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Eukaryotic Initiation Factor 6 is rate-limiting in translation, growth and transformation

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Abstract

Cell growth and proliferation require coordinated ribosomal biogenesis and translation. Eukaryotic Initiation Factors (eIF) control translation at the rate-limiting step of initiation^{1,2}. So far, only two eIFs connect extracellular stimuli to global translation rates³; eIF4E acts in the eIF4F complex and regulates binding of capped mRNA to 40S subunits, downstream of growth factors⁴; eIF2 controls loading of the ternary complex on the 40S subunit and is inhibited upon stress stimuli^{5–6}. No eIFs have been found to link extracellular stimuli to the activity of the large 60S ribosomal subunit. eIF6 binds 60S ribosomes precluding ribosome joining *in vitro*^{7–9}. However studies in yeasts showed that eIF6 is required for ribosome biogenesis rather than translation^{10–13}. We show that mammalian eIF6 is required for efficient initiation of translation, *in vivo*. eIF6 null embryos are lethal at preimplantation. Heterozygous mice have 50% reduction of eIF6 levels in all tissues, and show reduced mass of hepatic and adipose tissues due to a lower number of cells and to impaired G1/S cell cycle progression. eIF6^{+/-} cells retain sufficient nucleolar eIF6 and normal ribosome biogenesis. The liver of eIF6^{+/-} mice displays an increase of 80S in polysomal profiles, indicating a defect in initiation of translation. Consistently, isolated hepatocytes have impaired insulin-stimulated translation. Heterozygous mouse embryonic fibroblasts (MEFs) recapitulate the organism phenotype and have normal ribosome biogenesis, reduced insulin-stimulated translation, and delayed G1/S phase progression. Furthermore, eIF6^{+/-} cells resist to oncogene-induced transformation. Thus, eIF6 is the first eIF associated with the large 60S subunit that regulates translation in response to extracellular signals.

The eIF6 gene was deleted by homologous recombination using embryonic stem (ES) cell technology (Supplementary Fig. 1a). The portion of the gene containing the first two exons and the first two introns was substituted by a cassette containing the neomycin resistance gene. The presence of the neomycin resistance cassette did not affect expression of wt eIF6 and of adjacent genes (Supplementary Fig. 2). Germline transmission was achieved and intercrossing

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of eIF6 heterozygous mice did not produce any eIF6^{-/-} offspring. We did not observe eIF6^{-/-} mice as early as day 3.5 d.p.c. indicating that eIF6 is essential for proper development at the stage of preimplantation (Supplementary Table 1). The lethality of eIF6^{-/-} embryos is consistent with the early expression of the protein in the blastocysts (Supplementary Fig. 1d).

Heterozygous eIF6^{+/-} mice were viable and indistinguishable from wt counterparts up to 30 days after birth. At three months of age, heterozygous mice, independently from gender and genetic background, weighed less than their wt littermates (Fig. 1a). The head-anus length of eIF6^{+/-} and wt mice was identical, suggesting that the reduction of body mass in eIF6^{+/-} mice could be due to smaller size of specific organs (Fig. 1b). Biometric measures of eIF6^{+/-} and wt organs at autopsy revealed that heart and brain were equal to wt, whereas all other organs from eIF6^{+/-} mice showed a slight trend to mass reduction (not shown). When the weight of the organs was normalized to the body weight of the corresponding animal, 1-)for most organs the differences disappeared, 2-)the liver (Fig. 1c) and the adipose tissue (Supplementary Fig. 3) exhibited a reduction in their weight/body ratio. A reduction in liver mass could be due to a reduction in cell number, or to a reduction in cell size. Histological staining showed that the liver of eIF6^{+/-} mice was normal (Supplementary Fig. 4b, 4d), and further morphometric analysis excluded differences in cell size (Supplementary Fig. 4a, 4c). In contrast, we observed fewer cycling cells in the liver of eIF6^{+/-} mice (Fig. 1d, e) suggesting that its reduced mass was due to a reduction in cell number. We performed a similar analysis on adipocytes derived from mesenchymal stem cells: in summary we found that the reduction of adipose tissue was due to a decreased proliferation of pre-adipocytes, whereas the lipid content/cell was constant (Supplementary Fig. 3d). We conclude that the reduction in liver and fat tissue is likely due to a cell autonomus defect marked by reduced proliferative capability.

Quantitative Western blotting analysis of eIF6 levels showed that in all heterozygous organs the levels of eIF6 were reduced to half of the wild type (Fig. 1f; Supplementary Fig. 3c for fat tissue). In spite of reduction of eIF6 levels, we did not detect obvious phenotypes, in vivo, in other organs, including the skin (Supplementary Fig. 5), where we reported binding of eIF6 (p27BBP) to beta4 integrin¹⁴. The reduction of eIF6 in all organs from heterozygous mice suggests that under specific circumstances eIF6 haploinsufficiency may become rate-limiting.

We performed polysomal profiles of livers from eIF6^{+/-} and eIF6^{+/+} littermates. Approximately 30% of the het/wt couples analysed did not present detectable differences in polysomal profiles. In the remaining 70% het/wt couples, in eIF6^{+/-} mice polysomal profiles showed an increased 80S peak, accompanied by decreased polysomes (Fig. 2a), suggesting that in mammalian liver, eIF6^{+/-} hepatocytes have a defect in initiation of translation rather than in ribosome biogenesis. We tested this hypothesis using insulin to stimulate basal translation on primary hepatocytes *ex vivo*¹⁵. We found that insulin administration led to an increase of methionine incorporation in eIF6^{+/+} hepatocytes, but had little or no effect in eIF6^{+/-} hepatocytes (Fig. 2b).

We further explored primary MEFs (Mouse Embryonic Fibroblasts) from eIF6^{+/-} mice. Remarkably, primary MEFs from eIF6^{+/-} mice showed a phenotype identical to liver hepatocytes with an accumulation of 80S peak (Fig. 2c) and a reduction in insulin-stimulated translation (Fig. 2d), demonstrating that eIF6 haploinsufficiency alters translation in different cell types. We previously reported that PMA (Phorbol 12-myristate 13-acetate) stimulated methionine incorporation in conditions of eIF6 overexpression⁹. We tested whether PMA-stimulated methionine incorporation was reduced in eIF6^{+/-} cells, and found it was (Supplementary Figure 6). In order to understand if basal translation was affected in conditions of eIF6 haploinsufficiency, we pooled data from different experiments. Briefly, basal translation is slightly affected in primary hepatocytes, but not in MEFs, consistently with the

observation that, *in vivo*, only liver development is affected by eIF6 haploinsufficiency (Supplementary Figure 7).

We decided to test if the reduction in methionine incorporation observed in conditions of eIF6 haploinsufficiency could be due to an indirect inactivation of the mTOR pathway, which regulates translation at the level of initiation. We found that in MEFs, the activation of the PI3K-mTOR pathway, measured through the phosphorylation of Akt, rpS6 and 4E-BP1 was normal (Supplementary Fig. 8). The levels of insulin receptor were also normal (Supplementary Fig. 9).

MEF reconstituted with eIF6 to wt levels of eIF6 (Supplementary Fig. 10) regained insulin responsiveness (Fig. 2e), ruling out indirect effects due to chronic eIF6 depletion. In order to establish whether translation impairment might be due to the anti-association activity of eIF6, we exploited a Ser235Ala mutant, unable to be released from 60S, *in vitro*, upon phosphorylation as we previously described⁹. Heterozygous MEFs reconstituted with eIF6^{Ser235Ala} mutant (Supplementary Fig. 10) were not stimulated by insulin and behaved as eIF6^{+/-} cells (Fig. 2f).

We evaluated in detail whether eIF6 haploinsufficiency can lead to deficits in ribosome biogenesis. Briefly, the ratio of free 40S and 60S in eIF6^{+/-} MEFs was equal to eIF6^{+/+} MEFs, both in the cytoplasm and in the nucleus (Supplementary Fig. 11). The fact that eIF6^{+/-} MEFs show altered translation and normal ribosome biogenesis can be explained by specific reduction of eIF6 in the cytoplasm, but not in nucleoli. Strikingly, the reduction of eIF6 that we observed in the cytoplasm of eIF6^{+/-} cells was more conspicuous than the reduction in the nucleus (Supplementary Fig. 12a). In addition, the (apparent) amount of nucleolar associated eIF6 was identical in wild type and heterozygous cells (Fig. 3a). In order to answer whether subtle alterations in ribosome biogenesis could be observed in conditions of haploinsufficiency, we analysed by Northern blotting the integrity of rRNA processing pathway (Fig. 3b), in MEFs and liver. Data show that the steady-state level of rRNA precursors 47/45S, 32S and mature 28S is identical in wt and eIF6^{+/-} cells (Fig. 3b–c). MEFs from early passages (P2/P3) are rapidly growing in culture allowing also quantitative analysis of rRNA biogenesis¹⁶ by pulse-chase with radiolabelled uridine. We found that eIF6^{+/-} MEFs process rRNA normally (Supplementary Fig. 12c). We also analysed by qt RT-PCR the levels of eIF6 mRNA, and two ribosomal proteins rpL5 and rpS19. Data showed that primary eIF6^{+/-}MEFs have 50% reduction of eIF6 mRNA, but no other ribosome associated proteins, both at the level of the mRNA and at the level of protein (Supplementary Fig. 12d, e). Finally, we asked whether a further reduction of eIF6 could lead to a defect in rRNA biogenesis. Eighty percent depletion of eIF6 by RNAi in NIH3T3 cells (Supplementary Fig. 13) showed normal rRNA synthesis (Fig. 3d) and detectable eIF6 in the nucleolus (Fig. 3e). Taken together, these data suggest that the reduction of cytoplasmic eIF6 leads to an increase in the steady-state levels of 80S, accompanied by an impairment of the capability of cells to upregulate protein synthesis upon insulin stimulation, but a small amount of eIF6 is sufficient for rRNA biogenesis. In other terms, eIF6 is an initiation factor, *in vivo*.

We analysed the consequences of eIF6 depletion on cell cycle progression in normal and highly proliferative cells. In early passages eIF6^{+/-} MEFs proliferated similarly to wt, had equal replicative senescence (Supplementary Fig. 14a, b), rate of apoptosis (Supplementary Fig. 14c, d) and cell size (Supplementary Fig. 14e). In these conditions, cell cycle distribution by FACS analysis of exponentially growing MEFs derived from eIF6 wild type and matched heterozygous did not show gross differences (Fig. 4a). Next, we analysed cell cycle progression in cells synchronized by serum starving and stimulated by serum refeeding. Data obtained by ³H-Thymidine labelling show that eIF6^{+/-} MEFs enter S-phase 18 hours after stimulation (Fig. 4b), compared to the 12 hours required by wt cells. These data show that eIF6

haploinsufficiency impacts on translation and S-phase entry under specific conditions and raise the question on further eIF6 dependent phenotypes. eIF6 is overexpressed in cancer cells^{17, 18}. We decided to test whether eIF6 haploinsufficient MEFs were efficiently transformed by infection with retroviral vectors encoding DNp53 and H-ras^{V12}, or Myc and H-ras^{V12} respectively. eIF6^{+/-} MEFs formed fewer transformed colonies in soft agar when infected with oncogene-carrying retroviruses, as compared to wt (Fig. 4c, d). We had similar results in the focus formation assay (Supplementary Fig. 15a, b). The large difference in the transformation efficiency was not due to differences in retroviral infection and integration (Supplementary Fig. 15c).

Whether eIF6 is a true Initiation Factor has been unclear for a long time. The effect of eIF6, *in vitro*, ranges from no effects on translation to mild stimulation at low doses accompanied by repression at high doses⁷⁻⁹. The difference in the results and in the systems employed did not allow a final conclusion. Tif6 (yeast homologue of mammalian eIF6) depletion led to the loss of 60S biogenesis and rRNA processing defects¹⁰⁻¹³. In yeasts, most of Tif6 seems to be localized in the nucleus^{19,20}, and in pre-ribosomal particles²¹, thus raising the question on the physiological relevance of the regulated eIF6 antiassociation activity. The results we obtained in eIF6 knockout mice demonstrate that eIF6 may be rate-limiting for translation, *in vivo*, where it may behave as a stimulatory initiation factor downstream insulin/growth factors. From this perspective eIF6 is the first 60S associated initiation factor able to modulate translation downstream of extracellular signaling, *in vivo*. These data do not contradict a role of eIF6 in ribosome biogenesis, but suggest that ribosome biogenesis requires only sequestering a (minor) nucleolar pool of eIF6.

It remains to be understood if eIF6 regulates global translation or specific classes of mRNA. It was recently proposed that eIF6 is the mediator regulating miRNA-mediated repression²² in *C. elegans* and HeLa cells although not in *Drosophila*²³. In conditions of eIF6 heterozygosity we did not observe differences in expression of miRNA targets (Supplementary Fig. 16). Further studies are required.

eIF6 is consistently overexpressed in a variety of cancers including colon¹⁷ carcinomas and leukemias¹⁸. In this last context, eIF6 upregulation was interpreted as a by-product of transformation, namely increased growth demanded of ribosomes and translation. Our data support a model in which the levels of eIF6 may directly affect tumorigenesis. In any case, our observations strengthen the concept that translational control is crucial for malignancy, as it was recently proposed for eIF4E, downstream of the mTOR pathway⁴.

METHODS SUMMARY

All the analysis were performed at least three times on different genetic backgrounds. For the generation of knockout mice, we deleted exon 1 and 2.

Hepatocytes and primary MEFs were prepared from littermates and kept in culture for 3 hours and 2–3 weeks respectively. Infection and rescue by eIF6-lentivirus was as detailed in full methods and supplementary.

Analysis of rRNA biogenesis was performed by sucrose gradient centrifugation of dissociated ribosomes, and by pulse-chase rRNA labelling with ³H-Uridine, and Northern Blotting (supplementary). Analysis of translation was performed by sucrose gradient centrifugation and metabolic labelling with ³⁵S-Methionine²⁴.

Blastocysts and MEFs were fixed in 2% paraformaldehyde. Blastocysts were then stained by whole-mount immunofluorescence. Cells were attached on coverslips and stained by indirect immunofluorescence.

Primary fibroblasts were infected with retroviruses carrying myc + oncogenic H-ras^{V12} or DNp53 + oncogenic H-ras^{V12}. Foci were counted 2–3 weeks after the primary infection²⁵. SDS-PAGE and Western Blotting analysis were performed on total extracts in RIPA buffer (10 mM Tris-HCl pH 7.2, 1% Sodium Deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA pH 8.0) as detailed elsewhere¹⁶. Nuclear-cytoplasmic fractionation was performed by ultracentrifugation on a sucrose cushion as described in Supplementary Methods. The purity of the fractions was checked by antibodies against GAPDH (cytoplasm) or H2B (nucleus). We loaded equivalent amount of cells.

Cell number and viability were analysed as previously described²⁶. S-phase entry was analysed by ³H-Thymidine assay on primary embryo fibroblasts at early passages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. eIF6 reduction leads to diminished body weight and affects liver growth at proliferation level

Age-matched mice were weighted and organs growth was followed after birth. **a**, Body weight of females and males recorded from 30 to 93 days after birth. **b**, Length of mice at 93 days, expressed as mean $\pm$ s.d. (n=8). **c**, Relative weight of indicated organs was calculated as the ratio between organ/body weight. Values represent mean $\pm$ s.d. (n=8). **d**, Percentage of PCNA-positive cells in 9-days old livers (n=4) and **e**, Corresponding representative immunohistochemical staining. Scale bar, 100 μ m. **f**, Representative result of western blot analysis of eIF6 expression in organs from $eIF6^{+/+}$ and $eIF6^{+/-}$ adult mice; bottom, densitometric analysis normalised to actin levels. *, $P \leq 0.01$, **, $P \leq 0.05$.

Figure 2. eIF6 reduction results in 80S complex accumulation and a blunted translational response to insulin

a, Polysomal profiles from livers showing the different amount of 80S ribosomal complex and polysomes between $eIF6^{+/+}$ and $eIF6^{+/-}$, *in vivo*. **b**, ^{35}S -Methionine labelling experiments in isolated primary hepatocytes stimulated with 100 nM insulin (Ins). Basal rate of translation is indicated as 100%. Increase of translation is not detected in $eIF6^{+/-}$ hepatocytes upon insulin stimulation. **c**, Polysomal profile indicates 80S accumulation in heterozygous cells and **d**, Representative ^{35}S -Methionine labelling of MEFs shows a blunted response to insulin in $eIF6^{+/-}$. **e**, lentiviral-induced re-expression of wt $eIF6$ results in *de novo* insulin stimulation of methionine incorporation, whereas (f) lentiviral-induced re-expression of $eIF6^{Ser235Ala}$ does not. All data represent mean $\pm$ s.d. of three independent experiments. *, $P \leq 0.01$, **, $P \leq 0.05$.

Figure 3. eIF6 reduction in the cytoplasmic compartment does not affect 60S ribosomal biogenesis
a, eIF6 distribution analysed in the nucleolus by anti-eIF6 immunostaining of primary MEFs. Nucleolar staining with anti-nucleophosmin (NPM) merged with anti-eIF6 (MERGE) shows nucleolar eIF6, in $eIF6^{+/-}$ MEFs compared to wt. Scale bar, 3 μ m. **b**, Northern blot analysis of steady-state levels of 25S precursors in het. MEFs and livers, and corresponding ethidium bromide staining (**c**). **d**, siRNA of eIF6 in NIH3T3 (**f**) neither abolishes nucleolar staining (**g**) nor rRNA processing (**h**).

Figure 4. eIF6 reduction impairs G1/S progression in synchronised cells and causes reduced transformation

a, FACS analysis of asynchronous MEFs. No significant differences in cell cycle distribution are observed between *eIF6*^{+/+} and *eIF6*^{+/-} MEFs. **b**, ³H-Thymidine labelling of synchronised cells shows a delay in S-phase entry. **c–d**, Soft agar assay of asynchronous MEFs transformed with DNp53 plus H-ras^{V12} (n=6) and with Myc plus H-ras^{V12} (n=6). Number of colonies expressed as mean ± s.d indicates that cellular potential to undergo transformation is impaired in *eIF6*^{+/-} MEFs.