The m Subunit of Murine Translation Initiation Factor eIF3 Maintains the Integrity of the eIF3 Complex and Is Required for Embryonic Development, Homeostasis, and Organ Size Control*

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Mammalian eIF3 is composed of 13 subunits and is the largest eukaryotic initiation factor. eIF3 plays a key role in protein biosynthesis. However, it is not fully understood how different subunits contribute to the structural integrity and function of the eIF3 complex. Whether eIF3 is essential for embryonic development and homeostasis is also not known. Here, we show that eIF3m null embryos are lethal at the peri-implantation stage. Compound heterozygotes (eIF3mflox/flox) or FABP4-Cre-mediated conditional knock-out mice are lethal at mid-gestation stages. Although the heterozygotes are viable, they show markedly reduced organ size and diminished body weight. Acute ablation of eIF3m in adult mouse liver leads to rapidly decreased body weight and death within 2 weeks; these effects are correlated with a severe decline of protein biogenesis in the liver. Protein analyses reveal that eIF3m deficiency significantly impairs the integrity of the eIF3 complex due to down-regulation of multiple other subunits. Two of the subunits, eIF3f and eIF3h, are stabilized by eIF3m through subcomplex formation. Therefore, eIF3m is required for the structural integrity and translation initiation function of eIF3. Furthermore, not only is eIF3m an essential gene, but its expression level is also important for mouse embryonic development and the control of organ size.

Background: eIF3m is a non-core subunit of eIF3.
Results: eIF3m deficiency in mice results in animal death and instability of other eIF3 subunits; haploinsufficiency reduces organ size.
Conclusion: eIF3m is critical for eIF3 structure and function.
Significance: Understanding the mechanism of eIF3 complex formation and its role in embryonic development and homeostasis is crucial for determining the physiological function of this eukaryotic translation factor.

Protein synthesis is critical for all living organisms. Many eIFs are involved in the initial steps of protein translation. For instance, the eIF4F complex, which is composed of eIF4E, eIF4A, and eIF4G, binds directly to the 5’-cap structure of mRNA. eIF4G functions as a scaffold that links 5’-cap-binding eIF4E to poly(A)-binding protein. The interaction of eIF4G and eIF3 recruits the 40 S ribosomal subunit to the mRNA (1–3). The formation of the translation initiation complex can be activated by nutrients, growth factors, or hormones through mTOR (mammalian target of rapamycin) signaling (4). Regulation of the protein initiation machinery may promote longevity, death, or diseases, including cancer (4–8).

Among mammalian eIFs, eIF3 is the largest complex. It contains 13 subunits, which are named alphabetically as eIF3a–eIF3m (9). In addition to its role in recruiting the ribosome to mRNA, eIF3 stabilizes the binding of the eIF2-GTP-Met-tRNA^Met ternary complex to the 40 S ribosomal subunit (10). Furthermore, in response to nutrients, hormones, and mitogens, the protein kinases mTOR and S6K1 regulate translation initiation by associating with eIF3 (11). Recent interactome analyses suggest that eIF3 may also link the protein synthesis machinery to the protein degradation machinery for efficient protein quality control (12).

Cryo-electron microscopy has revealed that eIF3 exhibits a five-lobed structure (13). Three evolutionarily conserved subunits (eIF3a, eIF3b, and eIF3c) and three non-conserved subunits (eIF3e, eIF3f, and eIF3h) constitute its functional core (14). Other subunits also likely play important roles but have remained less characterized. For example, eIF3 is important for the tight association of eIF3 and the 40 S ribosomal subunit in vitro (15). eIF3m is a non-core subunit that interacts directly with eIF3f and eIF3h (9, 16). It is highly expressed in human colon cancer cells and has been implicated in cancer progression (17). eIF3m does not exist in budding yeast eIF3 (9, 18), but its counterpart in fission yeast is essential for viability (19). More importantly, in fission yeast, eIF3m and eIF3e define two distinct eIF3 complexes that share other subunits, and the eIF3m-containing complex appears to associate with the bulk...
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of mRNAs (19). Despite these studies, the detailed role of eIF3m in the eIF3 complex remains unclear. Furthermore, the importance of mammalian eIF3m in embryonic development and homeostasis is completely unknown.

In this study, we used a knock-out approach to address the in vivo function of eIF3m in mice. We demonstrate that murine eIF3m is an essential gene for both embryonic development and homeostasis. Furthermore, it maintains the integrity of the eIF3 complex by stabilizing the core subunits eIF3c, and eIF3f, eIF3h.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-eIF3m antibody was generated in rabbit using the full-length protein expressed in and purified from *Escherichia coli*. Primary antibodies against eIF4G (catalog no. 24985), eIF4E (catalog no. 20675), eIF3a (catalog no. 25385), eIF3c (catalog no. 20685), eIF3h (catalog no. 34135), and eIF3j (catalog no. 32615) were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies against eIF3b (sc-16377), eIF3i (sc-271539), eIF3d (sc-271516), and GFP (sc-8334) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-eIF3f antibody (catalog no. A303-005A) was obtained from Bethyl Laboratories (Montgomery, TX). Anti-Cre antibody (catalog no. 69050-3) was obtained from Novagen (San Diego, CA). Anti-GAPDH antibody (catalog no. 10494-1-AP) was purchased from Proteintech (Chicago, IL). Anti-α-tubulin (catalog no. T5168), anti-FLAG (catalog no. F3165), and anti-HA (catalog no. H9658) antibodies were obtained from Sigma.

Animals—Mice were maintained in a specific pathogen-free animal facility. eIF3m floxed mice were established at the Model Animal Research Center of Nanjing University and maintained on the 129Sv background. Ela-cre mice were maintained on the FVB background. Fabp4-cre mice were purchased from the Model Animal Research Center of Nanjing University and maintained on the C57BL/6 background. Genotyping analyses were performed by PCR on genomic DNA extracted from tail tips. Pregnancies were obtained by natural mating and were timed from the day of the vaginal plug, which was defined as embryonic day (E)2.5. For acute liver-specific knock-out, 8–10-week-old mice were injected through the tail vein with ~5 × 10⁶ plaque-forming units of adenovirus in 0.1 ml of PBS as described (20, 21).

Cell Culture—Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml streptomycin, 100 units/ml penicillin, and 0.3 mg/ml l-glutamine at 37 °C in 5% CO₂. Primary mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos as described (22). Experiments were performed at early passages (passages 1–6). HEK293T cells were transfected using the conventional calcium phosphate method. Virus infections were performed as described (23).

Lentivirus Package and Adenovirus Preparation—Lentivirus was packaged in HEK293T cells as described (24). Briefly, HEK293T cells were transfected with vesicular stomatitis virus G packaging plasmid Delta8.9 and transfer vector. At 48 h post-transfection, the culture medium was harvested and prepared for ultracentrifugation. The pellet of lentivirus was resuspended in PBS. Adenovirus particles were prepared in AD-293 cells and concentrated using CsCl gradient centrifugation as described (25). Purified virus particles were stored at −80 °C.

Immunoprecipitation and Immunoblotting—For co-immunoprecipitation, cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM KCl, 0.5% Nonidet P-40, 1 mM EDTA, 10% glycerol, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM PMSF, 3 mM DTT, and protease inhibitors). Anti-FLAG M2 resin (Sigma) was added to the lysate and incubated for 2 h at 4 °C. The resin was washed three times with lysis buffer and three times with wash buffer (20 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.5% Nonidet P-40, 1 mM EDTA, 10% glycerol, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM PMSF, 3 mM DTT, and protease inhibitors). The immunoprecipitates were then eluted with FLAG peptide as described (26). For immunoblotting, proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were developed in chemiluminescence reagent (PerkinElmer Life Sciences) and exposed in a Fujifilm LAS 4000 imager.

Quantitative RT-PCR—Total RNA was extracted using TRIzol reagent (Invitrogen), and the SuperScript III first strand synthesis system with oligo(dT) primers (Invitrogen) was used for the reverse transcription reaction. Quantitative RT-PCR was performed using an Applied Biosystems 7500 HT sequence detection system with a Power SYBR Green PCR Master Mix kit (Applied Biosystems). GAPDH served as the internal control.

Histological Staining—Tissue samples were excised and immediately fixed in 4% paraformaldehyde in PBS, and 5-μm sections of paraffin-embedded tissue samples were stained with hematoxylin and eosin for morphological analysis. The stained samples were photographed with an Olympus BX51 microscope equipped with an Olympus DP71 cooled CCD camera.

Statistics—All data are presented as means ± S.D. Student’s unpaired t test was used for statistical analyses. Differences were considered significant at p < 0.05.

RESULTS

eIF3m Deficiency in Mice Results in Embryonic Lethality—To gain insight into the physiological role of eIF3m, we examined its expression pattern in different organs of adult mice. Using GAPDH as the loading control, high levels of eIF3m were observed in the lung, spleen, testis, and fat tissue. Moderate levels of expression were observed in the liver, stomach, and kidney, and relatively low levels were observed in the brain, heart, and muscle (Fig. 1A).

To investigate the importance of eIF3m in mammalian development, we generated eIF3m knock-out mice using a Cre/loxp strategy. Exons 2–5 of eIF3m were flanked by two loxP sites and a neomycin resistance gene cassette (Fig. 1B and C). Male mice of the eIF3m floxed strain (eIF3mflox/+) were crossed with Ela-cre females (27) to obtain heterozygotes (eIF3m+/-). We then intercrossed the heterozygous mice to generate eIF3m null mice. The eIF3m+/- mice and eIF3m+/- offspring were genotyped at term and displayed the expected Mendelian ratios (Fig. 1D). However, the intercrossing of eIF3m+/- mice did not pro-
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Ablation of eIF3m in the Adult Mouse Liver Leads to Dramatically Decreased Protein Expression and Animal Death—We next investigated the importance of eIF3m in regulating homeostasis in adult mice. We used tail vein delivery of Cre recombinase adenovirus to achieve liver-specific knock-out of eIF3m in adult mice (20, 21). One week after adenoviral Cre administration, all four eIF3mflx/flx mice exhibited decreased body weight compared with the three wild-type control mice (Fig. 4A). Furthermore, all of the eIF3mflx/flx mice died within 2 weeks (Fig. 4B). To identify the cause of death, we extracted cell lysates from different tissues of an animal that died at 8 days post-injection. Ponceau S staining indicated a severe decline of total protein levels in the liver compared with uninjected eIF3mflx/flx littermates (Fig. 4C). Immunoblotting also confirmed specific ablation of eIF3m in the liver (Fig. 4C). Thus, eIF3m is required for homeostasis in adult mice due to its role in regulating liver protein synthesis.

Ablation of eIF3m Attenuates the Levels of the eIF3c, eIF3f, and eIF3h Subunits—To understand how eIF3m affects eIF3 complex function, we infected MEFs from eIF3mfloxfloxflox mice with virus to express Cre or GFP (as control). We examined the

duce any eIF3m<sup>−/−</sup> mice, suggesting that eIF3m is essential for normal embryonic development. We analyzed the embryos at E9.5 and again did not observe any eIF3m<sup>−/−</sup> embryos (Fig. 1E). Therefore, eIF3m is indispensable for early embryonic development.

As the very early embryonic death of eIF3m-deficient mice precluded disease-related studies, we explored whether compound mice (28) of eIF3m<sup>−/−</sup> are viable. Insertion of the phosphoglycerate kinase promoter-driven neomycin cassette into intron 5 may have resulted in transcriptional interference; thus, the floxed allele may have been a hypomorphic eIF3m allele. Therefore, we crossed the eIF3mfloxflox mice with the eIF3m<sup>+/−</sup> mice to generate compound heterozygotes (eIF3mflox<sup>+/−</sup>). The compound mice were expected to have lower eIF3m levels compared with the heterozygotes (eIF3m<sup>+/−</sup>) and may have shown disease phenotypes if the litters were viable. However, postnatal eIF3mflox<sup>+/−</sup> mice were not obtained (Fig. 1F). We again examined embryos at E9.5 and found that eIF3mflox<sup>+/−</sup> mice displayed severe developmental defects and growth retardation (Fig. 1G). These results indicate that eIF3m is an essential gene; sufficient expression levels of eIF3m are thus critical for normal mouse embryonic development.

**Ablation of eIF3m Attenuates the Levels of the eIF3c, eIF3f, and eIF3h Subunits**—To understand how eIF3m affects eIF3 complex function, we infected MEFs from eIF3mfloxfloxflox mice with virus to express Cre or GFP (as control). We examined the...
protein levels of different eIF3 subunits by immunoblotting at 3 days post-infection, at which time the viability of Cre-expressing cells was still comparable to that of control cells. Coincident with the decline of eIF3m levels in the Cre virus-infected population, we observed that the levels of eIF3c, eIF3f, and eIF3h were similarly reduced (Fig. 5A). By contrast, other subunits,
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FIGURE 5. eIF3m deficiency selectively down-regulates eIF3f, eIF3h, and eIF3c and impairs the integrity of eIF3. A, immunoblotting for the indicated proteins in total cell lysates. eIF3m<sup>-/-</sup> MEFs were infected with Cre adenovirus or GFP for 72 h. eIF3m deficiency did not affect the mRNA levels of eIF3f, eIF3h, and eIF3c. eIF3m<sup>-/-</sup> MEFs were infected with lentiviral GFP or Cre for 120 h. Total RNA was extracted and used for quantitative RT-PCR. The results are from two independent experiments. C, diagram depicting subunit interactions within the mammalian eIF3 complex. The diagram was modified from models of Zhou et al. (16) and Pick et al. (9). Subunits are not drawn to scale. D, immunoprecipitation of the eIF3 complex. eIF3m<sup>-/-</sup> MEFs were infected with lentivirus for 72 h to express the indicated proteins. The eIF3 complex was immunoprecipitated (IP) with anti-FLAG beads. The indicated eIF3 subunits were detected by immunoblotting. Flag-b, FLAG-tagged eIF3b.

Including eIF3a, eIF3b, eIF3d, eIF3i, and eIF3j, were not affected (Fig. 5A). In contrast to eIF3m, quantitative RT-PCR indicated that the mRNA levels of eIF3c, eIF3f, and eIF3h were not down-regulated in Cre virus-treated eIF3m<sup>-/-</sup>/<sup>-/-</sup> MEFs (Fig. 5B). Therefore, the reduced levels of eIF3c, eIF3f, and eIF3h were attributed to repressed protein translation and/or increased degradation.

Mass spectrometry studies have shown that eIF3m, eIF3f, and eIF3h interact to form a tertiary subcomplex, which is further recruited into the eIF3 complex through the eIF3h-eIF3c interaction (Fig. 5C) (9, 16). Furthermore, eIF3c is known to be critical for linking the eIF3c-eIF3d-eIF3e-eIF3l-eIF3k subcomplex formation, which in turn bind and stabilize eIF3c (Fig. 5C) (9, 16). We thus speculated that the reduced eIF3c in the eIF3m<sup>-/-</sup> cells would further impair the integrity of eIF3. To validate this hypothesis, we ectopically expressed FLAG-tagged eIF3b in eIF3m<sup>-/-</sup>/<sup>-/-</sup> MEFs to levels comparable to the endogenous levels of eIF3b (Fig. 5D). After Cre-mediated knock-out, we performed co-immunoprecipitation to isolate the entire eIF3 complex (Fig. 5D) (19). The immunoblot indicated that the indirect association of eIF3d with eIF3b was markedly reduced in eIF3m<sup>-/-</sup> cells, although the direct eIF3b-eIF3i interaction was not affected (Fig. 5, C and D).

**eIF3m Stabilizes eIF3f through Direct Interaction**—The stability of protein complex subunits in vivo frequently requires proper subcomplex and/or complex formation (30–32). Therefore, eIF3m may stabilize the eIF3h and eIF3f subunits through subcomplex formation, which in turn bind and stabilize eIF3c (Fig. 5, A–C) (19). Alternatively, as eIF3 is critical for protein synthesis (9, 13), the reduced levels of the eIF3 complex in eIF3m knock-out cells (Fig. 5D) may result in inefficient protein translation, thereby reducing the levels of eIF3c and eIF3f. We reasoned that in the former case, eIF3m would be able to stabilize eIF3f or eIF3h but not eIF3c upon overexpression. In the latter case, no effect should be observed for any of the subunits because their overexpression is unlikely to alter the overall level of the eIF3 complex.

When HA-tagged eIF3c, eIF3d, eIF3f, or eIF3h was co-expressed with FLAG-eIF3m in HEK293T cells, only eIF3f and eIF3h exhibited clear up-regulation compared with their expression levels in the absence of FLAG-eIF3m (Fig. 6A). To further clarify that the effect was interaction-dependent, we constructed two C-terminal truncation mutants of eIF3m (N1 and N2). Co-immunoprecipitation analysis revealed that both mutants were incapable of binding eIF3f (Fig. 6B). Compared with wild-type eIF3m, neither mutant increased the levels of HA-eIF3f (Fig. 6C). These results indicate that the eIF3m-eIF3f interaction is required for the stabilization effect.

**DISCUSSION**

Our results indicate that eIF3m is an essential gene for mouse embryonic development and homeostasis. First, whole animal knock-out of eIF3m resulted in early embryonic lethality. Because we were unable to identify any eIF3m<sup>-/-</sup> embryos at E9.5 (Fig. 1E), embryonic death might occur during the peri-implantation period, as occurs upon the knock-out of other essential genes (28, 33). Second, the eIF3m<sup>-/-</sup>/<sup>-/-</sup> Fabp4-cre embryos started to die at ~E9.5 and failed to develop to term (Fig. 3F). These results suggest that fetal fat tissue is essential for early embryonic development, in addition to its well recognized postnatal roles (34). Fabp4-Cre was expressed on the dorsal side of E9.5 embryos and later on the peripheral ganglia, cartilage primordial, and vertebrae; these expression areas are in addition to FABP4-Cre expression in brown adipose tissue (35). Because adipocytes, neurons, chondrocytes, and osteocytes share a common lineage (36), an alternative possibility for the embryonic death was the loss of certain stem cell populations. Third, the ablation of eIF3m in the adult liver resulted in animal death within 2 weeks. This was likely due to the abolishment of liver protein synthesis, a critical process in the maintenance of liver homeostasis (Fig. 4).

A sufficient level of eIF3m expression is also important for organ size. Although eIF3m<sup>-/-</sup>/<sup>-/-</sup> mice were viable and normal, eIF3m<sup>-/-</sup> embryos exhibited severe growth retardation at E9.5 and failed to further develop (Fig. 1, F and G). By contrast, the eIF3m<sup>−/−</sup> embryos, whose eIF3m levels were expected to be higher than those of eIF3m<sup>-/-</sup> embryos but lower than wild-type levels, were fully viable. Nevertheless, after birth, the
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FIGURE 6. eIF3m stabilizes eIF3f and eIF3h through direct interaction. A, eIF3m up-regulated eIF3f and eIF3h but not eIF3c upon overexpression. HEK293T cells were transfected to express the indicated eIF3 subunits for 48 h and subjected to immunoblot analysis. GAPDH served as the loading control. B, mapping of regions of eIF3m that are critical for the interaction with eIF3f. HEK293T cells were transfected to coexpress HA-eIF3f with the indicated FLAG-tagged proteins for 48 h. Cell lysates were then subjected to immunoprecipitation (IP) using anti-FLAG beads. Firefly luciferase (Luc) served as a negative control. The eIF3m mutants (N1 and N2) are shown in the diagrams. C, the interaction-defective eIF3m mutants failed to up-regulate HA-eIF3f upon overexpression. HEK293T cells were cotransfected to express the indicated proteins for 48 h and then subjected to immunoblot analysis. GAPDH served as the loading control. PCI, proteasome-COP9-initiation factor 3 domain (268–357 amino acids).

eIF3m<sup>+/−</sup> mice consistently displayed reduced body weight compared with their wild-type littersmates (Fig. 2). Coincident with the decreased eIF3m expression, several organs were significantly smaller in eIF3m<sup>−/−</sup> mice, including the liver, kidney, and fat pad (Fig. 2A and Fig. 3, A–C). Notably, our results suggest that the reduced organ size was due to decreased cell number and not reduced cell size (Fig. 3, D and E). Thus, we propose that the level of eIF3m is an important determinant of the number of rounds of cell division required during organ construction. Consistent with this hypothesis, eIF3m has been reported to be important for the translation of proliferation-related proteins such as Cdc25A (17). It will be interesting to clarify the molecular mechanisms underlying the relationship between the expression level of eIF3m and organ size.

In this study, we demonstrated that eIF3m is critical for the stability of eIF3c, eIF3f, and eIF3h (Fig. 5). Consequently, the ablation of eIF3m severely impaired the integrity of the eIF3 complex (Fig. 5D). eIF3f and eIF3h are able to form a heterodimer and heterotrimer with eIF3m (9, 16). Our results suggest that subcomplex formation helps protect eIF3f and eIF3h from degradation (Fig. 6), similar to the actin regulatory WAVE complex (32). eIF3c binds directly to eIF3h but not eIF3m in the eIF3 complex (Fig. 5C) (9, 16). Therefore, down-regulation of eIF3c is likely an indirect effect resulting from the ablation of eIF3m (Fig. 5).

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