Biological relevance and therapeutic potential of the hypusine modification system

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Abstract: Hypusine modification of the eukaryotic initiation factor 5A (eIF-5A) is emerging as a crucial regulator in cancer, infections and inflammation. Although its contribution in translational regulation of proline-repeat-rich proteins has been sufficiently demonstrated, its biological role in higher eukaryotes remains poorly understood. To establish the hypusine modification system as a novel platform for therapeutic strategies, we aimed to investigate its functional relevance in mammals by generating and using a range of new knockout mouse models for the hypusine modifying enzymes deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) as well as for the cancer-related isoform eIF-5A2. We uncovered that homozygous depletion of DHS or DOHH causes lethality in adult mice with different penetrance compared to haploinsufficiency. Network-based bioinformatic analysis of proline-repeat-rich proteins, which are putative eIF-5A targets, revealed that these proteins are organized in highly connected protein-protein-interaction networks. Hypusine-dependent translational control of essential proteins (hubs) and protein complexes inside these networks might explain the lethal phenotype observed after deletion of hypusine modifying enzymes. Remarkably, our results also demonstrate that the cancer-associated isoform eIF-5A2 is dispensable for normal development and viability. Together, our results provide first genetic evidence that the hypusine modification in eIF-5A is crucial for homeostasis in mammals. Moreover, these findings highlight functional diversity of the hypusine system compared to lower eukaryotes and indicate eIF-5A2 as a valuable and safe target for therapeutic intervention in cancer.

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Biological relevance and therapeutic potential of the hypusine modification system

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Running title: Genetic manipulation of the hypusine modification system in mammals

Key words: eukaryotic initiation factor 5A (eIF-5A), translation elongation factor, post-translational modification (PTM), mouse genetics, cancer therapy
CAPSULE

Background: Hypusine modification of the eukaryotic initiation factor 5A (eIF-5A) represents a conserved posttranslational modification that regulates translation.

Results: Deletion of hypusine modification enzymes exerts strong phenotypes. eIF-5A2 deleted animals are viable and fertile.

Conclusion: Both enzymatic steps of hypusine modification are essential for mammalian homeostasis, whereas the cancer-related isoform eIF-5A2 is dispensable.

Significance: eIF-5A2 might represent a safe therapeutic target.

ABSTRACT

Hypusine modification of the eukaryotic initiation factor 5A (eIF-5A) is emerging as a crucial regulator in cancer, infections and inflammation. Although its contribution in translational regulation of proline-repeat-rich proteins has been sufficiently demonstrated, its biological role in higher eukaryotes remains poorly understood. To establish the hypusine modification system as a novel platform for therapeutic strategies, we aimed to investigate its functional relevance in mammals by generating and using a range of new knockout mouse models for the hypusine modifying enzymes deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DOHH) as well as for the cancer-related isoform eIF-5A2. We uncovered that homozygous deletion of DHS or DOHH causes lethality in adult mice with different penetrance compared to haploinsufficiency. Network-based bioinformatic analysis of proline-repeat-rich proteins, which are putative eIF-5A targets, revealed that these proteins are organized in highly connected protein-protein-interaction networks. Hypusine-dependent translational control of essential proteins (hubs) and protein complexes inside these networks might explain the lethal phenotype observed after deletion of hypusine modifying enzymes. Remarkably, our results also demonstrate that the cancer-associated isoform eIF-5A2 is dispensable for normal development and viability. Together, our results provide first genetic evidence that the hypusine modification in eIF-5A is crucial for homeostasis in mammals. Moreover, these findings highlight functional diversity of the hypusine system compared to lower eukaryotes and indicate eIF-5A2 as a valuable and safe target for therapeutic intervention in cancer.

INTRODUCTION

Control of translational processes is essential to maintain cellular function and organismal integrity (1). In addition to the important role in normal physiology, there is accumulating evidence that alterations in the translation machinery (e.g. deregulated translation factors) lead to changes in protein biosynthesis and the development or progression of various diseases like cancer and viral infections (2,3). Given that translation factors are frequently regulated by posttranslational modifications which are mediated by enzymes, those modifications can be harnessed therapeutically (4).

In this context, the eukaryotic initiation factor 5A (eIF-5A) represents a particularly interesting target for therapeutic intervention since it carries a highly specific posttranslational modification, the unusual amino acid hypusine, which is unique in this protein (5,6). The biosynthesis of hypusine is catalyzed from lysine in a two-step enzymatic reaction. First, the deoxyhypusine synthase (DHS) transfers a 4-aminoilbutyl moiety of spermidine to the ε-amino group of Lys50 to form a deoxyhypusine-containing intermediate. Second, the deoxyhypusine hydroxylase (DOHH) catalyzes the hydroxylation of the deoxyhypusine residue to generate hypusine-containing eIF-5A (Fig. 1A). Hypusine is mandatory for eIF-5A activity and function (7,8) and only hypusinated eIF-5A was shown to control translation elongation as well as the nuclear export of specific mRNAs (9,10). Moreover, recent studies linked eIF-5A to the translational control of proteins containing consecutive proline residues (11).

Like other translation factors, eIF-5A and its modifying enzymes, DHS and DOHH, are highly conserved in eukaryotes as well as archaea. Prokaryotes express an orthologous factor, the elongation factor P (EF-P) (12).
Although EF-P is not hypusine-modified, it is activated by a similar modification (lysinylation) at a comparable amino acid position (Lys34) and promotes translation of polyproline motifs in the same way as eIF-5A (13-15). Based on work in yeast it seems that eIF-5A and its hypusine modification are essential in lower eukaryotes. However, a fundamental unanswered question is whether the hypusine axis is crucial for viability of mammals.

The observation that eIF-5A is also involved in diseases like cancer, infections and diabetes highlights eIF-5A and particularly its hypusine modification as an attractive target for the development of new therapeutic strategies. Upregulation of the eIF-5A1 isoform and the hypusine forming enzymes can be found in many types of tumors, and inhibition of hypusination by siRNA or small molecules showed anti-proliferative effects in numerous tumor entities (16,17). However, the hypusine system may have pleiotropic functions, as seen for the tumor suppressor activity of eIF-5A1 and DHS in lymphoma development (18). Moreover, perturbation of hypusination also preserves islet function in diabetes and blocks the replication cycle of HIV by affecting the export of specific mRNAs (19). Intriguingly, many cancers and tumor cell lines display elevated levels of eIF-5A2, an isoform of eIF-5A that shows no expression in normal tissue except brain and testis (20). In addition to its oncogenic activity (21,22), eIF-5A2 was demonstrated to promote invasion and metastasis, and overexpression correlates with worse prognosis in various cancers (23,24).

Together, a growing body of research now indicates that the enzymes of the hypusine system fulfill certain criteria for being a potential therapeutic target (25,26) and consequentially, lead substances have been developed (27,28). However, a key question that still needs to be addressed before the hypusine system can be considered a novel target for therapies is to understand its function in mammalian homeostasis. In this regard, development of mouse models for manipulation of the hypusine system could provide the most valuable tool for addressing this question (29). Based on that consideration we generated and characterized conditional knockout mouse models for Dhs, Dohh and eIF-5A2 that allowed either the general inhibition of both steps of hypusine modification or the selective depletion of the cancer-associated eIF-5A2 isoform in a particular temporal setting.

**EXPERIMENTAL PROCEDURES**

*Animal studies*

All animal experimental procedures were approved by the responsible Hamburg state authority according to German animal protection law. Mice were maintained in specific pathogen-free conditions at the University Medical Center Hamburg-Eppendorf animal facilities.

*Generation of conditional knockout mice*

Embryonic stem cell clones derived from C57BL/6N mice for the conditional knockout of the Dhs (clones EPD0628_1_B06, EPD0628_1_C06, EPD0628_1_F05) or eIF-5A2 (clone HEPD0734_3_A07) gene were obtained from the International Knockout Mouse Consortium (30) and verified by Southern blot and/or long range PCR. Clones were thawed, injected into E3.5 blastocysts of C57BL/6J mice, and transferred into the uterine horns of foster mothers. Male chimeric offspring was mated to C57BL/6J females and the resulting offspring analyzed for transmission of the targeted allele. Transgene-positive male offspring was mated to Flp-deleter (B6;SJL-Tg(ACTFLPe)9205Dym/J (31) in order to remove the selection cassette. Resulting offspring were selected for the floxed allele, hereinafter referred to as a superscript “p”. Wildtype alleles are indicated with a superscript “+”. To generate a complete knockout of Dhs or eIF-5A2, CMV-Cre-deleter (32) were mated to mice of the Dhs<sup>-/p</sup> or eIF-5A2<sup>-/p</sup> genotypes. After Cre-mediated deletion the knockout alleles are indicated with a superscript “-”. Mice of the resulting Dhs<sup>+/c</sup> or eIF-5A2<sup>+/c</sup> genotypes were further mated to individuals of the same genotype. To enable a 4-OHT-inducible knockout of Dhs or Dohh, mice of the B6.Cg-Tg(CAG-cre/Esr<sup>1</sup>)<sup>Samc/J113</sup> strain (Jackson Laboratory, Bar Harbor, ME) were mated to mice of the Dhs<sup>-/p</sup> or Dohh<sup>-/p</sup> genotype. Mice of the resulting Dhs<sup>-/p</sup>;CAG-cre/Esr<sup>1</sup> or Dohh<sup>-/p</sup>;CAG-cre/Esr<sup>1</sup> genotypes were further mated to individuals of the same genotype (Fig. 1B). Tamoxifen (4-OHT)-inducible knockout was induced by
administering 4-OHT-containing feed (0.4 g/kg, LASvendi, Soest, Germany) for up to three weeks. Mice were monitored regularly and weighed at least every week (Fig. 1C).

**Southern blot analysis**

Southern blot was carried out as described before (33).

**Long range PCR**

Genomic DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen, Venlo, NL) according to manufacturer’s instructions. Long range PCR was performed using oligonucleotides listed in Table 1 and Long PCR Enzyme Mix (Thermo Fisher Scientific, Waltham, MA). PCRs were performed at 94°C for 90 s, followed by 10 cycles of 95°C for 15 s, 54°C for 30 s and 68°C for 7 min and 25 cycles of 95°C for 15 s, 54°C for 30 s and 68°C for 7 min + 5 s/cycle and final elongation at 68°C for 10 min.

**Genotyping using genomic DNA from tail clippings, organs, embryos or culture cells**

Samples were digested overnight at 55°C using Proteinase K (Thermo Fisher Scientific, Waltham, MA) according to manufacturer’s instructions. PCR analyses of genomic DNA were performed using DreamTaq Green PCR Mastermix (Thermo Fisher Scientific, Waltham, MA) and 2 μl of lysate in a total volume of 20 μl. The oligonucleotide sequences are listed in Table 1.

**Quantitative PCR**

RNA was isolated from cell or tissues using TriFast (Peqlab, Erlangen, Germany) according to manufacturer’s instructions. cDNA was prepared by reverse transcription of 1 μg total RNA using random hexamer primer and M-MuLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR for Dhs, Dohh, elf-5A1 and elf-5A2 was performed in a 7500 Fast Real-Time PCR-System (Life Technologies, Carlsbad, CA, USA) using the oligonucleotides or Quant iTect primermix (Qiagen, Venlo, Netherlands) listed in Table 1 and Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies, Carlsbad, CA, USA). PCRs were performed at 95°C for 3 min, followed by 40 cycles of 95°C for 15s and 60°C for 30s. PCRs were conducted in triplicates and normalized against 18S rRNA or Gapdh as reference gene using the 2^ΔΔCT method (34).

Expression of elf-5A2 in normal mouse tissue was examined using the TissueScan qPCR Array MDRT101 (OriGene Technologies, Rockville, MD, USA). Values were normalized to the expression of the housekeeping gene Gapdh and depicted as fold change based on the bone marrow.

1-dimensional (1D) and 2-dimensional (2D) Western Blot analysis

1D and 2D Western blot analyses were carried out as described before (16,33) using anti-elf-5A1 (Novus Biologicals, Littleton, CO, USA) and anti-elf-5A2 (Abcam, Cambridge, UK).

**Generation and culture of mouse embryonic fibroblasts (MEFs)**

Mouse embryonic fibroblasts (MEFs) were isolated from Dhs<sup>op/op</sup>×Dhs<sup>op</sup>CAG-cre/Esr1<sup>+</sup> and Dhs<sup>op/op</sup>×Dhs<sup>op</sup>CAG-cre/Esr1<sup>+</sup> matings as described previously (33). The cells were cultured in DMEM (all cell culture media and additives from Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 25 μM b-mercaptoethanol, and 4 mM L-glutamine (37°C, 5% CO₂, humidified atmosphere). For inducible knockout experiments, 10<sup>6</sup> cells were seeded on a 10 cm culture dish, maintained at subconfluence and treated with 100 nM 4-OHT for up to 10 days (Sigma-Aldrich, St. Louis, MO, USA) or 50 μM GC7 for 2 days (Biosearch Tech., Novato, CA, USA).

**Morphological and histopathological analysis of mouse tissue**

Mice were transcardially perfused with a mixture of 4% paraformaldehyde (and 1% glutaraldehyde) in 0.1 M PB buffer at pH 7.4 for tissue preparation and bone marrow was morphologically analyzed as described before (33). Briefly, after postfixation overnight at 4°C tibiae were decalcified for 4 days in 10% (w/v) EDTA in PBS and cut in 150-μm sagittal sections with a vibratome. The sections were then postfixed in 1% (v/v) OsO₄, dehydrated and embedded in epon. Semi-thin sections (0.5 μm) were labeled with methylene blue and...
examined under the light microscope (Zeiss). Other tissues were processed to paraffin blocks using an ASP300S dehydration machine (Leica, Wetzlar, Germany) and an EG1160 tissue embedding system (Leica, Wetzlar, Germany). Paraffin blocks were cut into 4 µm sections, which were stained with hematoxylin and eosin or with turnbull’s blue reagents following standard laboratory procedures.

**Immunohistochemistry**

For immunohistochemical staining the Ventana Benchmark XT machine (Ventana, Tuscon, Arizona, USA) was used. Briefly, deparaffinized sections were boiled for 30 to 60 min in 10 mM citrate buffer, pH 6.0, for antigen retrieval. Primary antibodies were diluted in 5 % goat serum (Dianova Immundiagnostic, Hamburg, Germany), 45 % Tris buffered saline pH 7.6 (TBS) and 0.1 % Triton X-100 in antibody diluent solution (Zytomed, Berlin, Germany). Sections were then incubated with primary antibody against anti-Ki67 for proliferating cells (1:250; Abcam, Cambridge, UK) and anti-caspase-3 for apoptotic cells (1:1000; R&D Biosystems, Wiesbaden, Germany) for 1 hr. Anti-rabbit histofine Simple Stain MAX PO Universal immunoperoxidase polymer (Nichirei Biosciences, Wedel, Germany) were used as secondary antibodies. Detection of secondary antibodies and counter staining was performed with an ultraview universal DAB detection kit from Ventana (Ventana, Tuscon, Arizona, USA) according to the standard settings of the machine. All sections were cover-slipped using TissueTek® glove mounting media (Sakura Finetek, Staufen, Germany), and dried in an incubator at 60°C. Pictures were taken using a light microscope (Axioskop 40, Zeiss, Jena, Germany or Olympus BH2, Hamburg, Germany) equipped with a digital camera (AxioCam Icc3 Zeiss, Jena Germany).

**eIF-5A1 immunofluorescence of immortalized 3T3 Dohh-knockout cells or primary Dhs-knockout MEFs**

Knockout of Dohh or Dhs was induced with 100 nM 4-OHT for 7 days in 3T3 Dohhpp;CAG-cre/Esr1+ (33) or in MEFs Dhspp;CAG-cre/Esr1+, respectively. The cells were then seeded onto poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated 10 mm diameter round glass cover slips in 24-well plates and left to adhere for one more day in the presence or absence of 4-OHT. After washing with PBS, the cells were fixed with 3% (w/v) paraformaldehyde (PFA) in PBS for 20 min at RT. After washing, residual PFA was quenched with 50 mM NH4Cl in PBS for 10 min. For permeabilization, the cells were treated for 5 min with 0.1 % (v/v) Triton X-100 in PBS, then the samples were blocked with 0.5%(w/v) BSA in PBS for 30 min. Immunostaining was performed with mouse anti-eIF-5A1 (Clone 26/eIF-5a; BD Biosciences, Franklin Lakes, NJ, USA) and Alexa Fluor 488-labelled goat-anti-mouse antibody (Life Technologies, Carlsbad, CA, USA). Cell nuclei were stained with Hoechst 33342 (Life Technologies). Images were taken as single confocal sections of 1 µm using a Nikon C2+ confocal laser scanning microscope (Nikon Instruments, Tokyo, Japan). For image acquisition and preparation, Nikon NIS Elements software and Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA) were used.

**Construction and analysis of protein-protein interaction networks**

For the construction of protein-protein interaction (PPI) networks for PPP (Pro-Pro-Pro)- and PPG (Pro-Pro-Gly)-rich proteins, genes with >1 PPP units and >1 PPG units were extracted from Mandal et al. (35). The number of PPP- and PPG units were defined by the whole number of consecutive prolines divided by 3, as described recently (35). That means that, 3, 4 and 5 consecutive prolines were all counted as 1 PPP unit and 6, 7, and 8 consecutive prolines as 2 PPP units and so on. STRING database v10.0 was used as resource for contraction of PPI networks (36). PPI networks were visualized and analyzed using the software platform Cytoscape 3.2.1 (http://www.cytoscape.org/) with the plugins MCODE for cluster analysis and BinGO for gene ontology analysis of protein clusters (37-39). Global network topological analysis, including clustering coefficients, average number of neighbors (degree) and network heterogeneity as well as local network analysis using betweenness centrality value calculation were carried out with NetworkAnalyzer and the Cytoscape plugin CytoNCA (40,41). The edges in all PPI networks were treated as undirected. Clustering coefficient for a
network is the average of the clustering coefficient over all nodes and quantifies the local interconnectivity of a network. Node degree is the number of edges linked to a node. Network heterogeneity reflects tendency of a network to contain highly connected nodes (hubs). Betweenness centrality is a measure of the fraction of shortest paths passing through a node in the network, and nodes with higher betweenness are more globally central in the network (42).

Statistics

For statistical analyses GraphPad software (GraphPad Software, La Jolla, CA, USA) was used. The statistical tests used in each analysis are stated in the corresponding figure legends.

RESULTS

Dhs is crucial for embryonic development as well as for viability in adult mice

To study the biological relevance and functional requirement of the hypusine modification in embryonic development (43,44) and for viability, we used a recently described conditional knockout strain for Dohh (33) and established a new conditional knockout mouse model for Dhs (B6.Dhps\textsuperscript{tm2aEUCOMM}Wtsi). For the generation of the latter strain, embryonic stem cell clones were obtained from the International Knockout Mouse Consortium (30). Detailed explanation for the Dhs gene targeting strategy that leads to deletion of exons two to seven resulting in a nonfunctional truncated Dhs are depicted in Fig. 2A. Information about the genotyping procedure is provided in Fig. 2B. For Cre-mediated knockout we intercrossed B6.Dhps\textsuperscript{tm2aEUCOMM}Wtsi to two different Cre-deleter strains allowing us to analyze how Dhs impairs either embryonic development or affects the viability of an adult organism after 4-OHT mediated Cre-induction (Fig. 1B-C). After removal of the selection cassette we induced a general knockout using the CMV-Cre mouse model (Fig. 1B) (32). The loss of Dhs leads to embryonic lethality in a pure C57BL/6 background as we could not detect any homozygous Dhs\textsuperscript{+} offspring in our crossings (Fig. 3A). This observation is well in line with data obtained in a mixed background published by others (43,44). Heterozygous Dhs\textsuperscript{+} animals were viable and did not show any obvious phenotype. Intriguingly, when we investigated mRNA levels in several organs of Dhs\textsuperscript{+} mice, we detected a significant upregulation of Dhs as well as Dohh and eIF-5A1 compared to wildtype Dhs\textsuperscript{+}+ animals (Fig. 3B-D). Moreover, eIF-5A1 protein levels were also elevated (Fig. 3E-F), suggesting a compensatory feedback mechanism and upregulation of the hypusine-system in a Dhs-compromised setting protecting against the loss of DHS activity. We next asked whether heterozygous deletion of Dhs results in a reduced hypusine modification level in eIF-5A1. Two-dimensional Western blots revealed an accumulation of two additional eIF-5A1 spots in tissue from Dhs\textsuperscript{+}+ mice (Fig. 3G). According to previous data (33,45), these spots represent the unhypusinated (Fig. 3G, red arrow) and the more acidic unhypusinated and acetylated form of eIF-5A1 (Fig. 3G, blue arrow). Nevertheless, high amounts of hypusinated and therefore functional eIF-5A1 remained detectable (Fig. 3G, black arrow), explaining the normal phenotype of Dhs heterozygous animals. These observations indicate that under physiological conditions, a reduced pool of fully modified eIF-5A1 is sufficient to support normal cellular function. As previous studies proposed a pro-apoptotic function for unhypusinated eIF-5A1 (46), our results further suggest that accumulation of low levels of unhypusinated eIF-5A1 does not affect embryonic development and viability.

First step of hypusine modification is essential for viability

Given the crucial function of Dhs in embryonic development, we next investigated whether Dhs is also essential for viability of an adult organism. For this reason, we utilized a tamoxifen (4-OHT)-inducible conditional CAG/Cre-Esr1\textsuperscript{+} mouse strain (Fig. 1B-C), allowing us to induce a general knockout of Dhs in most tissues (47). First, we employed Dhs\textsuperscript{+}+ and Dhs\textsuperscript{+/-} mouse embryonic fibroblasts to investigate functionality of the Cre-mediated Dhs knockout in vitro. Analysis outlined in Fig. 3H revealed a clear reduction of wildtype Dhs transcript after 4-OHT treatment and impaired hypusination of eIF-5A1 in those cells (Fig. 3I), showing loss of DHS activity. Next, we induced Dhs deletion in 5-7 weeks old mice using 4-OHT-containing feed.
Intriguingly, after 4-OHT administration Dhs<sup>p</sup> animals behaved similarly to Dhs<sup>p/+</sup> mice, whereas the bodyweight of Dhs<sup>p/p</sup> animals drastically decreased (Fig. 4A) and mice died or had to be euthanized due to a wasting syndrome (weight loss >20%) 5-14 days after induction of the knockout (median 12 days; Fig. 4B). Notably, the Dhs<sup>p/p</sup> mice expressed a truncated form of Dhs suggesting a successful knockout (Fig. 4D) that leads to the accumulation of the unhyprusinated eIF-5A1 precursor protein as shown in heart and spleen by 2-dimensional Western blot (Fig. 4C). However, those animals also displayed a significant upregulation of the hypusine system in different organs at a terminal stage involving a remarkable increase in mRNA of Dhs, Dohh and eIF-5A1 (Fig. 4D), compared to their normal Dhs<sup>p/+</sup> counterparts, as well as a detectable level of hypusinated eIF-5A1 (Fig. 4C). This argues for a partial knockout in organs of Dhs<sup>p/p</sup> animals due to an incomplete mosaic-like expression of the Cre recombinase in CAG/Cre-Esr1<sup>+</sup> mice, as is known for this particular Cre-deleter strain (47). Furthermore, this implicates the activation of a compensatory positive feedback mechanism in remaining wildtype cells, similarly as observed in heterozygous Dhs<sup>p/+</sup> mice (Fig. 3B-F), to keep the hypusine modification in a range that allows viability.

**Deletion of Dhs affects bone marrow and spleen cellularity**

To identify putative morphological abnormalities accounting for the observed strong lethal phenotype associated with the loss of Dhs, we performed comprehensive histological analyses. Although these analyses did not point to a single recurrent tissue defect causing lethality, histopathological examination unveiled gross changes in various organs of Dhs<sup>p/p</sup> animals compared to their normal wildtype Dhs<sup>p/+</sup> counterparts. The hematopoietic system seemed to be severely affected since the bone marrow and the spleen showed reduced overall cellularity in Dhs<sup>p/p</sup> animals. As shown in Fig. 4E, this was mainly due to a reduction of all three cell lines in the bone marrow and in the red pulpa of the spleen. Moreover, in two out of four Dhs-deficient animals, the kidney showed enhanced incorporation of ferrous iron (Fig. 4E), however, histological examination revealed no obvious alteration in kidney tissue architecture (data not shown). In addition, the epithelial structures of the intestine were markedly destroyed in one out of four Dhs<sup>p/p</sup> animals. Thus, it can be assumed that loss of Dhs confers lethality by induction of multiple phenotypic changes particularly in high proliferative tissues (bone marrow and intestine) in adult mice.

Together, these data indicate that Dhs-mediated hypusination of eIF-5A1 is essential for embryonic development and mandatory to ensure viability of an adult organism. Moreover, a potential positive feedback mechanism is activated protecting heterozygous Dhs mice from gene dosage effects, underlining the importance of the hypusine system for cellular integrity.

Dohh is equally important for an adult organism, but consequences of a Dohh knockout are less pronounced compared to the Dhs knockout

The second step of hypusine modification in eIF-5A1 was shown to be not essential in lower eukaryotes like *S. cerevisiae* (48). However, we have shown recently that deletion of Dohh and subsequent loss of hypusine modification induced an embryonic lethal phenotype in mice (33).

To investigate the role of Dohh in adult mice, we intercrossed our previously described B6.DOHH<sup>tm2a/bal</sup> animals (33) with the CAG/Cre-Esr1<sup>+</sup> mouse strain and used 4-OHT to induce Dohh knockout in 4-7 weeks old mice as described for the Dhs knockout mouse model (Fig. 1B-C). Notably, heterozygous Dohh<sup>p/+</sup> animals behaved like wildtype controls, but the bodyweight of Dohh<sup>p/p</sup> mice stagnated and animals finally died 1-5 weeks after knockout induction due to a wasting condition (median 20 days; Fig. 5A-B). While we observed an expected decrease in Dohh mRNA in spleen and kidney from those animals at a terminal stage (Fig. 5C), we could not detect a consistent compensatory upregulation of the hypusine system like in Dhs-deficient mice (Fig. 5D). As a consequence, loss of Dohh resulted in a shift towards the unhypusinated eIF-5A1 precursor protein as seen in the spleen of Dohh<sup>p/p</sup> animals (Fig. 6A, red arrow), even though hypusinated eIF-5A1 was still detectable (Fig. 6A, black arrow). In agreement with our recent *in vitro* results (33) and in contrast to the ablation of
Dhs, unhypusinated and acetylated eIF-5A1 was not observed (Fig. 6A).

Although homozygous deletion of Dohh was lethal in all animals, rigorous histopathological examinations of adult Dohh knockout mice (homozygote deletion of Dohh was induced in seven mice at the age of 5-7 weeks and in two mice at the age of 6-7 month) revealed no relevant recurrent tissue alterations (Fig. 6B-F) except histological signs for a tubulus necrosis in the kidney of one animal (Fig. 6B) and increased incorporation of ferrous iron in the spleen of both analyzed adult (6-7 month old) animals (Fig. 6D). Furthermore, we have detected moderate necrosis in the liver of two knockout mice (group of 5-7 weeks old mice) (Fig. 6E) and moderate focal liver inflammation in one knockout animal (group of 6-7 month old mice) (Fig. 6F). Compared to the Dhs knockout animals, bone marrow was apparently not affected in Dohh deleted mice (Fig. 6C). All other animals died without obvious cause and no consistent pathology could be identified.

Together with the observation that an early general knockout of Dohh is embryonically lethal as published by our group recently (33), we here conclude that Dohh is equally as important for early embryonic development and adult organisms as Dhs, yet the lethal phenotype of Dohh-deficient animals seems to be less pronounced. However, it must be pointed out that the exact reason for the observed lethal phenotype has to be elucidated.

eIF-5A is also localized in the nucleus after deletion of Dhs or Dohh

Since modifications of eIF-5A1 influence its cellular localization (49,50), the accumulation of different eIF-5A1 forms may therefore account for the different phenotype of the Dhs and the Dohh knockout. While the knockout of Dohh causes an accumulation of unhypusinated eIF-5A1 precursor (Fig. 6A, red arrow), Dhs-deficiency provokes its additional acetylation (Fig. 4C, blue arrow). Investigating the localization of eIF-5A1 by immunofluorescence in a Dhs- and Dohh-dependent setting in vitro, we found that the 4-OHT induced knockout of Dhs and Dohh leads to nuclear accumulation of eIF-5A1 (Fig. 7A). Since fully hypusinated eIF-5A1 was recently shown to be located in the cytoplasm (where it regulates translation of specific proteins (11)), the observed nuclear eIF-5A in the Dhs and Dohh deficient setting likely reflects the unhypusinated (acetylated and non-acetylated) eIF-5A. Figure 7B summarizes a hypothesis explaining why eIF-5A1 is differently modified in these scenarios. In a Dohh-deficient background, DHS is constantly linked to eIF-5A1 due to the missing hydroxylation of deoxyhypusine (Dhp50) and subsequently prevents binding of acetyltransferases like HDAC6, SIRT2, p300 and CBP (49,51). On the other hand, Dhs-depletion prevents DOHH binding and further allows acetylation of eIF-5A1.

eIF-5A2 is not essential for normal development and steady state viability

As the general use of DHS- and DOHH-inhibitors seems to require more refined analyses due to their essential role in higher eukaryotes, we reasoned whether eIF-5A2, which is only expressed in few tissues of the body, but highly abundant in cells of various tumor types, might represent a better target for therapy. To address this question and to investigate the effects of eIF-5A2 deficiency in development and in adult mice, we generated eIF-5A2 conditional knockout mice (B6.eIF5A2tm2aEUCOMMwtsi) (Fig. 8A-B) and intercrossed them with the CMV-Cre-deleter strain to induce an early general knockout (Fig. 1B). In contrast to Dhs- and Dohh-deficient animals, both heterozygous eIF-5A2+/− and homozygous eIF-5A2−/− mice were viable (Fig. 9A-B) and did not show any abnormalities regarding bodyweight (data not shown) or survival over the indicated time period. Expression of eIF-5A2 is thought to be limited to few human tissues like lung, brain, testis or prostate (52). Quantitative PCR revealed a comparable expression pattern in mouse tissues (Fig. 9C). As expected, eIF-5A2 mRNA and protein are not detectable in testis and brain tissue of eIF5-A2−/− mice (Fig. 9D and 9E), indicating the functional and successful knockout. Moreover and in contrast to a loss of DHS, a consistent compensatory overexpression of the hypusine modification system was not detectable in eIF5-A2−/− mice (Fig. 9F-H). Taken together, these data suggest that loss of the eIF-5A2 isoform is not essential for embryonic development or for steady state viability in an adult organism since eIF-5A2 knockout mice are viable, fertile and do not show an obvious phenotype. Fig. 10
summarizes phenotypes induced by deletion of the hypusine modification systems.

*eIF-5A targets are organized in highly connected protein-protein-interaction networks*

It was recently shown that fully hypusinated eIF-5A1 is a sequence-specific elongation factor, regulating the translation of proteins containing consecutive prolines in the form of repetitive PPP (Pro-Pro-Pro) and/or PPG (Pro-Pro-Gly)-sequence units (11). Translation of proteins working together in complexes is regulated in a “proportional synthesis strategy” in order to provide the cell with the correct stoichiometric amount for each member of the protein complex (53). Based on these observations, we hypothesize that eIF-5A in being a sequence-specific elongation factor might regulate the optimal translation of PPP- and PPG-repeat-rich protein complexes and that the observed phenotype after homozygous deletion of Dhs or Dohh is based on disruption of those complexes. Bioinformatic network analysis using STRING database and the Cytoscape software platform with the aim to find network clusters and essential proteins (hubs) was performed for yeast and mouse genes expressing PPP- or PPG-units. To reduce the complexity of the networks and to focus on proteins with long consecutive proline sequences we analyzed genes with more than one PPP- or PPG-unit. Exact number of proteins that have been used for network construction and number of nodes in the networks are summarized in Table 2. As depicted in Fig. 11 and 12 PPP- and PPG-repeat-rich proteins are organized in networks with a considerable variance in the structure of the networks between both species. We exploited three fundamental network parameters (clustering coefficient, average degree and network heterogeneity) to determine the network interconnectivity (Table 2). Together, the global networks for murine genes show a higher degree of interconnectivity. The average number of neighbors (average degree) and the network heterogeneity have shown higher values for the murine interactome. Interestingly, PPP-rich proteins in yeast forming a highly connected network with higher clustering coefficient compared to murine PPP-rich proteins. Furthermore, detailed information for each node inside the networks is available in Supplementary Tables 1 to 4.

*Identification protein complexes and biological processes in proline-repeat-rich proteins*

To detect densely connected subnetworks (clusters) potentially representing biological modules or protein complexes, we performed MCODE cluster analysis for yeast and murine PPP- or PPG-rich networks. This resulted in a higher number of protein clusters for *M. musculus* as compared to *S. cerevisiae* (Fig. 11 and Fig. 12), further supporting the higher complexity of proline-repeat-rich proteins in multicellular eukaryotes compared to yeast. We identified 28 and 21 clusters for PPP- and PPG-rich for murine proteins, respectively. In *S. cerevisiae*, MCODE analysis revealed 4 and 1 clusters for PPP-rich and PPG-rich proteins, respectively. Detailed information about the proteins forming these clusters is available in Supplementary Tables 1 to 4. To predict the biological functionality of the clusters, we used BinGO to determine enriched biological processes. Highest scoring gene ontology (GO) terms are depicted in Fig. 11 and Fig. 12 for each cluster. Stingly, functional activity of these clusters partially overlapped between *S. cerevisiae* and *M. musculus*. These include biological processes involved in cytoskeletal organization and regulation, mRNA metabolism and processing as well as regulation of transcription. However, GO terms associated with cellular differentiation, chromatin modification and DNA replication appeared exclusively in protein clusters extracted from *M. musculus* (Fig. 12), pointing further to specialized function of proline-repeat-rich proteins in multicellular eukaryotes.

*Identification of highly connected network hubs*

Given that the most connected proteins in network are the most important for cellular survival (54), we used betweenness centrality analysis to identify those network hubs for murine PPP- or PPG-rich proteins, respectively. Betweenness centrality describes the level of control that one node exerts over the interaction of other nodes in a network (55). Information about the betweenness centrality score, for each protein in the global networks for murine PPP- or PPG-rich proteins
is available in Supplementary Table 3 and 4. The ten highest scoring proteins are listed in Table 3. Furthermore, we performed literature search for known phenotypes associated with a knockout of these proteins in M. musculus (Table 3). These data suggested that the observed phenotypes in Dhs and Dohh knockout animals are based on might related to dysregulated translation of these proteins after inhibition of the hypusine modification.

**DISCUSSION**

The highly specific and conserved post translational hypusine modification system has a crucial function in translation regulation (9,11), and aberrant activation or inhibition is implicated in disparate disorders like cancer and infectious diseases. Notably, hypusine modification of eIF-5A displays an attractive platform for therapeutic intervention. First proof-of-principal studies have already shown promising results in that direction (17,56-58). However, this modification occurs in all eukaryotic cells and has been shown to be essential for proliferation of lower eukaryotes and mammalian cell lines (59). As these data have been mostly generated in yeast and in vitro, they do not entirely reflect the in vivo situation in mammals. Not surprisingly, there are concerns about whether such a crucial modification can be safely targeted by drugs. From a clinical point of view, further preclinical studies analyzing the biological relevance of hypusinated eIF-5A are needed to evaluate the hypusine axis as a drug-target and to predict possible side effects. The well-known observation that approximately 20-30% of all new therapeutic strategies fail in early clinical trials due to unexpected safety concerns clearly emphasizes that need (60).

Given that mouse models represent an appropriate tool to predict possible side effects (61), we generated several conditional knockout mouse models and here present the first comprehensive analysis of the biological consequences of the inhibition of the hypusine system in adult mammals (summarized in Fig. 10). Based on our results, we propose that particularly the isoform eIF-5A2 represents a promising target for the treatment of malignant tumors. In contrast, inhibition of the hypusine-mediating enzymes (i.e. DHS and DOHH) seems to have a smaller therapeutic index, but nevertheless is tolerated up to the level of haploinsufficiency. Therefore, they might be targeted without relevant side effects in a pathological condition where cells depend on an increased rate of hypusine modification, compared to normal cells.

Of note, we observed drastic effects upon a complete deletion of either Dhs or Dohh. This is in line with a number of previous observations, indicating that proliferation of eukaryotic cells depends on accurate modification of eIF-5A1. One possible explanation for the strong embryonic and adult phenotype observed after deletion of Dhs and Dohh is that insufficient hypusine modification of eIF-5A1 affects the correct translation of a huge number of proline-repeat-rich proteins on the level of single proteins and in terms of protein complexes. We have shown here that murine proline-repeat-rich proteins are organized in networks with an increasing connectivity and complexity in multicellular eukaryotes compared to yeast. In fact, recent data suggest that control of phenotypes is partially regulated by multi-protein complexes rather than by single genes (62,63) and that protein complexes can be used to predict phenotypic effects (64). We believe that disruption of these complexes causes at least some of the knockout phenotypes described in this study. Differences in biological processes enriched in protein complexes from M. musculus compared to S. cerevisiae may reflect the fact that in higher eukaryotes the hypusine modification is involved in more complex cellular function and that both hypusine modifying enzymes are essential. In addition, centrality analysis of global murine networks revealed highly connected hubs (single highly connected proteins) in the networks with crucial biological function for the development and viability. Literature analysis uncovers that a knockdown of most of the ten highest scoring network hubs lead to embryonic lethality in mice caused by diverse defects (Table 3). Strikingly, dysregulation of each of the top three proteins (Abi1, Crebp, and Notch1) is associated with defects in hematopoiesis (65-67). This meets our observation of reduced cellularity in bone marrow and spleen after Dhs depletion, indicating a severe defect in hematopoiesis as a consequence of reduced hypusine modification. Together, the observed phenotypes of Dhs and Dohh knockout animals
can be interpreted as a synergistic effect of functional loss of single essential proteins as well as multi-protein complexes due to inhibition of the hypusine modification of eIF-5A. Further in vitro and in vivo studies are needed to prove the functional relevance of these bioinformatics-driven results. Furthermore, since DHS and DOHH might have other cellular targets than eIF-5A, we cannot entirely exclude hypusine-independent effects after deletion of Dhs and Dohh. Studies using mutated hypusine-deficient eIF-5A (eIF-5A-K50 mutant) will be needed to further address this question.

Interestingly, the knockout of Dohh, although lethal, exerted a lower phenotype penetrance compared to the depletion of Dhs, most likely owing to the different modification states of the resulting non-hypusinated eIF-5A1. In contrast to fully hypusinated eIF-5A1 which is localized in the cytoplasm where it regulates translation of specific proteins (11), the observed unhypusinated acetylated and non-acetylated eIF-5A forms in the Dhs and Dohh deficient setting, respectively, seem to be located in the nucleus. Therefore, we assume that the phenotypical differences in Dhs- and Dohh-knockout animals are caused by yet unknown distinct nuclear functions of the acetylated and the non-acetylated unhypusinated eIF-5A1 precursor. This observation points to modification-dependent, translation-independent functions of the non-hypusinated eIF-5A as it has been demonstrated for other translational factors or ribosomal proteins (68,69). However, the molecular functions of the differently modified non-hypusinated eIF-5A1 forms have to be elucidated. As current studies highlight that eIF-5A2 has additional transcriptional activity (24), it can be assumed that eIF-5A1 might have similar nuclear activity, which is responsible for its strong phenotype.

It is common knowledge that tumor cells accelerate their translational activity to adapt to the increasing cellular demands (2). Accordingly, previous work unveiled that the hypusine modification system is frequently overexpressed in cancer tissue (16,17,70), supporting the hypothesis that eIF-5A facilitates translation of genes with tumor promoting activity as described for other translation factors (71). Indeed, certain oncogenes like c-Abl contain proline stretches and should therefore be translationally regulated by eIF-5A1. Furthermore, our studies on glioblastoma cell lines have shown a higher sensitivity of tumor cells against inhibition of hypusine synthesis compared to normal human astrocytes (16), suggesting that certain malignant cells depend on an activated hypusine-dependent translation that is above the activation level in normal cells. An intriguing observation made using by another tumor model is that haploinsufficiency of ribosomal proteins attenuates Myc-dependent malignant transformation without affecting normal cells (72). This clearly highlights the dependence of tumor cells on elevated translational activity. Therefore, we propose that a pharmacological inhibition of the hypusine-modifying enzymes below a level that is required for tumor cell proliferation and disease progression might represent a reliable therapeutic intervention. Interestingly, the concept of submaximal inhibition of hypusine synthesis has been suggested recently for inflammatory diseases (44). However, further studies to define the correct therapeutic index are needed to prevent devastating effects on normal homeostasis. Given the less pronounced phenotype of DOHH depletion, an inhibition of the second step of hypusine modification might represent the preferential target. In that regard, recent studies have demonstrated antiproliferative effects of DOHH inhibition in cancer cells (17,70,73).

In contrast to eIF-5A1, the expression of eIF-5A2 is limited to tissue such as testis and few parts of the adult brain (52), but is highly abundant in cancers such as ovarian, lung and melanoma (74-76). The eIF-5A2 gene is located on 3q26, a chromosomal region that is frequently amplified in cancer. Moreover, the expression of eIF-5A2 in those entities correlates with survival (77,78), disease stage as well as metastasis (79), suggesting that eIF-5A2 function is crucial for tumor development and maintenance, but not for normal tissue homeostasis. As eIF-5A2 shares almost 84% homology with eIF-5A1, it can be speculated that eIF-5A2 is also implicated in translation elongation and responsible for the selective transport and translation of specific mRNA subsets. This is supported by the observation that eIF-5A2 is detectable in the riboproteome of prostate cancer cells compared to normal prostate cells (80).
However, while eIF-5A1 was shown to preferentially regulate the translation of proline-rich proteins (11), the spectrum may be different for eIF-5A2. Furthermore, there are data that propose that eIF-5A2 might also control transcriptional processes in the nucleus, yet more studies are necessary to address this question (24). In this regard, Zender and coworkers have shown that nuclear eIF-5A2 exerts oncogenic activity (21). Therefore, it is conceivable that the two isoforms differ in their function. This hypothesis is supported by our previous observation that the protein interaction networks of both isoforms showing isoform-specific protein-protein interaction patterns (81). While eIF-5A1 seems to control basic cellular processes, eIF-5A2 is involved rather in disease regulation and essential during tumorigenesis, making it more attractive as a therapeutic target. Encouraging reports in direction of isoform-specific drug targeting have been described for other essential genes. Muller and coworkers introduced the concept of collateral vulnerability for the inhibition of enolase 2 in glioblastoma cells (82). While targeting enolase 2 in tumor cells inhibited cell growth, normal astrocytes were protected by the expression of enolase 1. Other promising examples for isoform-specific tumor targeting have been demonstrated for pyruvate kinase M (83) and phosphoinositide-3 kinase (84).

Indeed, recent data from in vitro studies support the hypothesis that eIF-5A2 has oncogenic potential (21,22). In particular, inhibition of eIF-5A2 by shRNA blocked invasion in a melanoma cell line and inhibited growth of ovarian cancer (22,76). Moreover, foci-formation capacity was markedly reduced in liver cancer cells transfected with eIF-5A2 shRNA (21). Intriguingly, in terms of oncogenic activity the presence of eIF-5A1 in these cancer cells could not compensate for the loss of the second isoform. This leads to the assumption that certain tumor cells are dependent on functional eIF-5A2 and the specific interference with eIF-5A2 activity displays an effective strategy to block proliferation and invasion in eIF-5A2-overexpressing cancers.Remarkably, we found that knockout of eIF-5A2 is not lethal in adult mice, and that animals do not show any conspicuous phenotype, suggesting that the expected toxic effects may be quite low in normal tissues after eIF-5A2 interference. This finding differs from data from C. elegans where phenotypical alterations after deletion of both isoforms were shown (85). Nevertheless, the exact role of 5A2 still needs to be elucidated. This research will be of great interest to address eIF-5A2’s detailed molecular function and may enable therapeutic targeting of downstream molecules or pathways in eIF-5A2-dependent tumor entities.

Together, our knockout mouse models have revealed important aspects of the biology of the hypusine modification system in mammals and constitute a useful resource for further investigation into the molecular function of the hypusine modification under normal and pathological conditions. Furthermore, our data suggest that the hypusine biosynthesis can be harnessed for therapeutic intervention. Even more intriguing is the observation that the cancer-related isoform eIF-5A2 is not crucial for normal homeostasis in mammals. Therefore, we propose that specific inhibitors of eIF-5A2 will exhibit selective toxicity towards malignant cells, making them promising therapeutic targets in the treatment of eIF-5A2-dependent tumors.

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FOOTNOTES

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The abbreviations used are: eIF-5A, eukaryotic initiation factor 5A; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase; EF-P, elongation factor P; Lys, lysine; 4-OHT, 4-hydroxytamoxifen; MEF, mouse embryonic fibroblasts; 1-D, 1-dimensional; 2-D, 2-dimensional; Esr1, estrogen receptor 1; CAG, CMV early enhancer/chicken beta actin promoter;

FIGURE LEGENDS

**Figure 1.** Overview of the hypusine modification and experimental design to study its role in mammals. (A) Schematic representation of hypusine synthesis in eIF-5A catalyzed by the DHS and DOHH enzymes. Red numbers in brackets indicate the knockout strategy for the respective gene as outlined in panel B. Lys: lysine; Dhp: deoxyhypusine; Hyp: hypusine. (B) Conditional knockout strategy using two Cre-deleter mouse strains for either an early constitutive(1) and/or a 4-OHT-inducible knockout of the respective gene. Cre mouse strains: (1)=B6.C-Tg(CMV-cre)1Cgn/J; (2)=B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J113. (C) Time schedule for the 4-OHT-inducible knockout(2) of the genes outlined in panel B.

**Figure 2.** Generation of a conditional Dhs knockout mouse strain using ES-cell clones harboring a targeted mutation of the Dhs gene. (A) Schematic representation of the strategy for achieving a knockout of the Dhs gene. The seven exons designed for deletion are depicted in a lighter color. Blue arrows indicate regions encoding amino acids that are essential for binding of NAD, green arrows indicate regions essential for binding of spermidine (86). The targeted mutation of the Dhs gene consists of a lacZ-reporter cassette (SA: splice acceptor, IRES: internal ribosome entry site, lacZ: β-galactosidase gene, pA: polyadenylation signal), a neomycin-selection cassette (Pneo: eukaryotic β-actin promotor, neo2: neomycin resistance gene), three loxp and two FRT sites. (B) Genotyping strategy for Cre-mediated knockout of the Dhs gene. Animals harboring the floxed Dhs gene were mated to Cre-deleter mice (B6.C-Tg(CMV-cre)1Cgn/J strain or B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J113 strain plus oral 4-OHT administration). Usage of three oligonucleotides (Dhs-3’-arm, Dhs-for, Dhs-rev) allows simultaneous detection of all possible alleles (+, p, -), including the deleted allele (-), which is achieved by Cre-mediated recombination.

**Figure 3.** Homozygous deletion of Dhs is embryonically lethal in mice while heterozygous Dhs knockout animals are viable and show compensatory upregulation of the hypusine system. (A) Representative litter size of the indicated breeding. Note that no Dhs+/− pups were born after Dhs+/− x Dhs+/− breeding. (B-D) mRNA levels of Dhs, Dohh and eIF-5A1 in different organs of wildtype or heterozygous mice as assessed by quantitative real-time PCR. Expression values were plotted relative to the expression of the housekeeping gene 18S rRNA. (E-F) Expression of eIF-5A1 protein in different organs using Western blot. Protein expression was quantified relative to the loading control alpha-Tubulin using the Odyssey Infrared Imaging System. (G) 2-dimensional Western blot detecting
Genetic manipulation of the hypusine modification system in mammalians different eIF-5A1 forms in the outlined organs. (H-I) Analysis of mouse embryonic fibroblasts (MEFs) isolated from Dhs-deficient Dhs<sup>+/−</sup> and Dhs<sup>−/−</sup>-CAG-cre/Esr1<sup>−/−</sup> mice as well as wildtype Dhs<sup>+/+</sup>; CAG-cre/Esr1<sup>−/−</sup> animals after 4-OHT treatment in vitro. (H) mRNA expression status of Dhs after 100 nM 4-OHT-treatment at the indicated time points relative to the housekeeping gene 18S as assessed by quantitative real-time PCR. (I) 2-dimensional Western blot for eIF-5A1 after treatment with 4-OHT (100 nM; 8 days) or incubation with 50 mM GC7 (2 days). Significances were calculated using the unpaired t-test and marked with an asterisk if significant (**: p<0.001; ***: p<0.01; *: p<0.05). : wildtype allele, : deleted allele. Colored arrows in the representative blots correspond to the different eIF-5A1 forms as outlined in the schematic plot on the left: black = fully hypusinated Lys<sup>50</sup>; pH 5.2; red = unmodified Lys<sup>50</sup>, pH 5.1; blue = unmodified Lys<sup>50</sup> plus acetylated Lys<sup>57</sup>; pH 5.0.

Figure 4. Loss of Dhs confers lethality in adult mice and affects organ integrity. (A)-(E) Analysis of adult Dhs<sup>+/+</sup>, Dhs<sup>−/−</sup> and Dhs<sup>−/−</sup>-CAG-cre/Esr1<sup>−/−</sup>-positive mice after 4-OHT administration resulting from a breeding according to the scheme in panel B-C of Figure 1. (A) Bodyweight of animals after 4-OHT-mediated knockout in vivo at the indicated time points. Red arrow indicates duration of 4-OHT treatment. (B) Kaplan-Meier plot showing overall survival of mice after 4-OHT-mediated knockout in vivo at the indicated time points. Red arrow indicates duration of 4-OHT treatment. (C) 2-dimensional Western blot for eIF-5A1 after 4-OHT treatment in vivo. Heart and spleen were isolated from mice showing more than 20% weight loss. Colored arrows in the representative blots correspond to the different eIF-5A1 forms as outlined in the schematic plot on the left: black = fully hypusinated Lys<sup>50</sup>, pH 5.2; red = unmodified Lys<sup>50</sup>, pH 5.1; blue = unmodified Lys<sup>50</sup> plus acetylated Lys<sup>57</sup>; pH 5.0. (D) Quantitative real-time PCR assessing the mRNA expression status of Dhs, Dohh and eIF-5A1 in several tissues after 4-OHT treatment in vivo expressed relative to the housekeeping gene 18S rRNA. Organs were isolated from animals showing more than 20% weight loss. (E) Comprehensive histopathological analysis of various mouse organs after 4-OHT-mediated knockout in vivo. Organs were isolated from animals showing more than 20% weight loss. Staining with methylene blue or H&E showed reduced bone marrow cellularity (4/4 animals) and an impaired cellularity of the red pulp in the spleen (4/4 animals), respectively, in Dhs-deficient animals. Turnbull staining unveiled enrichment of ferrous iron in the kidney of Dhs-deficient mice (3/4 animals). Epithelium of the intestine was severely damaged in Dhs-deficient animals as demonstrated by H&E (1/4 animals). Significances were calculated using the unpaired t-test and marked with an asterisk if significant (**: p<0.001; ***: p<0.01; *: p<0.05). : wildtype allele, : floxed allele, n.d.: not detectable.

Figure 5. Dohh-mediated hypusination of eIF-5A1 is essential in adult mice. (A-D) Analysis of adult Dohh<sup>+/+</sup>, Dohh<sup>−/−</sup> and Dohh<sup>−/−</sup>-CAG-cre/Esr1<sup>−/−</sup>-positive mice after 4-OHT administration resulting from a breeding according to the scheme in Fig. 1(B-C). (A) Bodyweight of mice after 4-OHT-mediated knockout in vivo at the indicated time points. Red arrow indicates duration of 4-OHT treatment. (B) Kaplan-Meier plot showing overall survival of mice after 4-OHT-mediated knockout in vivo at the indicated time points. Red arrow indicates duration of 4-OHT treatment. (C-D) Real-time PCR assessing the mRNA expression status of Dohh and eIF-5A1 in spleen and kidney after 4-OHT treatment in vivo expressed relative to the housekeeping gene 18S rRNA. Organs were isolated from animals showing more than 20% weight loss.

Figure 6. Loss of Dohh reveals histological anomalies. (A) 2-dimensional Western blot for eIF-5A1 in spleen tissue after 4-OHT treatment in vivo. Spleen was isolated from mice showing more than 20% weight loss. Colored arrows in the representative blots correspond to the different eIF-5A1 forms as outlined in the schematic plot in Fig. 4(C): black = fully hypusinated Lys<sup>50</sup>, pH 5.2; red = unmodified Lys<sup>50</sup>, pH 5.1. (B) H&E staining of kidney sections after 4-OHT induction in vivo unveiled tubulus necrosis in one of the Dohh-deficient mice (1/4 animals; red arrow). Organs were isolated from animals showing more than 20% weight loss. (C) Methylene blue staining revealed no overt change in cellular composition of the bone marrow. (D) Histological analysis of the spleen using H&E staining did not show any major difference. Turnbull staining unveiled enrichment in ferrous iron in Dohh-deficient mice (2/2 animals). (E-F) H&E, Ki67 and caspase-3 staining of liver tissue compared to control in 5-7 weeks (E) and 6-7 month old mice (F). Red arrow indicates liver necrosis and green arrow indicates focal inflammation.
Figure 7. Knockdown of the hypusine modification enzymes affect localization and posttranslational modification of eIF-5A1. (A) Immunofluorescence for endogenous eIF-5A1 in a Dhs or Dohh-deficient cellular background, respectively. Upper panels depicts eIF-5A1 fluorescence alone, the lower panels show the overlay of the eIF-5A1 signal (green) and the Hoechst 33342 nuclear DNA stain (blue). Primary mouse embryonic fibroblasts (MEFs) from DhsR5C: CAG-cre/Esr1+ mice (left) and immortalized MEFs (3T3) from DohhR5C: CAG-cre/Esr1+ animals (right) were treated with or without 4-OHT (100 nM; 7 days) in vitro to induce the respective knockout. Red arrows indicate nuclear accumulation of eIF-5A1. (B) Predicted model explaining how loss of Dhs and Dohh could influence eIF-5A1’s modification.

Figure 8. Generation of a conditional eIF-5A2 knockout mouse strain using an ES-cell clone harboring a targeted mutation of the eIF-5A2 gene. (A) Schematic representation of the knockout strategy for achieving a conditional knockout of the eIF-5A2 gene. The two exons designed for deletion are depicted in a lighter color. Note that this region encodes the translational start point and the critical hypusine modification residue Lys30. The targeted gene of the eIF-5A2 gene consists of a lacZ-reporter cassette (SA: splice acceptor, IRES: internal ribosome entry site, lacZ: ß-galactosidase gene, pA: polyadenylation signal), a neomycin-selection cassette (PAct: eukaryotic ß-actin promoter, neoR: neomycin resistance gene), three loxP and two FRT sites. (B) Genotyping strategy for Cre-mediated knockout of the eIF-5A2 gene. Mice harboring the floxed eIF-5A2 gene were mated to Cre-deleter mice (B6.C-Tg(CMV-cre)1Cgn/J strain). Usage of three oligonucleotides (eIF-5A2-3′-arm, eIF-5A2-5′-arm, eIF-5A2-rev) allows detection of all possible alleles (+, p: green arrow), including the deleted allele (−: red arrow), which is achieved by Cre-mediated recombination.

Figure 9. eIF-5A2 knockout mice are viable and fertile. (A) Offspring analysis of the indicated breedings. (B) Kaplan-Meier plot showing overall survival of mice at the indicated time points after birth. (C) mRNA levels of eIF-5A2 in different tissues of wildtype mice as assessed by quantitative real-time PCR using the TissueScan qPCR Array MDRT101 (OriGene Technologies, Rockville, MD). Values were normalized to the expression of the housekeeping gene Gapdh and depicted as fold changes with bone marrow as the reference. (D) mRNA status of eIF-5A2 in different organs of mice as assessed by quantitative real-time PCR. Expression values were plotted relative to the expression of the housekeeping gene β-Actin. Expression values of eIF-5A2 in testis and brain of eIF-5A2-5A2 mice were normalized to Gapdh (P<0.001; *: p<0.05).

Figure 10. Summary of observed phenotypes after genetic manipulation of the hypusine modification system. Whereas a heterozygous knockout of any of the genes of the hypusine modification system does not affect the viability of mice, a homozygous deletion of Dhs, Dohh or eIF-5A1 causes lethality in embryonic and adult mice with different penetrance. In contrast, the cancer-associated isoform eIF-5A2 is dispensable for normal development and viability.

Figure 11. PPP- and PPG-rich proteins form functional networks in yeast. STRING-network analysis for yeast (Saccharomyces cerevisiae) genes encoding >1 PPP units (A) or >1 PPG units (B). The complete protein-protein-interaction networks are depicted in the center of (A) and (B), respectively. Closely connected clusters were directly extracted from each network using the MCODE algorithm. The highest-scoring clusters are represented around the whole networks for genes encoding > 1 PPP units (A) or > 1 PPG units (B). For each cluster the highest-significant GO biological process based on BinGO analysis is indicated. Additional information for complete networks and for all clusters is available in Supplementary Table 1 and 2.

Figure 12. Murine PPP- and PPG-rich proteins are organized in highly interconnected networks. STRING-network analysis for murine (Mus musculus) genes encoding >1 PPP units (A) or >1 PPG units (B). The complete protein-protein-interaction networks are depicted in the center of (A) and (B),
respectively. Closely connected clusters were directly extracted from each network using the MCODE algorithm. The ten highest-scoring clusters are represented around the whole networks for genes encoding >1 PPP units (A) or >1 PPG units (B). For each cluster the highest-significant GO biological process based on BinGO analysis is indicated. Additional information for complete networks and for all clusters is available in Supplementary Table 3 and 4.
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Table 1. Oligonucleotides used in this study

| Application | Oligonucleotide | Sequence / Qiagen QuantiTect Number |
|-------------|----------------|-----------------------------------|
| Long range PCR | 3’ Universal | CAC ACC TCC CCC TGA ACC TGA AAC |
| | 3’ Dhs Gene Specific | CAG GTT CTA TCG ATT CCA GTG TCC G |
| | 3’ Dhs Gene Specific | GTG GCC ACG GCT ACG AAG TGC TAG |
| | 3’ eIF-5A2 Gene Specific | GAT GAC TGC TGT GTG GAA TAG TAT CAT CTG |
| | 3’ eIF-5A2 Gene Specific | GAG GAG GAC CAT GAG ATG GTG AGG ACA TG |
| Genotyping | Dhs-for | CCT CTG CCC TCT CAC AGA CCT GCG |
| | Dhs-5'-arm | AGA GCG CCC AGG TCC AAG GCT ACG |
| | Dhs-3'-arm | AGC GTT AAG CTC CTA CAA AGC |
| | Dhs-rev | ACC ATC CGC AGG AGA CCA CAC CTA |
| | eIF-5A2-5'-arm | AGC GAT GCT TGG GAA CTG GAG G |
| | eIF-5A2-3'-arm | TGG AGT ATA CTT TGC CAT TCA GGC C |
| | eIF-5A2-rev | AAG GCC AGC CTG AGA CCT AT |
| Quantitative PCR | 18S-rRNA | QT01036875 |
| | Dhs | QT02529093 |
| | Dohh | QT00163268 |
| | eIF-5A1 | QT01757861 |
| | eIF-5A2 | QT00148225 |
| | Dhs del fwd | TTC AAC CGC GGC GTA GAT TA |
| | Dhs del rev | TCT GCT CCA TTC CTC ATG GC |
Table 2. PPP- and PPG-repeat-rich proteins showing different network topology in yeast and mouse. Overview of network parameters for protein-protein-interaction networks of murine and yeast proteins containing >1 PPP- or >1 PPG units.

| Network parameters | Saccharomyces cerevisiae | Mus musculus |
|--------------------|--------------------------|--------------|
|                    | >1 PPP units | > 1 PPG units | > 1 PPP units | > 1 PPG units |
| number of proteins* used for network construction in STRING | 76 | 14 | 1523 | 913 |
| number of nodes | 70 | 11 | 1256 | 710 |
| clustering coefficient | 0.392 | 0.136 | 0.238 | 0.251 |
| avg. number of neighbors (avg. degree) | 7.0 | 2 | 11.1 | 9.5 |
| network heterogeneity | 0.87 | 0.43 | 1.28 | 1.32 |

*a extracted from (35)
Table 3. Summary of the ten highest connected proteins (hubs) in networks from murine proteins containing >1 PPP- or >1PPG units and known knockout phenotype of the genes found in the literature.

| gene name | knockout phenotype |
|-----------|--------------------|
| Abl1      | Neonatal lethality (approx.75%); thymic and splenic atrophy, T and B cell lymphopenia (65) |
| Crebbp    | Embryonic lethality (E9-10.5); defects in hemopoiesis, blood vessel formation, and neural tube closure (66) |
| Notch1    | Embryonic lethality (before E11.5) (87), Neonatal induced KO: growth retardation, defect in thymocyte development (67) |
| Ep300     | Embryonic lethality (E9-11.5); defects in neurulation, cell proliferation, and heart development (88) |
| Abl2      | Viable; no hematopoietic abnormalities, reduced litter frequency, behavioural phenotypes (89) |
| Erbb2     | Embryonic lethality (E10.5); defects in cardiac and neural development (90) |
| Ptpn23    | Embryonic lethality (E9.5) (91) |
| Erbb4     | Embryonic lethality (E11); cardiac defects, alterations in hindbrain development (92) |
| Jund      | Viable; reduced postnatal growth, defects in male reproduction (93) |
| Smarca4   | Embryonic lethality (before E6.5) (94) |
| Abl1      | Neonatal lethality (approx.75%); thymic and splenic atrophy, T and B cell lymphopenia (65) |
| Notch1    | Embryonic lethality (before E11.5) (87), Neonatal induced KO: growth retardation, defect in thymocyte development (67) |
| Jund      | Viable; reduced postnatal growth, defects in male reproduction (93) |
| Mapk7     | Embryonic lethality (E9.5-10.5); abnormal embryonic cardiac and vascular development (95) |
| Ep300     | Embryonic lethality (E9-11.5); defects in neurulation, cell proliferation, and heart development (88) |
| Ddhx8     | No information for knockout mice available |
| Crebbp    | Embryonic lethality (E9-10.5); defects in hemopoiesis, blood vessel formation, and neural tube closure (66) |
| Cad       | No information for knockout mice available |
| Smarca4   | Embryonic lethality (before E6.5) (94) |
| Ptpn23    | Embryonic lethality (E9.5) (91) |
A

Gene function analysis in vivo using two Cre-mediated knockout strategies

| Gene | knockou strategy | timepoint | cross |
|------|-----------------|-----------|-------|
| Dhs  | (1) constitutive Cre-ko | germline | floxed dhs mice x CMV-cre animals |
|      | (2) tamoxifen-inducible Cre-ko | adult | floxed dhs mice x CAG-cre/Esr1+ animals |
| Dohh | (2) tamoxifen-inducible Cre-ko | adult | floxed dohh mice x CAG-cre/Esr1+ animals |
| eIF-5A2 | (1) constitutive Cre-ko | germline | floxed eIF-5A2 mice x CMV-cre animals |

B

C

Time schedule for (2)

* phenotype-depending

Figure 1
Figure 3
Figure 4

**A**

Graph showing relative body weight over weeks for different genotypes: Dhs+/+, Dhs+/p, and Dhs+/p. Bars indicate standard deviation. 4-OHT treatment is marked.

**B**

Graph showing survival (%) over days for different genotypes: Dhs+/+, Dhs+/p, and Dhs+/p. n = 6 for each group, p = 0.0005.

**C**

Diagram showing pH and protein levels for different tissues: heart, spleen, and bone marrow. 4-OHT and eIF-5A1 are marked.

**D**

Bar charts showing relative mRNA expression for Dhs, Dhs (truncated), Dohh, and eIF-5A1 in different tissues: gut, testis, heart, and kidney. qPCR results are shown for each tissue.

**E**

Images showing histological staining for different tissues: bone marrow, spleen, kidney, and gut. Staining methods include methylene blue, H&E, and turnbull.

**Figure 4**
**Figure 5**

(A) Graph showing relative bodyweight over time (weeks) for different genotypes: Dohh\(^{+/+}\), Dohh\(^{+/p}\), and Dohh\(^{p/p}\). 4-OHT treatment is indicated.

(B) Survival graph with time (days) on the x-axis and survival (%) on the y-axis. Dohh\(^{+/+}\), Dohh\(^{+/p}\), and Dohh\(^{p/p}\) genotypes are shown, with significant differences indicated (\(* p < 0.05\), *** p < 0.0001\).

(C) qPCR analysis for Dohh expression in spleen and kidney for different genotypes: +/+ and +/p are shown.

(D) qPCR analysis for eIF-5A1 expression in spleen and kidney for different genotypes: +/+ and +/p are shown.
Figure 6
Effect of Dhs/Dohh knockout on eIF-5A modification

Figure 7
**Figure 8**

A. **eIF-5A2 exon structure**

- Start (1)
- Stop (5)
- Lys^{50}

Modified eIF-5A2

- lacZ-reporter
- neo-resistance

Floxed eIF-5A2 (p)

- FLP

Deleted eIF-5A2 (-)

B. **eIF-5A2 exon structure**

- 5'-arm
- 3'-arm
- rev

- 500 bp
- 400 bp

568 bp
403 bp
381 bp
Figure 9
| Gene    | knockout | embryonic     | adult          |
|---------|----------|---------------|----------------|
| **Dhs** | * / **   | heterozygous  | viable         |
|         |          | homozygous    | lethal (d<6.5) |
|         |          |               | viable         |
|         |          |               | lethal (d5-14 ***)) |
| **Dohh**| ****     | heterozygous  | viable         |
|         |          | homozygous    | lethal (d<9.5) |
|         |          |               | viable         |
|         |          |               | lethal (d8-35 ***)) |
| **eIF-5A1** | *     | heterozygous  | viable         |
|         |          | homozygous    | lethal (d<7.5) |
|         |          |               | unknown        |
|         |          |               | unknown        |
| **eIF-5A2** |       | heterozygous  | viable         |
|         |          | homozygous    | viable         |
|         |          |               | viable         |

* Nishimura et al. 2011, ** Templin et al. 2011, *** post cre-induction **** Sievert et al. 2014
Figure 11

A  yeast proteins > 1 PPP units

B  yeast proteins > 1 PPG units

cluster 1
actin cytoskeleton organization

cluster 2
COPII-coted vesicle budding

cluster 3
mRNA metabolic process

cluster 4
regulation of transcription

Figure 11
Figure 12
Protein Synthesis and Degradation: Biological Relevance and Therapeutic Potential of the Hypusine Modification System

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