio NBPI-85055), rabbit anti-uL1m (PRAB4964), rabbit anti-uL3m (ProteinTech 16584-1-AP), rabbit anti-bL12m (ProteinTech 14795-1-AP), rabbit anti-uL13m (ProteinTech 16241-1-AP), rabbit anti-u23m (PRAB1716), rabbit anti-bL32m (PRAB4957), rabbit anti-mL44 (ProteinTech 16394-1-AP), rabbit anti-mL62 (10403-1-AP), rabbit anti-uS14m (ProteinTech 16301-1-AP), rabbit anti-bS15m (ProteinTech 17006-1-AP), rabbit anti-bS16m (ProteinTech 16735-1-AP), rabbit anti-mS27 (ProteinTech 17280-1-AP), rabbit anti-mS40 (ProteinTech 16139-1-AP), rabbit anti-MALSU1 (ProteinTech 22838-1-AP), rabbit anti-NGRN (ProteinTech 14885-1-AP), rabbit anti-GTPBP7 (ProteinTech 13742-1-AP), mouse anti-SDHA (Invitrogen 459200), mouse anti-GAPDH (Santa Cruz sc-32233), mouse anti-COX1 (Invitrogen 459600), mouse anti-COX2 (ab110258), rabbit anti-MFN2 (ProteinTech 12186-1-AP), rabbit anti-TIM23 (PRAB1527), rabbit anti-TIM44 (ProteinTech 13859-1-AP).

[³⁵S]Methionine *de novo* synthesis

Labeling was performed as described previously (16,17). Prior to labeling, cells were treated with 100 µg/ml emetine (Invitrogen) or anisomycin to inhibit cytosolic translation. Mitochondrial translation products were labeled with 0.2 mCi/ml [³⁵S]Methionine for 1 h (pulse labeling), separated on 10–18% Tris-Tricine gel, blotted, visualized by

Typhoon imaging system (GE Healthcare) and quantified using ImageQuant TL. For pulse-chase experiments media was changed after 1 h pulse labeling and cells were further incubated for 3, 6 or 24 h prior analysis.

Co-immunoprecipitation

Immunoprecipitation of FLAG-tagged proteins were performed as described (13) with some modifications. Mitochondrial lysates were prepared in buffer containing 50 mM Tris/HCl [pH 7.4]; 100 mM NH₄Cl; 10 mM MgCl₂; 10% glycerol; 1 mM PMSF and 1% digitonin. After centrifugation at 16 000 g at 4°C for 10 min supernatants were subjected to co-immunoprecipitation using either anti-FLAG M2 Affinity Gel (Sigma) or specific or control antibodies conjugated to ProteinA-sepharose columns (GE Healthcare) for 1h. Elution of co-purified proteins was achieved by FLAG peptides or by pH-shift.

Isokinetic sucrose gradient analysis

Lysed mitoplasts (500 μg in 3% sucrose, 100 mM NH₄Cl, 20 mM MgCl₂, 20 mM Tris-HCl, pH 7.5, 1% Digitonin, 1 × PI-Mix, 0.08 U/μl RiboLock RNase Inhibitor) or affinity purified native complexes were separated by linear sucrose gradient centrifugation (5–30% (w/v) in 100 mM NH₄Cl, 20 mM MgCl₂, 20 mM Tris-HCl pH 7.5, 1 × Protease inhibitor cocktail (Roche)) at 79 000 g, 4°C for 15 h using SW41 Ti (Beckman Coulter). Fractions (1-16) were collected applying BioComp fractionator and analysed by western blot.

RNA isolation and northern blot

RNA was isolated from whole cell extracts using TRIzol reagents (Invitrogen), following the manufacturer's instructions. RNA separation on a denaturing formaldehyde/formamide 1.2% agarose gel was performed as previously described (18). [³²P]-radiolabeled probes were generated utilizing T4 Polynucleotide Kinase (Thermo Scientific) according to the manufacturer's instructions. Imaging was performed with Typhoon imaging system (GE Healthcare).

Quantitative mass spectrometry analyses

SILAC experiments were performed as described previously (13). In brief, cells were cultured for five passages in DMEM containing either 'light' or 'heavy' (¹³C₆¹⁵N₄-arginine ¹³C₆¹⁵N₂-lysine, Cambridge Isotope Laboratories, Tewksbury, MA, USA) labeled amino acids supplemented with 10% dialyzed FBS and 600 mg/l proline.

Equal amounts of isolated mitochondria from differentially SILAC-labeled HEK293T wild type cells and cells expressing GTPBP10^{FLAG} were mixed prior to immunoprecipitation. Purified protein complexes were separated on a 4–12% Nu-PAGE (Invitrogen) and gel slices were digested with trypsin (Sigma Aldrich) and subjected to quantitative mass spectrometry as described previously (19) using Orbitrap Fusion (ThermoFisher). Data were analyzed using Max Quant (version 1.6.0.1) utilizing the human Uniprot database (version 24.11.16) as a reference.

Proteins with a mean ratio ≥ 2 and a *P*-value ≤ 0.05 were considered to be specifically associated with GTPBP10 complexes.

Quantification and statistical analysis

Western blots and northern blots were quantified with Typhoon imaging system and ImageJ software. The protein and RNA levels are presented as percentages relative to WT control. Error bars indicate the SEM from the mean of *n* experiments (see figure legends for details).

RESULTS

GTPBP10 is a mitochondrial protein homologous to bacterial Obg proteins

As Obg proteins are highly conserved GTPases and present in both, prokaryotes and eukaryotes, we performed cluster analysis showing that both human Obg proteins GTPBP5 as well as GTPBP10 group together with *E. coli* ObgE (Figure 1A). Both proteins share ~30% identities with ObgE (GTPBP5: 32.7% and GTPBP10: 31.2%) and show high similarities in the Obg fold region at the N-terminus and in the GTPase domain, whereas the C-terminal domain appears to be more diverse (Figure 1B). In contrast to GTPBP5, which localizes to mitochondria, GTPBP10 was shown to be present in the nucleolus (10). However, a recent genome-wide CRISPR 'death screen' suggested that GTPBP10 is required for mitochondrial OXPHOS function (20). Based on this observation we investigated the localization of GTPBP10 biochemically performing a Proteinase K treatment on intact mitochondria and mitoplasts. Interestingly, GTPBP10 followed the same pattern as the matrix marker uS14m and was protected against Proteinase K even in the absence of the outer mitochondrial membrane (Figure 1C). To assess whether GTPBP10 is an integral membrane protein or a soluble matrix protein we performed sodium carbonate extraction at different pH (Figure 1D). At lower pH a significant portion of GTPBP10 remained in the membrane-containing pellet fraction, but was almost completely extracted at a higher pH similarly to TIM44, a component of the mitochondrial protein import motor associated with the inner mitochondrial membrane (21,22). These results demonstrate that GTPBP10 is a mitochondrial matrix protein peripherally attached to the inner mitochondrial membrane.

GTPBP10 is required for mitochondrial gene expression

As GTPBP10 is present in mitochondria and has been suggested to be required for mitochondrial OXPHOS function (20), we asked whether GTPBP10 is maintained in OXPHOS deficient cells lacking mtDNA (ρ^0). Interestingly, GTPBP10 is significantly reduced in ρ^0 cells (Figure 2A), which is reminiscent to other proteins required for mitochondrial gene expression including MRPs and ribosome biogenesis factors such as MALSU1, DDX28 or mtRBFA (23,24).

Based on this observation, we speculated that GTPBP10 is involved in mitochondrial gene expression. To address this further we aimed to analyze the consequences of the

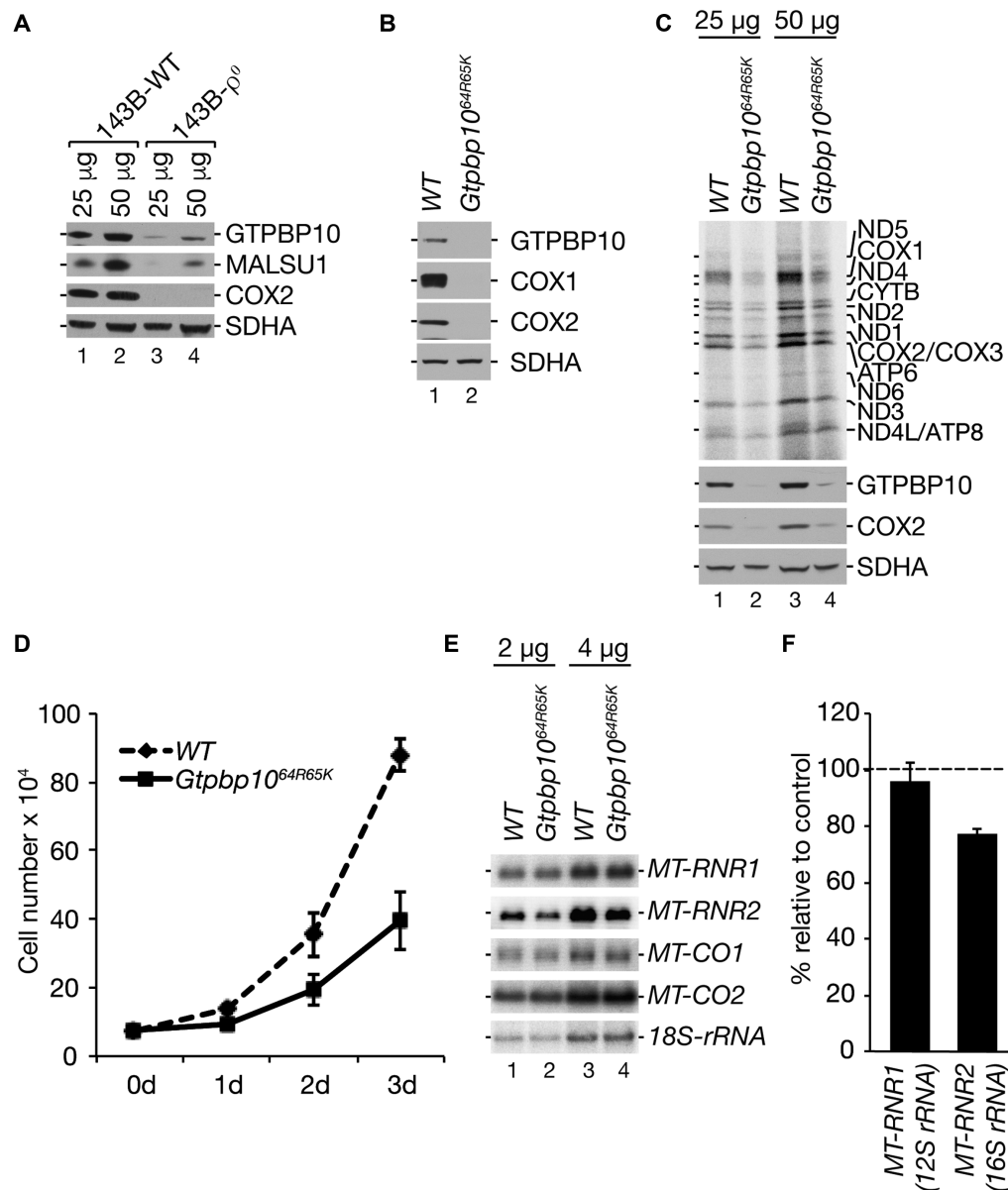


Figure 2. GTPBP10 is involved in mitochondrial gene expression. (A) GTPBP10 is unstable in the absence of mtDNA. Protein steady state levels from 143B wild type (WT) or 143B- ρ^0 cells were analyzed by western blotting. (B) Altered mitochondrial gene expression in *Gtpbp10*^{64R65K} cells. Steady state levels of mtDNA-encoded proteins (COX1 and COX2) isolated from HEK293T WT or *Gtpbp10*^{64R65K} cells. SDHA is used as a loading control. (C) *Gtpbp10*^{64R65K} cells exhibit diminished mitochondrial translation. [³⁵S]methionine *de novo* synthesized mtDNA-encoded proteins from HEK293T WT cells or *Gtpbp10*^{64R65K} cells were visualized by autoradiography (upper panel) or with designated antibodies (lower panel). SDHA is used as a loading control ($n = 3$). (D) Ablation of GTPBP10 reduces growth rate. Equal numbers of HEK293T WT and *Gtpbp10*^{64R65K} cells were seeded on day 0 (0d) and counted after 1 day (1d), 2 days (2d) and 3 days (3d) (mean \pm SD, $n = 3$). (E and F) GTPBP10 is required for 16S rRNA stability. (E) Steady state levels of mtDNA-encoded RNAs extracted from HEK293T WT and *Gtpbp10*^{64R65K} cells were analyzed by Northern blot with indicated probes. *MT-RNR1*: 12S rRNA; *MT-RNR2*: 16S rRNA; *MT-CO1*: mRNA encoding COX1; *MT-CO2*: mRNA encoding COX2. *18S-rRNA* was used as a loading control. (F) *MT-RNR1* and *MT-RNR2* were quantified using ImageJ and normalized to *18S-rRNA* (mean \pm SEM; $n = 3$).

loss of function of GTPBP10 on mitochondrial protein synthesis. In order to generate a stable knockout cell line we applied CRISPR/Cas9 technology, however, we failed to gain a complete loss of GTPBP10 as residual protein levels were still detectable, but were significantly reduced in the obtained clone (Figure 2B). Sequencing analysis revealed a deletion of six nucleotides (190–195) leading to the deletion of two amino acids at position 64 (R) and 65 (K) in the Obg fold domain (Supplementary Figure S1 and Figure

1B). As especially lysine at position 65 is conserved between human GTPBP10 and ObgE it is tempting to speculate that this deletion compromises function and thus leads to the instability of GTPBP10 and therefore represents a good tool to analyze the function of GTPBP10 in human mitochondria further. In order to address the effect of functional loss of GTPBP10 in this mutant cell line (*Gtpbp10*^{64R65K}) we performed *de novo* mitochondrial translation assays and observed an overall significant reduction of newly synthe-

sized mtDNA-encoded proteins leading to reduced protein steady state levels as represented by COX1 and COX2 (Figure 2B and C). The ablation of GTPBP10 and its accompanied decreased mitochondrial protein synthesis lead to a significant reduced cell growth as demonstrated by cell counts for one to three days in glucose containing media (Figure 2D). We asked further whether the reduced levels of mtDNA-encoded proteins were due to decreased levels of mitochondrial transcripts and analyzed isolated RNA from wild type and *Gtpbp10*^{64R65K} cells via Northern blot (Figure 2E). Interestingly, the levels of the mitochondrial rRNA of the mtSSU, 12S rRNA (*MT-RNR1*) and of mitochondrial mRNAs encoding COX1 (*MT-CO1*) and COX2 (*MT-CO2*) remained stable suggesting that GTPBP10 does not affect mitochondrial gene expression at the level of transcription. However, the mitochondrial rRNA of the mtLSU, 16S rRNA (*MT-RNR2*) was significantly reduced suggesting defects in mtLSU and thus in mitoribosome stability or biogenesis, which might explain the diminution in mitochondrial translation (Figure 2F).

GTPBP10 associates specifically with the mtLSU at a late maturation state

As the 16S rRNA was significantly reduced in *Gtpbp10*^{64R65K}, we speculated that GTPBP10 plays a role in the biogenesis of the mtLSU. In order to address this possibility, first, we analyzed whether GTPBP10 interacts with the mitoribosome. Applying sucrose density gradient centrifugation GTPBP10 mostly migrated in the less dense fractions, however, a portion of GTPBP10 clearly co-fractionated with proteins of the mtLSU, but not with the monosome (Figure 3A). To assess a possible interaction of GTPBP10 with the mtLSU, we performed co-immunoprecipitation experiments using C-terminal FLAG-tagged mL62 (13), a structural component of the central protuberance of the mtLSU (25–27). Utilizing mL62^{FLAG} as bait, we were able to isolate the mtLSU and the 55S monosome as demonstrated by the co-isolation of MRPs of the mLSU and mtSSU (Figure 3B). Interestingly, GTPBP10 was also significantly enriched in the elution fraction of mL62^{FLAG}, which is in agreement with the co-sedimentation of GTPBP10 with the mtLSU on sucrose gradients. To exclude the association of GTPBP10 with the 55S monosome or the mtSSU we performed similar immunoprecipitation experiments using mS40^{FLAG}, a component of the mtSSU (Figure 3C). Under those conditions we expect to co-isolate the mtSSU and the 55S monosome, but no free mtLSU. Indeed, we did not detect any GTPBP10 in this elution fraction indicating that GTPBP10 exclusively interacts with the mtLSU, but not with the mtSSU or the assembled 55S monosome. To directly address whether GTPBP10 interacts only with the completely assembled mtLSU or also with assembly intermediates and other ribosome biogenesis factors, we generated a stable cell line inducibly expressing a C-terminal FLAG-tagged GTPBP10 to determine its interactome via co-immunoprecipitation experiments. To avoid overexpression of GTPBP10^{FLAG}, we titrated the concentration of the inducer tetracycline to a minimum (Supplementary Figure S2). Western blot analysis of

co-immunoprecipitation experiments using GTPBP10^{FLAG} as bait confirmed the association of GTPBP10 with the mtLSU as all the tested MRPs of the mtLSU were enriched in the eluate of GTPBP10^{FLAG} (Figure 3D). In agreement with our previous observations GTPBP10^{FLAG} did not co-isolate components of the mtSSU confirming that GTPBP10 does not interact with the mtSSU or with the 55S mitoribosome. Interestingly, we detected also a number of known ribosome biogenesis factors including GTPBP7 (MTG1), NGRN and MALSU1 (11,20,28,29).

To comprehensively determine the interactome of GTPBP10^{FLAG} we performed quantitative mass spectrometry analyses. We identified 51 of 52 MRPs of the mtLSU (Figure 3E, Supplementary Table S1). Only bL36m was not detected, which seems to be a common problem as it was also not detected in other mitoribosome analyses (30). None of the MRPs of the mtSSU were enriched under our set thresholds. Most interestingly, we identified a number of mtLSU assembly factors and mtRNA granule proteins, which are involved in the processing and maturation of the 16S rRNA indicating that GTPBP10 is indeed involved in the biogenesis of the mtLSU (Table 1). The most enriched associated factors of GTPBP10^{FLAG} were SMCR7L and MALSU1. Both proteins were shown to be part of a late mtLSU assembly intermediate as shown by cryo-EM analyses (31), suggesting that GTPBP10 might act late in the mtLSU biogenesis. In agreement with such a hypothesis is the association with MTERF4, which targets NSUN4, also identified in the complex with GTPBP10, to the mtLSU at a late maturation state to form the 55S monosome (32,33). To support this observation, we performed a FLAG-immunoprecipitation of GTPBP10 and analyzed native isolated complexes via sucrose gradient (Figure 3F). We tested a number of mitoribosomal proteins of the mtLSU including uL1m and uL23m, which can be usually also detected in less dense fractions presumably presenting assembly intermediates (Figure 3A). However, apart from GTPBP10 itself, we did not observe any other protein including uL1m and uL23m in the less dense fractions suggesting that GTPBP10 does not associate with early mtLSU assembly intermediate complexes. All tested MRPs, which were co-purified with GTPBP10 sediment in the higher dense fractions indicating that GTPBP10 associates either with a very late assembly intermediate or with the matured mtLSU.

Loss of GTPBP10 affects mitoribosome assembly

As GTPBP10 associates exclusively with the mtLSU, it is tempting to speculate that GTPBP10 has a role in the biogenesis of the mtLSU potentially at a late assembly stage. Hence, we aimed to analyze the consequences of the loss of GTPBP10 on the biogenesis of the mtLSU and the 55S monosome. First, we tested GTPBP10 ablation on the protein steady state level and observed a decrease in certain mitoribosomal proteins, especially from the mtLSU, namely uL3m, uL13m and mL62m (Figure 4A and C). Interestingly, we noticed a decrease of NGRN as well, but not of GTPBP7 or MALSU1 (Figure 4B and C), which were all identified to be in complex with GTPBP10 (Figure 3D and F) and are suggested to be involved in the as-

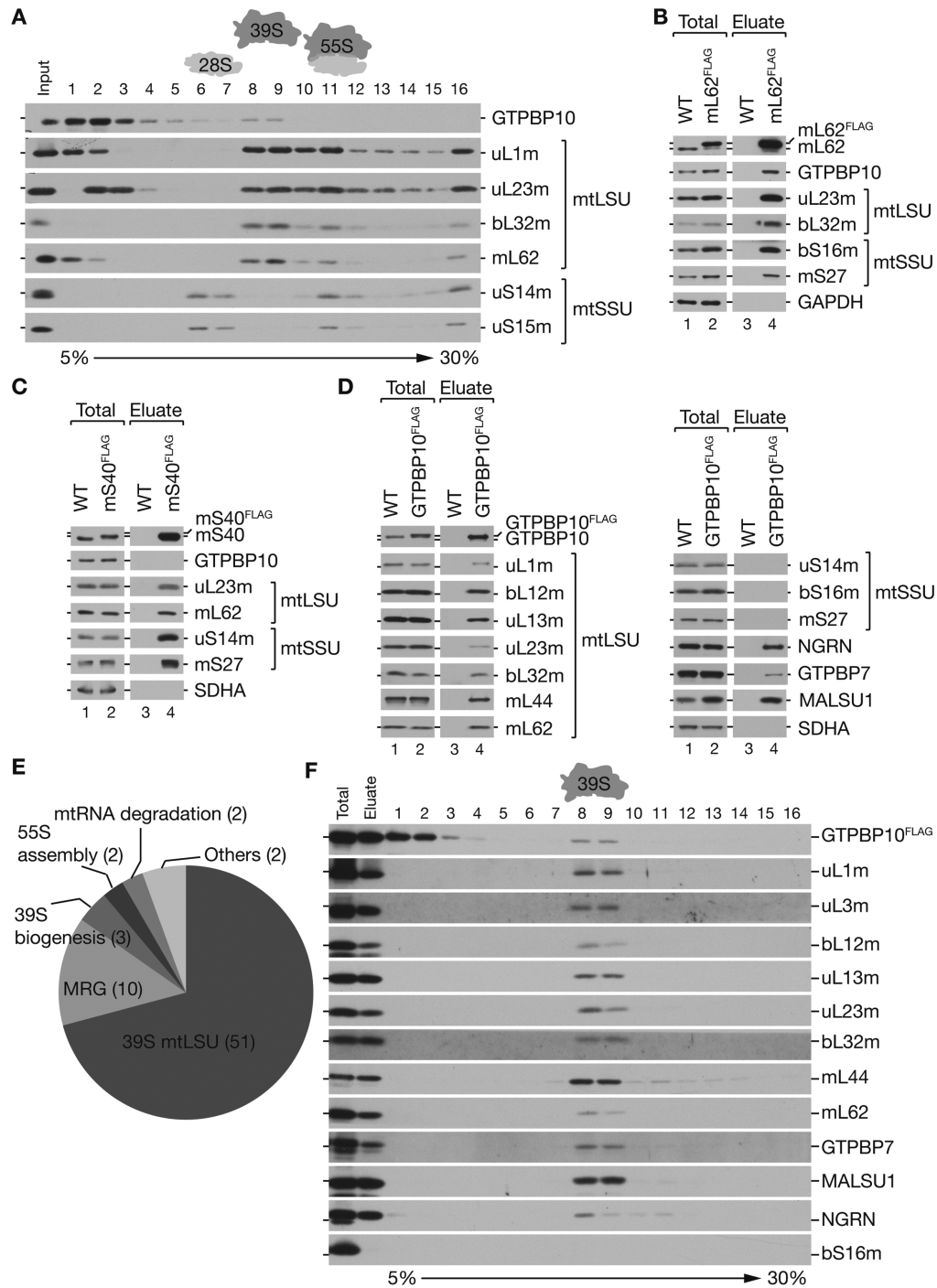


Figure 3. GTPBP10 interacts with the mtLSU and assembly factors. (A) GTPBP10 co-fractionates with mtLSU. Native protein complexes were isolated from HEK293T WT mitoplasts and separated by 5–30% sucrose gradient centrifugation. Fractions (1–16) were visualized by western blot with antibodies against mtLSU (uL1m, uL23m, bL32m, mL62) and mtSSU (uS14m, uS15m). (*n* = 4). (B–C) GTPBP10 associates specifically with mtLSU. Co-immunoprecipitation of FLAG-tagged mL62 (B) and mS40 (C). GAPDH and SDHA were used as negative controls for unspecific binding. Total, 3%; Eluate, 100% (*n* = 3). (D) Mitochondrial proteins and biogenesis factors of the mtLSU co-purify with GTPBP10^{FLAG}. Mitochondrial protein complexes containing GTPBP10 were co-purified via FLAG-tagged GTPBP10. Total, 3%; Eluate, 100% (*n* = 3). (E) The interactome of GTPBP10^{FLAG}. Equal amounts of differentially labeled mitochondria from HEK293T WT and GTPBP10^{FLAG} cells were mixed and applied to FLAG-immunoprecipitation. Native eluted complexes were analyzed by quantitative mass spectrometry. Diagram represents the results from four experiments (including label switch). *P* < 0.05; mean ratio ≥ 2 (*n* = 4). (F) GTPBP10 associates with mtLSU at a late assembly stage. FLAG-immunoprecipitation via GTPBP10^{FLAG} was performed as in (D). Native eluate was subjected to 5–30% Sucrose gradients. Fractions were analyzed by western blot with specific antibodies against mitoribosomal proteins of the mtLSU and mtSSU, and assembly factors. Total (FLAG-immunoprecipitation input), 1%; Eluate (FLAG-immunoprecipitation eluate = gradient input), 10% (*n* = 3).

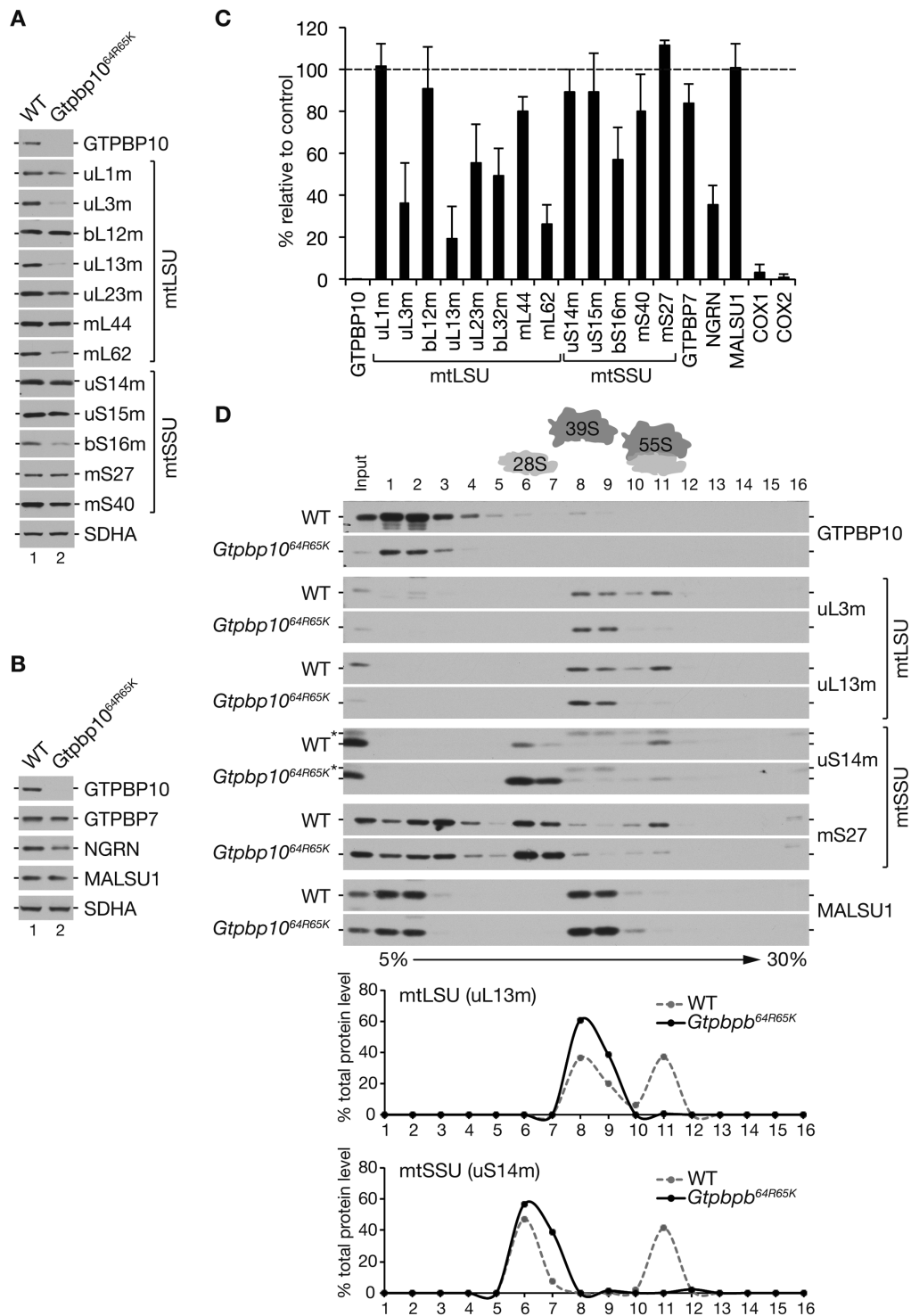


Figure 4. GTPBP10 is required for 55S monosome formation. (A-C) *Gtpbp10*^{64R65K} leads to reduced protein levels of selected MRPs (A) and ribosome biogenesis factors (B). (C) Quantification of steady state analysis of MRPs and biogenesis factors in *Gtpbp10*^{64R65K} cells relative to HEK293T WT control. SDHA was used as a loading control. ($n = 3$, mean \pm SEM). (D) Ablation of GTPBP10 reduces monosome formation. Protein complexes from HEK293T WT and *Gtpbp10*^{64R65K} mitoplasts were separated on 5–30% sucrose gradients and fractions (1–16) were analyzed by western blot with specific antibodies against mtSSU and mtLSU components and MALSU1. Protein distributions for uL13m (mtLSU) and uS14m (mtSSU) are presented as percentage of the total protein abundance. (*) indicates residual signals of bL32m.

Table 1. GTPBP10-associated factors

Name	Accession	Mean ratio	P-value
SMCR7L	L0R8F8	4.68	0.00015
MTERF4	Q7Z6M4	4.38	0.00031
MALSU1	Q96EH3	4.29	0.00038
DDX28	Q9NUL7	4.10	0.00055
NSUN4	Q96CB9	3.85	0.00102
TRUB2	O95900	3.36	0.00330
YARS2	Q9Y2Z4	3.32	0.00365
NGRN	Q9NPE2	3.19	0.00513
RNMTL1/MRM3	Q9HC36	3.13	0.00593
GTPBP7/MTG1	Q9BT17	3.13	0.00590
PTCD1	O75127	3.10	0.00635
FASTKD2	Q9NYY8	3.04	0.00770
WBSCR16/RCC1L	Q96I51	2.98	0.00874
SUPV3L1	Q8IYB8	2.95	0.00944
MTERF3	Q96E29	2.89	0.01210
TRMT61B	Q9BVS5	2.64	0.02145
RPUSD4	Q96CM3	2.56	0.02982
HSPA9/mtHSP70	P38646	2.56	0.02582
RPL22L1	Q6P5R6	2.56	0.02608
PNPT1/PNPASE	Q8TCS8	2.35	0.04580
PMPCA/MPPA	Q10713	2.34	0.04656

Mean ratio ≥ 2 ; P-value ≤ 0.05 .

Identified proteins of the mtLSU (51/52) are excluded from this table (see Supplementary Table S1).

sembly of the mtLSU. Our data indicate that GTPBP10 is required for the stability of certain mitoribosomal proteins and of mtLSU biogenesis factor NGRN and that the loss of GTPBP10 impairs mitoribosome maturation. To support this hypothesis we analyzed the formation of the 55S monosome by co-immunoprecipitation of uL1m, a core component of the mtLSU, which remains stable in the absence of GTPBP10 (Figure 4A and C). As expected in the absence of GTPBP10 the level of monosome formation decreases as demonstrated by the reduced level of co-immunoprecipitated mtSSU protein mS27 whereas components of the mtLSU including uL3m and uL13m remain associated to uL1m (Supplementary Figure S3).

In addition, we analyzed the consequences of the loss of GTPBP10 on mtLSU assembly and monosome formation via sucrose gradient centrifugation (Figure 4D). In agreement with the aforementioned co-immunoprecipitation experiments of uL1m, GTPBP10 ablation leads clearly to a drastic reduction of the 55S monosome, explaining the significant decrease in mitochondrial gene expression. All mitoribosomal proteins tested in these experiments were accumulating in the mtLSU or mtSSU, respectively. Thus, GTPBP10 influences mitoribosome biogenesis at a very late stage and loss of GTPBP10 leads to the accumulation of mtSSU and mtLSU with reduced 55S monosome formation.

GTPBP10 mutants are impaired in mtLSU binding

As GTPBP10 is homologous to bacterial ObgE, containing Obg domain as well as a functional GTPase domain (10), we asked whether both domains are required for mitoribosome biogenesis. To address this question, we generated two inducible stable cell lines expressing a FLAG tagged Obg domain mutant, namely GTPBP10^{G82E-FLAG} and a FLAG tagged GTPase domain mutant GTPBP10^{S325P-FLAG}. Both variants represent mutants with the replacement of a sin-

gle highly conserved amino acid in the respective domain (Figure 1B). Respective mutations in Obg proteins were reported to have an effect on ribosome biogenesis and cellular function in different bacterial strains (8,34). Interestingly, the induced expression of both mutants led to a significant reduction in cell growth monitored by decreased cell numbers in comparison to HEK293T wild type or GTPBP10^{WT-FLAG} cells (Figure 5A). To address the cause of this growth defect we analyzed the protein steady state levels of mtDNA-encoded proteins and indeed, we observed a significant reduction in COX1 and COX2 levels in both mutants of which the Obg mutant GTPBP10^{G82N-FLAG} showed the strongest effect (Figure 5B). The observed decrease in COX1 and COX2 levels suggest impaired mitochondrial gene expression in GTPBP10 mutants. Hence, we analyzed the *de novo* synthesis of mtDNA-encoded proteins by [³⁵S]Methionine labeling. Although we monitored a reduction in mitochondrial protein synthesis upon overexpression of GTPBP10^{WT-FLAG}, we observed a more drastic decrease in mitochondrial translation in the GTPBP10 mutant cell lines which might lead to a progressively reduction in the steady state level of mtDNA-encoded proteins (Figure 5C). To exclude that the reduction in COX1 and COX2 are due to decreased stability of newly synthesized mtDNA-encoded proteins, we performed [³⁵S]Methionine pulse-chase labeling experiments (Figure 5D). As the stability of newly synthesized mtDNA-encoded proteins are only marginally affected in the GTPBP10 mutants compared to the wild type cell line it is reasonable to suggest that reduced COX1 and COX2 levels are mainly caused by decreased mitochondrial translation.

As GTPBP10 binds the mtLSU at a late assembly state it is tempting to speculate that mtLSU biogenesis is impaired upon expression of GTPBP10 mutants. Thus, we analyzed whether GTPBP10 mutants maintained the ability to bind to the mtLSU. To this end, we performed FLAG-

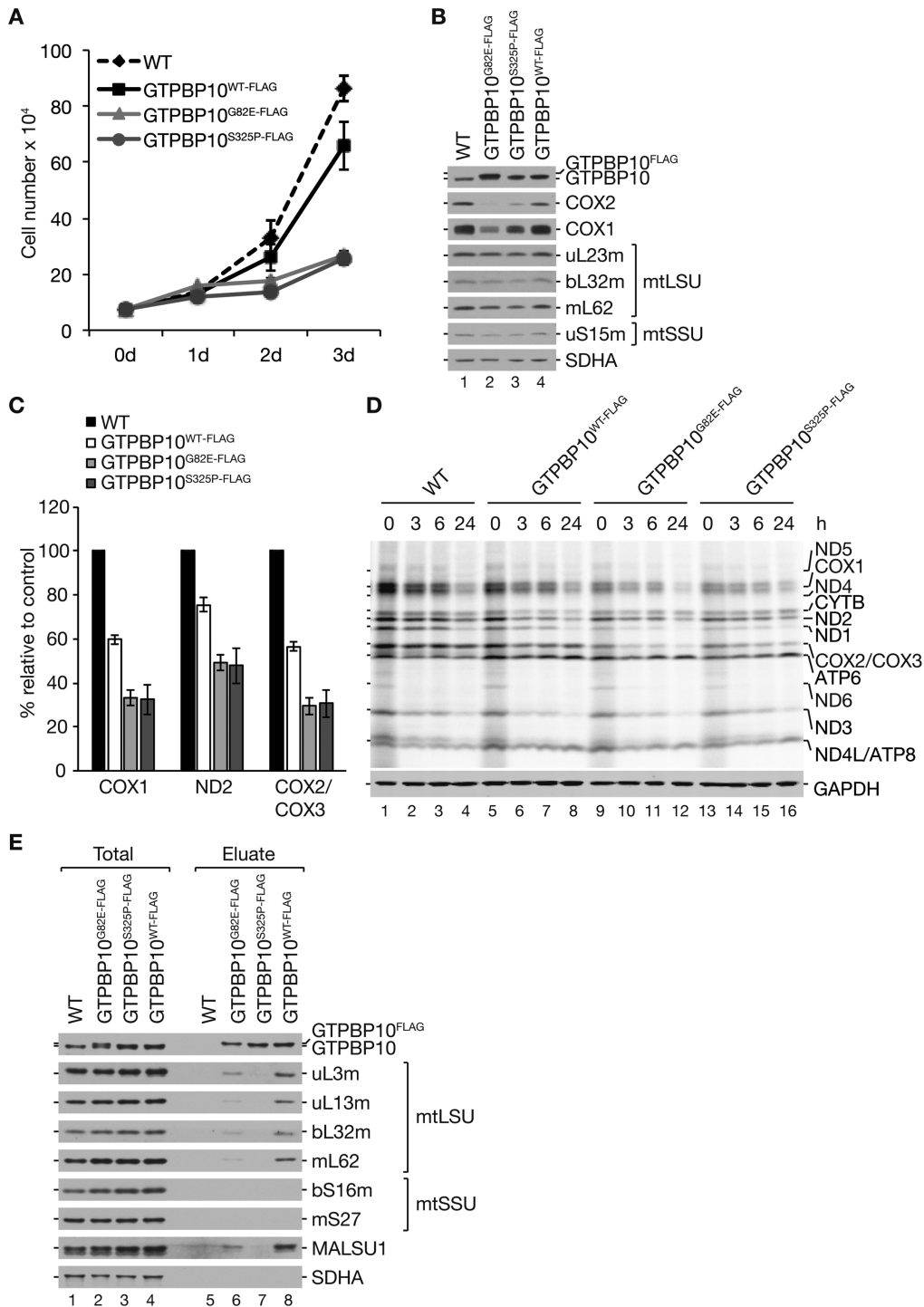


Figure 5. Expression of Obg and GTPase domain mutants negatively affect the function of GTPBP10. (A) GTPBP10^{G82E} and GTPBP10^{S325P} affect cell growth. Equal numbers of HEK293T WT, GTPBP10^{G82E-FLAG}, GTPBP10^{S325P-FLAG} and GTPBP10^{WT-FLAG} cells were seeded on day 0 (0d) and counted after 1 day (1d), 2 days (2d) or 3 days (3d) ($n = 3$, mean \pm SD). (B) GTPBP10^{G82E} and GTPBP10^{S325P} affect the steady state level of mtDNA-encoded proteins. Proteins were extracted from indicated cell lines and analyzed by western blot. SDHA was used as a loading control. (C and D) Synthesis and stability of mtDNA-encoded proteins in GTPBP10 mutants. Cells were pulse labeled for 1h in the presence of [³⁵S]Methionine (lanes 1, 5, 9 and 13) and chased for the indicated time points. Mitochondrial translation products from HEK293T WT cells or GTPBP10^{G82E-FLAG}, GTPBP10^{S325P-FLAG} and GTPBP10^{WT-FLAG} cells were visualized by autoradiography (D, upper panel). GAPDH was used as a loading control. Newly synthesized COX1, ND2 and COX2/COX3 were quantified after 1h pulse labeling using ImageQuant TL (C) (mean \pm SEM, $n = 4$). (E) GTPBP10 mutants show reduced mtLSU binding capacity. Lysed mitochondria from cell lines expressing FLAG-tagged GTPBP10^{G82E}, GTPBP10^{S325P} or wild type GTPBP10 were subjected to FLAG-immunoprecipitation. Samples were analyzed by western blot using indicated antibodies. SDHA was used as a negative control for unspecific binding. Total, 3%; Eluate, 10% ($n = 2$).

immunoprecipitation experiments with GTPBP10^{WT-FLAG} and the Obg and GTPase mutants. All FLAG-tagged versions of GTPBP10 were expressed at a similar level comparable to the endogenous level (Figure 5E). In contrast to GTPBP10^{WT-FLAG} both mutants showed a reduction in mtLSU association as demonstrated by decreased or non-detectable levels of co-immunoprecipitated mitoribosomal proteins of the mtLSU, where the GTPase mutant GTPBP10^{S325P-FLAG} revealed the most drastic reduction in mtLSU binding capacity. These data suggest that both the Obg domain as well as the GTPase domain are required for mtLSU binding and consequently for mitoribosome biogenesis and therefore for mitochondrial gene expression.

DISCUSSION

The two human homologues of bacterial ObgE, namely GTPBP5 and GTPBP10, were previously shown to localize to different cellular compartments. While GTPBP5 was demonstrated to be present within mitochondria, GTPBP10 was shown to localize to the nucleolus using fluorescence microscopy (10). Our studies provide evidence that GTPBP10 is a mitochondrial matrix protein, peripherally associated with the inner mitochondrial membrane, which is also in agreement with data by Arroyo *et al.* showing that GTPBP10 is required for OXPHOS function using a genome wide CRISPR ‘death screen’. Applying CRISPR/Cas9 technology we generated a GTPBP10 mutant with a deletion of arginine and lysine at position 64 and 65 within the Obg domain leading to the instability of the protein. This mutant cell line clearly demonstrated a mtDNA-expression defect, which also explains the requirement for OXPHOS function and concomitantly for cell growth for this factor. In addition, the overexpression of an Obg and a GTPase mutant variant also led to a reduction in mtDNA-expression with a progressively decrease in mtDNA-encoded proteins accompanied with a cell growth defect. What is the cause of this translation defect? Similarly to other mitochondrial GTP binding proteins such as GTPBP5 and GTPBP7 we show that GTPBP10 specifically associates with the mtLSU, but not with the mtSSU or the assembled monosome (11). For the association of GTPBP10 with the mtLSU both the Obg as well as the GTPase domain are required as mutants loose mtLSU binding capacity. Defining the interactome of GTPBP10 we identified besides the proteins of the mtLSU also components of the mitochondrial RNA granules and the 16S rRNA regulatory module such as DDX28, NGRN, WBSR16, RPUSD4, TRUB2, and FASTKD2 (20,23,35–38) suggesting the involvement of GTPBP10 in the biogenesis of the mtLSU. The ablation of GTPBP10 leads to a reduction of NGRN, which might also explain the partial decrease in the steady state level of 16S rRNA and some mitoribosomal proteins as NGRN depletion is associated with 16S rRNA diminishment (20). Interestingly, two of the most enriched proteins found in a complex with GTPBP10 were MALSU1, which was previously suggested to be a ribosome assembly or stability factor involved in mtLSU function (28,29), and SMCR7L. Both factors were identified in a late assembly intermediate of the mtLSU by cryo-EM (31). In addition MTERF4 and NSUN4 were also found to be

part of a GTPBP10 containing complex. As MTERF4 and NSUN4 together associate with a late matured mtLSU facilitating monosome formation (33), it is tempting to speculate that GTPBP10 is also involved in the late steps of mtLSU biogenesis, which would be also in agreement with its interaction with the late assembly factors MALSU1 and SMCR7L. Similar to the loss of either MTERF4 or NSUN4, GTPBP10 deficiency leads to a reduction in 55S monosome and thus to a decrease in mtDNA-expression at the translational level (32,33). What is the role of GTPBP10 in mtLSU biogenesis? The bacterial homologue ObgE has been suggested to be an anti-association factor preventing the formation of a monosome with irregular LSU particles (9). A temperature sensitive bacterial ObgE mutant affects rRNA processing and decreases 70S formation (8), reminiscent to the phenotypes in GTPBP10 deficient cells. As GTPBP10 associates with the mtLSU at a late assembly stage it is tempting to speculate that GTPBP10 might also act as a quality control factor for mtLSU maturation. Although both GTPBP5 and GTPBP10 complement Δ ObgE in *E. coli* (10), both are required in mitochondria as loss of either GTPBP5 (11) or GTPBP10 affect mitochondrial translation suggesting that they do not have overlapping function in human. Interestingly, GTPBP10 shows two deletions in the Obg domain in loop 1 and loop 3 in comparison to bacterial Obg proteins or human GTPBP5 (Figure 1B). As the human mitoribosome differs substantially from its bacterial counterpart these rearrangements in the Obg fold of GTPBP10 might reflect co-evolutionary changes and might explain the requirement for two Obg proteins in human mitochondria.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Peter Rehling and Sven Dennerlein for critical reading of the manuscript and for discussions, Peter Rehling for providing antibodies, and Abhishek Aich for his guidance in CRISPR/Cas9 technology. We are thankful to Monika Raabe and Annika Kühn for technical assistance in mass spectrometry.

FUNDING

Deutsche Forschungsgemeinschaft [SFB860-A10 to H.U., SFB860 to R.R.-D., SFB1190-Z02 to H.U., SFB1190 to R.R.-D.]; Max Planck Society (to H.U.). Funding for open access charge: Deutsche Forschungsgemeinschaft.

Conflict of interest statement. None declared.

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