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ORIGINAL ARTICLE

Essential role of eIF5A-1 and deoxyhypusine synthase in mouse embryonic development

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Abstract The eukaryotic initiation factor 5A (eIF5A) contains a polyamine-derived amino acid, hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine]. Hypusine is formed post-translationally by the addition of the 4-aminobutyl moiety from the polyamine spermidine to a specific lysine residue, catalyzed by deoxyhypusine synthase (DHPS), and subsequent hydroxylation by deoxyhypusine hydroxylase (DOHH). The eIF5A precursor protein and both of its modifying enzymes are highly conserved, suggesting a vital cellular function for eIF5A and its hypusine modification. To address the functions of eIF5A and the first modification enzyme, DHPS, in mammalian development, we knocked out the *Eif5a* or the *Dhps* gene in mice. *Eif5a* heterozygous knockout mice and *Dhps* heterozygous knockout mice were viable and fertile. However, homozygous *Eif5a1*$^{gt/gt}$ embryos and *Dhps*$^{gt/gt}$ embryos died early in embryonic development, between E3.5 and E7.5. Upon transfer to in vitro culture, homozygous *Eif5a*$^{gt/gt}$ or *Dhps*$^{gt/gt}$ blastocysts at E3.5 showed growth defects when compared to heterozygous or wild type blastocysts. Thus, the knockout of either the eIF5A-1 gene (*Eif5a*) or of the deoxyhypusine synthase gene (*Dhps*) caused early embryonic lethality in mice, indicating the essential nature of both eIF5A-1 and deoxyhypusine synthase in mammalian development.

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Abbreviations

eIF5A	Eukaryotic translation initiation factor 5A
DHPS	Deoxyhypusine synthase
DOHH	Deoxyhypusine hydroxylase
Eif5a	Mouse gene encoding eIF5A-1
Eif5a2	Mouse gene encoding the eIF5A-2 isoform
ES cells	Embryonic stem cells
TCA	Trichloroacetic acid
DTT	Dithiothreitol

Introduction

eIF5A is a putative translation initiation factor and the only cellular protein that contains the unique modified lysine, hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine] (Park et al. 1981). Hypusine is formed by the post-translational modification of one specific lysine residue of the eIF5A precursor, in two consecutive enzymatic steps (Chen and Liu 1997; Park 2006). The first enzyme, deoxyhypusine synthase (DHPS) (Joe et al. 1995) catalyzes the transfer of the aminobutyl moiety from the polyamine spermidine to form an intermediate, deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] residue. This deoxyhypusine residue is hydroxylated by deoxyhypusine hydroxylase (DOHH) (Park et al. 2006; Cano et al. 2009). There is abundant evidence that eIF5A and its hypusine residue are vital for the viability and growth of eukaryotic and mammalian cells (Byers et al. 1994; Hanauske-Abel et al. 1994; Park et al. 1994, 2010; Chen and Liu 1997; Nishimura et al. 2005; Chattopadhyay et al. 2008).

The hypusine/deoxyhypusine modification occurs in certain archaea and in eukaryotes, but not in eubacteria. eIF5A, DHPS and DOHH are highly conserved, suggesting a vital function for eIF5A and the deoxyhypusine/hypusine modification. The amino acid sequence surrounding the hypusine modification site is strictly conserved in eukaryotes (Wolff et al. 2007). Crystal structures of yeast and human eIF5A (PDB 3er0, 3cpf), four archaeal proteins (PDB 1eif, 2eif, 1iz6, 1bkb) and two *Leishmania* proteins (PDB 1x6o, 1xtd) are similar with only minor differences (Tong et al. 2009). These eIF5A homolog proteins consist of two domains: a basic N-terminal domain containing the hypusine modification site in an exposed loop and an acidic C-terminal domain with an apparent oligonucleotide-binding fold. There exist two eIF5A genes (encoding two isoforms, eIF5A-1 and eIF5A-2), a single DHPS gene and a single DOHH gene in mammals. DHPS is not found in most commonly studied bacteria. Only aIF5A and DHPS homolog genes but no DOHH homolog genes have been identified in the genome of archaea kingdom (Wolff et al. 2007). In yeast *S. cerevisiae*, whereas disruption of both of the eIF5A genes or the DHPS gene is lethal, a DOHH null strain is viable and grows at a rate slightly slower than the wild type strain (Park et al. 2006). Yet, DOHH or eIF5A hydroxylation appears to be important in the fission yeast *Saccharomyces Pombe*, since a mutation in DOHH gene (*Mmd1*) caused aberrant mitochondrial morphology and growth defect at non-permissive temperature (Weir and Yaffe 2004). In multi-cellular (higher) eukaryotes such as *Caenorhabditis elegans* (*C. elegans*) (Sugimoto et al. 2004) and *Drosophila melanogaster* (*D. melanogaster*), (Spradling et al. 1999; Patel et al. 2009) the DOHH gene appears to be essential. Thus, eIF5A and the two modification enzymes seem to have evolved sequentially to meet the specialized developmental needs of eukaryotic organisms and in an independent manner without a co-evolutionary linkage between them.

The precise cellular function of eIF5A has remained elusive for decades. eIF5A was initially isolated from reticulocyte lysate ribosomes as a factor that stimulates methionyl-puromycin synthesis (a model assay for translation initiation) (Kemper et al. 1976; Benne and Hershey 1978). eIF5A appears to bind to the ribosome in a hypusine-dependent manner, since the eIF5A mutant protein (yeast eIF5A K51R), with a Lys to Arg substitution at the hypusine modification site, does not associate with ribosomes (Jao and Chen 2006; Zanelli et al. 2006). The reported increase in the polysome/monosome ratios in eIF5A mutant yeast strains (Gregio et al. 2009; Saini et al. 2009) and also in mammalian cells depleted of eIF5A (Landau et al. 2010) points to a role for eIF5A in the elongation rather than the initiation step of translation. *S. cerevisiae* strains harboring eIF5A temperature-sensitive mutant proteins exhibit diverse cellular changes, suggesting a direct or indirect role for eIF5A in cell wall integrity, mRNA decay, actin polarization, apoptosis and cell cycle progression (Zuk and Jacobson 1998; Valentini et al. 2002; Chatterjee et al. 2006; Schrader et al. 2006). It is not yet clear how depletion or dysfunction of eIF5A in the mutant strains leads to such pleiotropic phenotypes. It has not been resolved whether eIF5A stimulates the peptidyl transferase reaction at the initial peptide bond formation as well as at all steps of elongation, and whether it stimulates translation of a subset of mRNAs. The true physiological function(s) of eIF5A and its mode of action are yet to be elucidated in mammals.

Whether the two eIF5A isoforms have different functions in mammalian physiology is not known. eIF5A-1 is constitutively expressed in all cells and tissues of mammals, and its expression is increased in rapidly proliferating cells and in cancer/neoplastic tissues. eIF5A-2 protein expression is normally extremely low (Clement et al. 2006) and appears to be regulated in a tissue-specific manner in testis and brain, suggesting a specialized function for eIF5A-2 in mammalian development. Furthermore, gene amplification or overexpression of eIF5A-2 has been reported to be associated with several human cancers, including ovarian, liver and colorectal cancers (Guan et al. 2004; Xie et al. 2008). eIF5A has also been implicated in HIV-1 replication as a cellular cofactor for *Rev* (Ruhl et al. 1993; Hoque et al. 2009). Recently, eIF5A and deoxyhypusine synthase have been implicated in inflammation and the progression of diabetes (Maier et al. 2010). In view of these proposed functions of eIF5A in mammals, we undertook to investigate the role of these two genes in mouse development. Our results demonstrate that *Eif5a* and *Dhps* are vital genes from the early stages of embryonic development in mice.

Materials and methods

ES cell culture

The heterozygous *Eif5a* gene-trapped ES cell line, RRE174, and the heterozygous *Dhps* gene-trapped ES cell line, RRM039, were purchased from Bay Genomics (San Francisco, CA). The ES cells were cultured in high glucose DMEM supplemented with 15% fetal bovine serum, penicillin–streptomycin, glutamine, 0.1 mM 2-mercaptoethanol, nonessential amino acids and 1000 Unit/ml Leukemia inhibitory factor (LIF).

Generation of gene trapped mice

Animal study protocols were approved by the NIH Animal Care and Use Committee. To generate chimeric mice, the

RRE174 and RRM039 ES cell lines were microinjected into blastocysts derived from C57BL/6 J and the microinjected blastocysts were transferred to pseudo-pregnant females by the Gene Targeting Core Facility, NIDCR, NIH. Chimeric male offsprings were mated to C57BL/6 J females. The heterozygous mutant mice, with germ-line transmission of the mutant allele, were identified by genotyping of tail DNA of F1 offspring with agouti coat color as described below.

Identification of insertion position of gene trap vector

Genomic DNA from ES cells, RRE174 and RRM039 was isolated by using a QIAGEN Genomic DNA kit (#19060) according to the manufacturer's protocol. To determine the precise insertion position of the gene trap vector in the *Eif5a* gene, 1 µl of the DNA solution was used as a template for PCR with the primer set of KN43F (5′-TC TGGAGCTCATCCCTCAGTC-3′) (intron1 of *Eif5a* gene) and KN03R (5′-CGACGGGATCCTCTAGAGTC-3′) (*β*-geo of the vector). The primer set of KN01F (5′-GCAGCAA GTCAACGCCATGGTG-3′) (exon1 of *Dhps* gene) and KN40R (5′-CCTGGCCTCCAGACAAGTAGATC-3′) (En 2 of the gene trap vector) was used for the *Dhps* gene-trap. The insertion positions of the vector were determined by sequencing of the PCR products.

PCR Genotyping of mouse tail DNA

Genomic DNA from mouse tail was prepared by HotSHOT method (Truett et al. 2000). The tail sample (2–3 mm) was heated in 50 µl of 50 mM NaOH at 95°C for 30 min and neutralized with 5 µl of 1 M Tris–HCl (pH 8.0). 1 µl of the DNA solution was used as a template for PCR genotyping with the primer set of KN48F (5′-TGCATCTGCAAGTG CACGTTGG-3′) and KN49R (5′-CACTCTGCCAGGAC TATAATTAG-3′) for the *Eif5a* WT (203 bp), and KN48F and KN50R (5′-TCTCAAAGTCAGGGTCACAAGG-3′) for the *Eif5a* mutant (267 bp). The primer set of KN51F (5′-CTGAGGTTTGGAGTCTTAGTGC-3′) and KN52R (5′-CATGTTGTGCTGCACGAGGTATC-3′) was used for the *Dhps* WT gene (212 bp) and KN51F and KN41R (5′-CCTCTTCACATCCATGCTGAGG-3′) for the *Dhps* mutant (385 bp).

Southern blot analysis

Genomic DNA (10 µg) from *Eif5a1* gene trap ES cells or mice and genomic DNA from *Dhps* gene trap cells or mice were digested with SacI and XbaI, respectively, and were transferred to Nytran Nylon membranes (Whatman) after electrophoresis in a 1% agarose gel. Hybridizations were performed in Hybrisol I hybridization solution (INTERGEN) with a ^{32}P-labeled probe. Templates of the probes were amplified by using the primer set of 5′-TGT TTTAGGGAGGAATGGGATAGATC-3′ and 5′-TTGAG AGGTGATTCTAACCTTG-3′ for the 3′-probe of *Eif5a-1* (285 bp), the primer set of 5′-AAGGCTCTCGAGTCGCA CTTC-3′ and 5′-GCGCCTCACCATGGCGTTGACTT G-3′ for 5′-probe of *Dhps* (320 bp) and the primer set of 5′-TTATCGATGAGCGTGGTGGTTATGC-3′ and 5′-GC GCGTACATCGGGCAAATAATATC-3′ for the *β*-gal probe (680 bp). The probes were radiolabeled using a Ladderman DNA Labeling Kit (TAKARA).

Culture of blastocysts

Blastocysts (E3.5) were flushed from the uteri of pregnant mice with PBS (phosphate-buffered saline) on day 3.5 and cultured in 0.2 mL DMEM containing 15% fetal calf serum, 1 mM glutamine, 0.05 mM 2-mercaptoethanol, 1 mM pyruvate, 0.1 mM nonessential amino acids (Life Technologies), 50 U/mL streptomycin, 100 U/mL penicillin G (Nishimura et al. 2002). Photographs were taken with an inverted microscope (Zeiss) at specific intervals indicated. After 7 days, the cells were washed with PBS and used for genotyping as described above.

BrdU incorporation assay and immunehistochemistry

A BrdU incorporation assay was performed using the 5-Bromo-2′-deoxyuridine Labeling and Detection Kit I (Roche Applied Science). Blastocysts were cultured as described above for 7 days, labeled with 10 µM BrdU labeling reagent for 30 min then fixed in 70% ethanol with 50 mM glycine (pH 2.0). For immunostaining, rabbit polyclonal antibodies against human eIF5A-1 (NIH353, 1:100 dilution) or against human deoxyhypusine synthase (1:100 dilution) and mouse anti-BrdU antibody were used as primary antibodies. Anti-rabbit IgG-Cy3 and anti-mouse IgG-fluorescein were used as secondary antibodies. The cells were counterstained with DAPI and analyzed by fluorescence microscopy.

Genotyping of embryos

Heterozygous male and female mice were mated naturally to obtain embryos of all different genotypes (*eIF5a*$^{+/+}$, *eIF5a*$^{+/gt}$ and *eIF5a*$^{gt/gt}$, *Dhps*$^{+/+}$, *Dhps*$^{+/gt}$ and *Dhps*$^{gt/gt}$). The post-implantation embryos were detached from the uterus at each developmental stage (E6.5, E7.5, and E8.5) and used for PCR genotyping by the HotSHOT method, as described above. The pre-implantation embryos and blastocysts (E3.5) were isolated from the uterus by flushing out with PBS and the genotype determined. The blastocysts were heated at 95°C for 15 min in 10 µl of 50 mM NaOH

and neutralized with 1 µl of 1 M Tris–HCl (pH 8.0). 2 µl of the DNA solution was used for genotyping by PCR using the same primer sets.

Results

Generation of *Eif5a* and *Dhps* gene trap mice

The ES cell lines RRE174 (with *Eif5a* disruption) and RRM039 (with *Dhps* disruption) were obtained from BayGenomics and were used for the generation of mutant mice. The insertion positions of the gene trap sequences, pGT2lxf and pGT0lxf, in the *Eif5a* gene and the *Dhps* gene, respectively, were determined by PCR using vector specific primers, as shown in Fig. 1a, b. The pGT2lxf trap sequence was inserted in intron 1 of the *Eif5a* gene and pGT0lxf sequence in exon 2 of the *Dhps* gene. After confirmation of gene disruption by sequencing, the ES cells were microinjected into C57BL/6 blastocysts and transplanted into pseudo-pregnant females to generate chimeric mice. After crossing the chimeric male mice with C57BL/6 females, the mutated *Eif5a* or *Dhps* allele was transmitted to their progeny and agouti mice with germ-line transmission of the mutated allele were selected. The validity of gene disruption was confirmed by Southern blotting using two probes (a β-gal specific probe and a gene specific probe). In the case of the *Eif5a* gene trap, SacI digests of the ES cell DNA and tail DNA of a representative heterozygous mouse (+/gt) displayed two bands hybridized to the 3′ probe, one at 3.9 kb (wt allele) and the other at 8.4 kb (gt allele), whereas only the 3.9 kb band was seen in the digest of the wild type (+/+) tail DNA. Gene disruption was also confirmed using β-gal probe which showed one 4.1 kb band in the digests of ES cell and the heterozygous mutant mouse but not in the wild type (+/+). Likewise, disruption of the *Dhps* gene in the ES cells and a heterozygous mutant mouse was confirmed by southern blotting of XbaI digested genomic DNA using the 5′ probe (detecting both the wt and gene trapped alleles) and the β-gal probe (specific for the gene trapped allele) The sizes of the hybridized bands in each case corresponded to those calculated from the gene sequences.

Heterozygous *Eif5a*$^{+/gt}$ mice or *Dhps* $^{+/gt}$ mice appeared to be normal in growth and fertility. No apparent defects or phenotypes were observed during the first 1 year. The

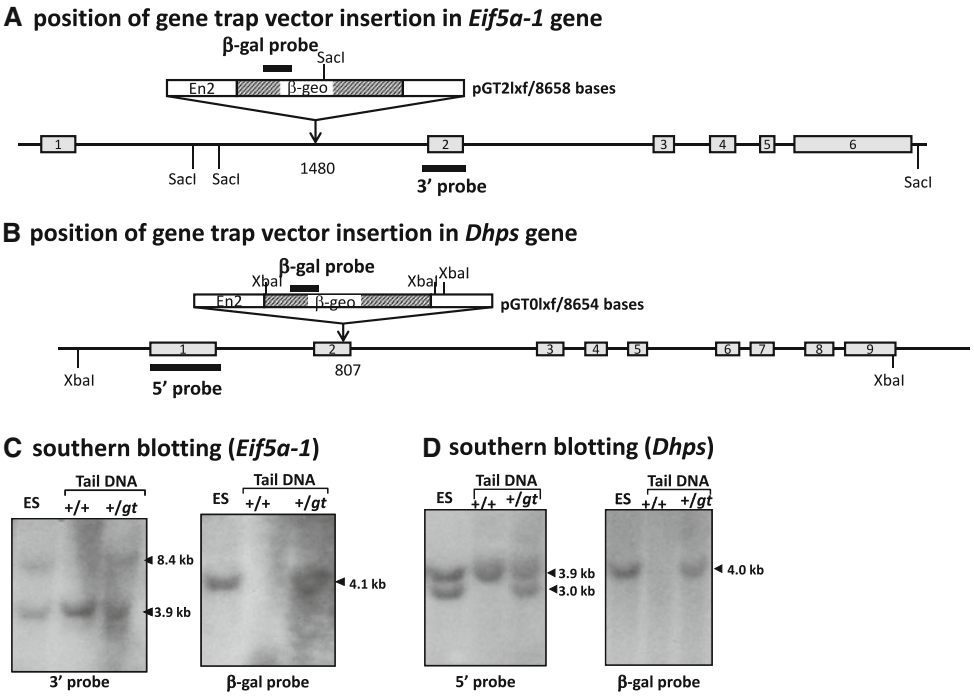

Fig. 1 Confirmation of insertion of a single copy of the gene trap vector in the *Eif5a* (**a, c**) and *Dhps* (**b**, **d**) genes by southern blots. Generation of *Eif5a1 and Dhps* gene trap mice. **a** Location of insertion of the gene trap vector in the *Eif5a* gene was determined by PCR as described under "Materials and methods" and is indicated by arrow at 1480 bp downstream from the transcription initiation site on exon1. **b** Location of insertion of the gene trap vector in the *Dhps* gene (at 807 bp) was determined as in (**a**). **c** Genomic DNA's from *Eif5a*$^{+/gt}$ ES cell RRE174, wild type mouse tail (+/+) and heterozygous mutant mice (+/gt) were digested with SACI. After electrophoretic separation and blotting, the membrane was probed with a radiolabeled gene specific probe (3′ probe) and a β-gal probe. **d** Genomic DNA from *Dhps* gene trapped ES cell RRM039, wild type and heterozygous mutant mice (+/gt) was digested with XbaI. After electrophoresis and blotting, the membrane was hybridized using the radiolabeled 5′-probe and the β-gal probe

levels of eIF5A-1 or DHPS proteins in the mutant ES cells or heterozygous mutant mice appeared somewhat reduced (data not shown), but the reduced levels appear to be sufficient to support normal growth.

Eif5a and *Dhps* genes are essential for mouse embryonic development

In order to determine whether the *Eif5a* or *Dhps* gene is essential for mouse development, we attempted to generate homozygous gt/gt mutant mice by intercrossing of heterozygous agouti pups with germ-line transmission. Genotypes of a litter of pups born from a heterozygous intercrossing were determined by PCR using two sets of primers, one specific for the wild type allele and the other for the gene targeted allele (Fig. 2a for *Eif5a* agouti pups and Fig. 2b for the *Dhps* agouti pups). In both cases, only wild type (+/+) and heterozygous pups (+/gt), but no homozygous knockout pups (gt/gt) were born suggesting embryonic lethality in the absence of expression of either the *Eif5a* or *Dhps* gene. We then analyzed the genotypes of blastocysts (E3.5). All three genotypes (+/+, +/gt and gt/gt) were detected in blastocysts (E3.5) from $Eif5a^{+/gt}$ intercross and those from a $Dhps^{+/gt}$ intercross (Fig. 2c, d), suggesting viability of gt/gt blastocysts up to 3.5 days of gestation. The gt/gt blastocysts appeared morphologically similar to those of wild type (+/+) and the heterozygous (+/gt) for both *Eif5a* (Fig. 3a) and *Dhps* (Fig. 3b). From multiple intercrossings of $Eif5a^{+/gt}$ and of $Dhps^{+/gt}$, three genotypes (+/+, +/gt, gt/gt) were present (Table 1A, B) confirming the viability of blastocysts up to 3.5 days in the absence of expression of the *Eif5a* or *Dhps* gene.

To clarify the developmental stage at which defects from an *Eif5a* deficiency or *Dhps* deficiency were manifested, we performed multiple heterozygous intercrossings to determine genotypes of pups and embryos at different days of gestation (Table 1A, $Eif5a^{+/gt}$ intercrossing and Table 1B, $Dhps^{+/gt}$ intercrossing). No pups with the gt/gt genotype were born from 10 intercrossings of $Eif5a^{+/gt}$ mutants (Table 1A) and from 11 intercrossings of $Dhps^{+/gt}$ mutants (Table 1B), indicating that homozygous disruption of either the *Eif5a* or *Dhps* gene is embryonic lethal. Furthermore, the average number of pups per litter (Table 1A, 6.5 for the $Eif5a^{+/gt}$ intercross and Table 1B, 4.73 for the $Dhps^{+/gt}$ intercross) was lower than those of pups from a wild-type intercrosses (typically 8–10). Embryos with gt/gt genotypes were not detected at stages E6.5, E7.5 and E8.5 for $Dhps^{+/gt}$ intercrosses. For $Eif5a^{+/gt}$ intercrosses, no *gt/gt* embryo was detected at E7.5 and E8.5. These findings suggest that the $Dhps^{gt/gt}$ embryos died between 3.5 and 6.5 days of gestation and that the $Eif5a^{gt/gt}$ embryos died between 3.5 and 7.5 days.

We compared the growth of wild type (+/+), heterozygous (+/gt) and homozygous (gt/gt) blastocysts (E3.5) in vitro on days 3, 5 and 7 (Fig. 3a, b). In all three genotypes, cells from the blastocysts attached to plastic dishes and started to spread. The wild type and the heterozygous embryos showed similar growth patterns, whereas growth was definitely retarded in the gt/gt blastocysts. Cell proliferation activity was further compared by BrdU incorporation assays (Fig. 4a for *Eif5a* blastocysts and b for *Dhps* blastocysts). After 7 days in culture, BrdU incorporation was similar in the +/+ and +/gt blastocysts. In contrast, cell proliferation was greatly reduced in the $Eif5a^{gt/gt}$ blastocysts (Fig. 4a) and no sign of growth was observed with $Dhps^{gt/gt}$ blastocysts in culture (Fig. 4b). Interestingly, the growth defect was more severe for the $Dhps^{gt/gt}$ blastocyst-derived cells than those from the $Eif5a^{gt/gt}$ blastocysts.

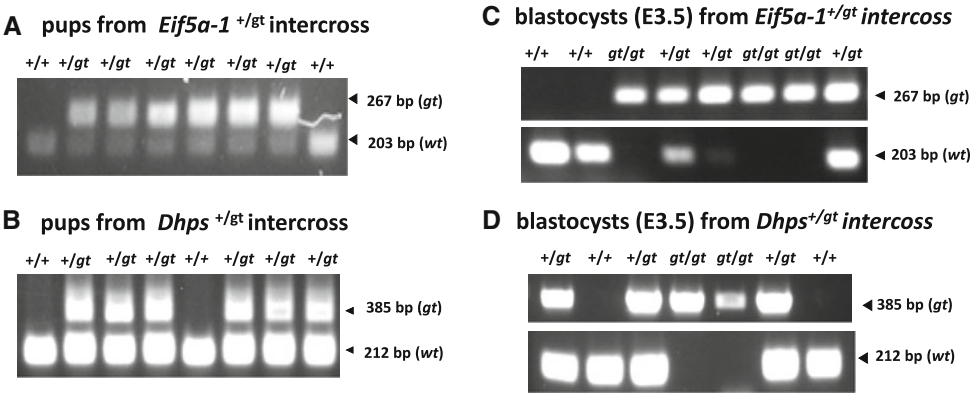

Fig. 2 Genotyping of pups and blastocysts (E3.5) from $Eif5a^{+/gt}$ intercross (**a, b**) and from $Dhps^{+/gt}$ intercross (**c**, **d**). Genomic DNA was isolated from tail snips of every litter mates (pups) from one $Eif5a^{+/gt}$ intercross (**a**) or one $Dhps^{+/gt}$ intercross (**c**), from every blastocysts isolated from another $Eif5a^{+/gt}$ intercross (**b**) and $Dhps^{+/gt}$ intercross (**d**) and were used as templates for PCR genotyping. For genotyping of pups shown in a and c, both sets of primers (indicated under "Materials and methods") were included in one PCR mixture to detect the targeted allele as well as the wild type allele in one tube. For genotyping of blastocysts (**b**, **d**), two separate PCR reactions were carried out with either mutant allele specific primer sets or with wild type specific primer sets

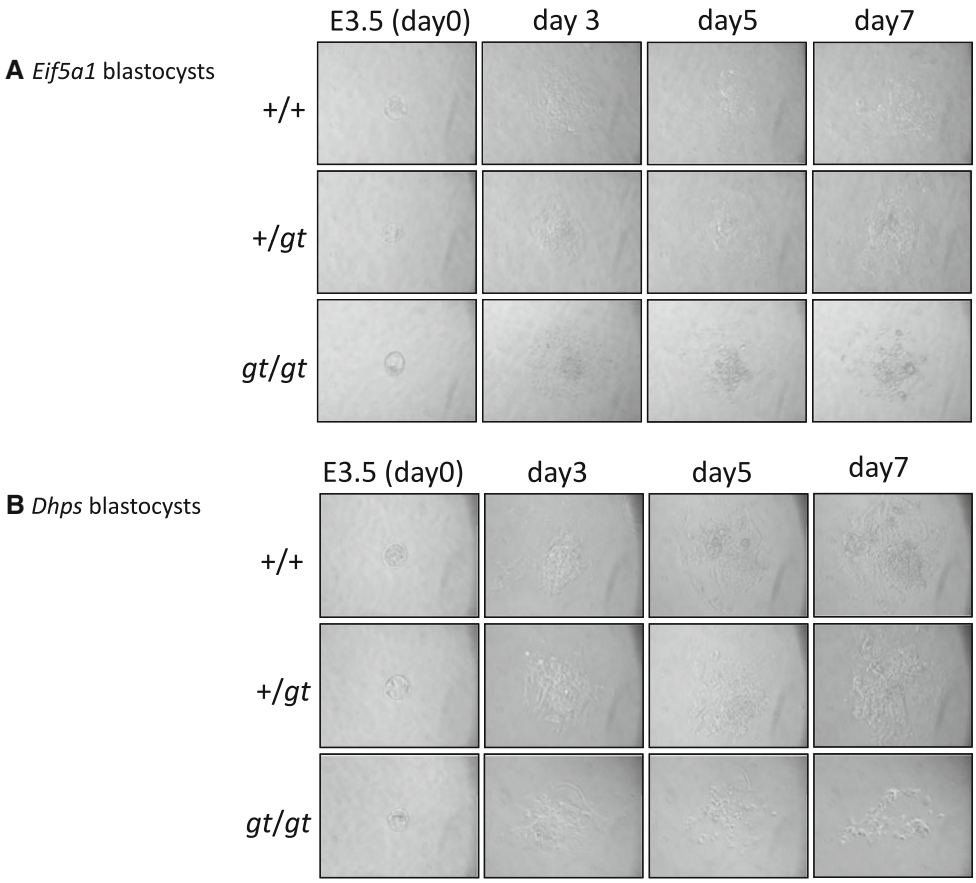

Fig. 3 Reduced cell proliferation of *Eif5a*$^{gt/gt}$ and *Dhps*$^{gt/gt}$ blastocysts (E3.5) compared to wild type (+/+) or heterozygous (+/gt) blastocysts upon culture in vitro. Blastocysts (E3.5) were flushed from the uterus of pregnant mice on day 3.5 and cultured in vitro as described under "Materials and methods". Photographs were taken with an inverted microscope (Zeiss) on 3, 5 and 7 days. After 7 days, the cells were washed with PBS (phosphate-buffered saline) and used for genotyping

Table 1 Early embryonic lethality of homozygous disruption of *Eif5a* (A) and *Dhps* (B) genes

Stage	Number/litter	Genotype		
		+/+	+/gt	gt/gt
A *Eif5A-1*$^{+/gt}$ intercross				
Mice (3 weeks)	65/10	23	42	0
E8.5	29/3	9	20	0
E7.5	9/1	1	8	0
E6.5	5/1	0	4	1
E3.5	52/9	11	19	21
B *Dhps*$^{+/gt}$ intercross				
Mice (3 weeks)	52/11	12	40	0
E8.5	8/1	2	6	0
E7.5	9/1	3	6	0
E6.5	25/3	9	16	0
E3.5	25/4	7	13	5

Heterozygous *Eif5a*$^{+/gt}$ male and female or *Dhps*$^{+/gt}$ male and female were mated. On day 3.5, 6.5, 7.5 and 8.5, embryos were flushed out or detached, genomic DNA was isolated for PCR genotyping. Multiple matings were performed for *Eif5a*$^{+/gt}$ (10 intercrosses) and *Dhps*$^{+/gt}$ (11 intercrosses) for genotyping of the pups. Sum of the numbers from multiple matings is shown for each genotype

Discussion

Our data demonstrate that the *Eif5A-1* and *Dhps* genes, encoding eIF5A-1 and deoxyhypusine synthase, respectively, are both essential in early embryonic development. *Eif5a*$^{gt/gt}$ and *Dhps*$^{gt/gt}$ embryos were normal until E3.5, a time at which DNA is being actively synthesized. Preexisting eIF5A-1 and deoxyhypusine synthase in gt/gt blastocysts derived from maternal reserves may have supported their DNA synthesis and also maintained these embryos viable until E3.5, but not beyond day 6.5 or 7.5. *Eif5a*$^{gt/gt}$ and *Dhps*$^{gt/gt}$ blastocysts died at the early stage of gastrulation. The depletion of hypusine-containing eIF5A-1 in these gt/gt blastocysts may limit protein synthesis required to enter the gastrulation stage where synthesis of many different proteins is necessary. These results are consistent with a primary role of eIF5A in protein synthesis.

The two eIF5A genes in yeast are reciprocally regulated depending on oxygen availability, but the two yeast genes are functionally identical, and expression of either gene is sufficient for supporting yeast growth. There also exist two *Eif5a* genes in mice but the functional and regulatory

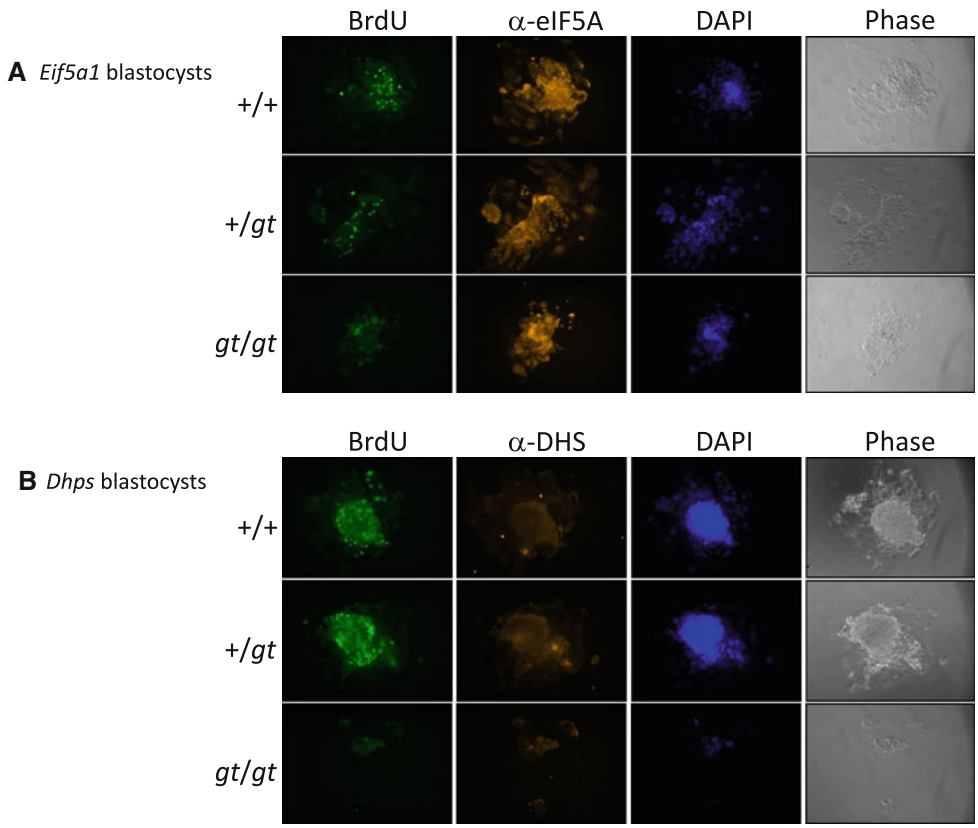

Fig. 4 Reduced levels of eIF5A-1 and DHPS proteins and reduced BrdU incorporation in *Eif5a*$^{gt/gt}$ and *Dhps*$^{gt/gt}$ blastocysts (E3.5) cultured in vitro. The cells from the 7-day in vitro culture of blastocysts (E3.5) were labeled with BrdU. Cell proliferation was estimated from incorporation of BrdU by using anti-BrdU and anti-mouse IgG-fluorescein. To determine the levels of eIF5A-1 and DHPS in these cells, the cells were treated with rabbit polyclonal primary antibodies, anti-eIF5A and anti-DHPS, respectively, and then stained using anti-rabbit IgG-Cy3. DAPI was used for counterstaining of nuclei

relationships between the two genes are not known. While the *Eif5a* gene is constitutively expressed in all mammalian cells and tissues, the *Eif5a2* gene is normally silent and may be expressed in a tissue-specific manner or upon induction. The finding that *Eif5a*$^{gt/gt}$ embryos are not viable beyond E7.5 indicates that the *Eif5a2* gene cannot substitute for the *Eif5a* gene in supporting embryonic development. It would be of interest to determine whether the *Eif5a2* gene itself has a specialized function and is essential for mouse development or not. It is interesting to note that *Eif5a*$^{gt/gt}$ blastocysts grew better than *Dhps*$^{gt/gt}$ blastocysts upon culture in vitro. Although it has not been directly measured, expression of the eIF5A-2 isoform in *Eif5a*$^{gt/gt}$ embryos may contribute to the slight extension of embryo life-span in vitro. In view of the speculative role of the non-hypusinated eIF5A precursor in apoptosis, it is also possible that unmodified eIF5A precursor protein that accumulated in the *Dhps*$^{gt/gt}$ embryo may be detrimental to its survival.

eIF5A has been implicated in the pathology of several diseases including cancer, HIV-1 infection, inflammation and diabetes. eIF5A-1 has been implicated in the regulation of cell proliferation in mammalian cells. Increased expression of eIF5A-1 or eIF5A-2 has been observed in several human cancer cells and tissues and has been proposed as a diagnostic or a prognostic marker in cancers. Gene amplification or overexpression of eIF5A-2 has been reported to be associated with several human cancers, including ovarian, liver and colorectal cancers, as a factor regulating cell motility and metastasis (Guan et al. 2004; Xie et al. 2008). However, there is dearth of studies directly addressing the role of eIF5A and its hypusine modification in animal development, cancer and physiology. Our future goal is to address the function of the two eIF5A genes in animals and to characterize the effects of underexpression or overexpression of the eIF5A isoforms and its modification enzymes, deoxyhypusine synthase and deoxyhypusine hydroxylase, in animal development, tumorigenesis and other pathologies.

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References

Benne R, Hershey JW (1978) The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes. J Biol Chem 253:3078–3087

Byers TL, Lakanen JR, Coward JK, Pegg AE (1994) The role of hypusine depletion in cytostasis induced by S-adenosyl-L-methionine decarboxylase inhibition: new evidence provided by 1- methylspermidine and 1, 12-dimethylspermine. Biochem J 303:363–368

Cano VS, Medrano FJ, Park MH, Valentini SR (2009) Evidence for conformational changes in the yeast deoxyhypusine hydroxylase Lia1 upon iron displacement from its active site. Amino Acids 38:479–490

Chatterjee I, Gross SR, Kinzy TG, Chen KY (2006) Rapid depletion of mutant eukaryotic initiation factor 5A at restrictive temperature reveals connections to actin cytoskeleton and cell cycle progression. Mol Genet Genomics 275:264–276

Chattopadhyay MK, Park MH, Tabor H (2008) Hypusine modification for growth is the major function of spermidine in Saccharomyces cerevisiae polyamine auxotrophs grown in limiting spermidine. Proc Natl Acad Sci U. S. A 105:6554–6559

Chen KY, Liu AY (1997) Biochemistry and function of hypusine formation on eukaryotic initiation factor 5A. Biol Signals 6:105–109

Clement PM, Johansson HE, Wolff EC, Park MH (2006) Differential expression of eIF5A–1 and eIF5A–2 in human cancer cells. Febs J 273:1102–1114

Gregio AP, Cano VP, Avaca JS, Valentini SR, Zanelli CF (2009) eIF5A has a function in the elongation step of translation in yeast. Biochem Biophys Res Commun 380:785–790

Guan XY, Fung JM, Ma NF, Lau SH, Tai LS, Xie D, Zhang Y, Hu L, Wu QL, Fang Y, Sham JS (2004) Oncogenic role of eIF-5A2 in the development of ovarian cancer. Cancer Res 64:4197–4200

Hanauske-Abel HM, Park MH, Hanauske AR, Popowicz AM, Lalande M, Folk JE (1994) Inhibition of the G1-S transition of the cell cycle by inhibitors of deoxyhypusine hydroxylation. Biochim Biophys Acta 1221:115–124

Hoque M, Hanauske-Abel HM, Palumbo P, Saxena D, D'Alliessi Gandolfi D, Park MH, Pe'ery T, Mathews MB (2009) Inhibition of HIV-1 gene expression by Ciclopirox and Deferiprone, drugs that prevent hypusination of eukaryotic initiation factor 5A. Retrovirology 6:90

Jao DL, Chen KY (2006) Tandem affinity purification revealed the hypusine-dependent binding of eukaryotic initiation factor 5A to the translating 80S ribosomal complex. J Cell Biochem 97:583–598

Joe YA, Wolff EC, Park MH (1995) Cloning and expression of human deoxyhypusine synthase cDNA. Structure- function studies with the recombinant enzyme and mutant proteins. J Biol Chem 270:22386–22392

Kemper WM, Berry KW, Merrick WC (1976) Purification and properties of rabbit reticulocyte protein synthesis initiation factors M2Balpha and M2Bbeta. J Biol Chem 251:5551–5557

Landau G, Bercovich Z, Park MH, Kahana C (2010) The role of polyamines in supporting growth of mammalian cells is mediated through their requirement for translation initiation and elongation. J Biol Chem 285:12474–12481

Maier B, Ogihara T, Trace AP, Tersey SA, Robbins RD, Chakrabarti SK, Nunemaker CS, Stull ND, Taylor CA, Thompson JE, Dondero RS, Lewis EC, Dinarello CA, Nadler JL, Mirmira RG (2010) The unique hypusine modification of eIF5A promotes islet beta cell inflammation and dysfunction in mice. J Clin Invest 120:2156–2170

Nishimura K, Nakatsu F, Kashiwagi K, Ohno H, Saito T, Igarashi K (2002) Essential role of S-adenosylmethionine decarboxylase in mouse embryonic development. Genes Cells 7:41–47

Nishimura K, Murozumi K, Shirahata A, Park MH, Kashiwagi K, Igarashi K (2005) Independent roles of eIF5A and polyamines in cell proliferation. Biochem J 385:779–785

Park MH (2006) The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). J Biochem (Tokyo) 139:161–169

Park MH, Cooper HL, Folk JE (1981) Identification of hypusine, an unusual amino acid, in a protein from human lymphocytes and of spermidine as its biosynthetic precursor. Proc Natl Acad Sci U S A 78:2869–2873

Park MH, Wolff EC, Lee YB, Folk JE (1994) Antiproliferative effects of inhibitors of deoxyhypusine synthase. Inhibition of growth of Chinese hamster ovary cells by guanyl diamines. J Biol Chem 269:27827–27832

Park J-H, Aravind L, Wolff EC, Kaevel J, Kim YS, Park MH (2006) Molecular cloning, expression, and structural prediction of deoxyhypusine hydroxylase: a HEAT-repeat-containing metalloenzyme. Proc Natl Acad Sci U S A 103:51–56

Park MH, Nishimura K, Zanelli CF, Valentini SR (2010) Functional significance of eIF5A and its hypusine modification in eukaryotes. Amino Acids 38:491–500

Patel PH, Costa-Mattioli M, Schulze KL, Bellen HJ (2009) The Drosophila deoxyhypusine hydroxylase homologue nero and its target eIF5A are required for cell growth and the regulation of autophagy. J Cell Biol 185:1181–1194

Ruhl M, Himmelspach M, Bahr GM, Hammerschmid F, Jaksche H, Wolff B, Aschauer H, Farrington GK, Probst H, Bevec D et al (1993) Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. J Cell Biol 123:1309–1320

Saini P, Eyler DE, Green R, Dever TE (2009) Hypusine-containing protein eIF5A promotes translation elongation. Nature 459: 118–121

Schrader R, Young C, Kozian D, Hoffmann R, Lottspeich F (2006) Temperature-sensitive eIF5A mutant accumulates transcripts targeted to the nonsense-mediated decay pathway. J Biol Chem 281:35336–35346

Spradling AC, Stern D, Beaton A, Rhem EJ, Laverty T, Mozden N, Misra S, Rubin GM (1999) The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes. Genetics 153:135–177

Sugimoto A, Hakuba N, Gyo K (2004) Reconstruction of mastoid cortex defect using bone morphogenetic protein: an experimental study in the guinea pig. Acta Otolaryngol 124:253–257

Tong Y, Park I, Hong BS, Nedyalkova L, Tempel W, Park HW (2009) Crystal structure of human eIF5A1: insight into functional similarity of human eIF5A1 and eIF5A2. Proteins 75:1040–1045

Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML (2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques 29:52–54

Valentini SR, Casolari JM, Oliveira CC, Silver PA, McBride AE (2002) Genetic interactions of yeast eukaryotic translation initiation factor 5A (eIF5A) reveal connections to poly(A)-binding protein and protein kinase C signaling. Genetics 160: 393–405

Weir BA, Yaffe MP (2004) Mmd1p, a novel, conserved protein essential for normal mitochondrial morphology and distribution in the fission yeast Schizosaccharomyces pombe. Mol Biol Cell 15:1656–1665

Wolff EC, Kang KR, Kim YS, Park MH (2007) Posttranslational synthesis of hypusine: evolutionary progression and specificity of the hypusine modification. Amino Acids 33:341–350

Xie D, Ma NF, Pan ZZ, Wu HX, Liu YD, Wu GQ, Kung HF, Guan XY (2008) Overexpression of EIF-5A2 is associated with metastasis of human colorectal carcinoma. Hum Pathol 39:80–86

Zanelli CF, Maragno AL, Gregio AP, Komili S, Pandolfi JR, Mestriner CA, Lustri WR, Valentini SR (2006) eIF5A binds to translational machinery components and affects translation in yeast. Biochem Biophys Res Commun 348:1358–1366

Zuk D, Jacobson A (1998) A single amino acid substitution in yeast eIF-5A results in mRNA stabilization. EMBO J 17:2914–2925