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eEF1A2 exacerbated insulin resistance in male skeletal muscle via PKC β and ER stress

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Abstract

Recent studies raise the possibility that eukaryotic translation elongation factor 1 alpha (eEF1A) may play a role in metabolism. One isoform, eEF1A2, is specifically expressed in skeletal muscle, heart and brain. It regulates translation elongation and signal transduction. Nonetheless, eEF1A2's function in skeletal muscle glucose metabolism remains unclear. In the present study, suppression subtractive hybridisation showed a decrease in *Eef1a2* transcripts in the skeletal muscle of diabetic Mongolian gerbils. This was confirmed at mRNA and protein levels in hyperglycaemic gerbils, and in db/db and high-fat diet-fed mice. Further, this downregulation was independent of *Eef1a2* promoter methylation. Interestingly, adeno-associated virus-mediated eEF1A2 overexpression in skeletal muscle aggravated fasting hyperglycaemia, hyperinsulinaemia and glucose intolerance in male diabetic gerbils but not in female gerbil models. The overexpression of eEF1A2 in skeletal muscle also resulted in promoted serum glucose levels and insulin resistance in male db/db mice. Up- and downregulation of eEF1A2 by lentiviral vector transfection confirmed its inhibitory effect on insulin-stimulated glucose uptake and signalling transduction in C2C12 myotubes with palmitate (PA)-induced insulin resistance. Furthermore, eEF1A2 bound PKC β and increased its activation in the cytoplasm, whereas suppression of PKC β by an inhibitor attenuated eEF1A2-mediated impairment of insulin sensitivity in insulin-resistant myotubes. Endoplasmic reticulum (ER) stress was elevated by eEF1A2, whereas suppression of ER stress or JNK partially restored insulin sensitivity in PA-treated myotubes. Additionally, eEF1A2 inhibited lipogenesis and lipid utilisation in insulin-resistant skeletal muscle. Collectively, we demonstrated that eEF1A2 exacerbates insulin resistance in male murine skeletal muscle via PKC β and ER stress.

Key Words

- ▶ eEF1A2
- ▶ insulin resistance
- ▶ skeletal muscle
- ▶ PKC β
- ▶ ER stress

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Introduction

As a major metabolic tissue, skeletal muscle constitutes 40% of the total body mass and plays a predominant role in postprandial glucose disposal in humans (DeFronzo

et al. 1981). Insulin resistance in skeletal muscle is an initiating defect and a hallmark of obesity and type 2 diabetes mellitus (T2DM) (DeFronzo & Tripathy 2009).

However, the pathogenesis of insulin resistance in skeletal muscle, especially essential genes involved in the process, is poorly understood.

Selection of differentially expressed (DE) genes by utilising a suitable diabetic animal model provides a convenient approach to explore novel players in diabetes. Previously, we developed a diabetic Mongolian gerbil line that spontaneously presented with hyperglycaemia, impaired glucose tolerance and diabetic pathophysiological lesions (Li *et al.* 2016). Using suppression subtractive hybridisation (SSH), eukaryotic translation elongation factor 1 alpha (*Eef1a2*) was selected as one of the DE genes in the skeletal muscle of diabetic gerbils, as detailed below.

eEF1A is a highly conserved GTPase that recruits aminoacylated tRNA to the ribosomal A-site during translation elongation. It has two isoforms in mammals: eEF1A1 and eEF1A2. Under physiological conditions, the expression of eEF1A2 is limited to the skeletal muscle, heart and brain, whereas eEF1A1 is widely expressed in other tissues (Abbas *et al.* 2015). The *in vitro* study indicates an indistinguishable activity of the two isoforms in translation elongation (Kahns *et al.* 1998). Of note, eEF1A is also implicated in non-translational functions. Likely due to the different spatial organisation and dimer-forming ability (Soares & Abbott 2013), the two isoforms play different non-translational roles in many cellular events. For instance, eEF1A1 is involved in virus replication, proteolysis and nuclear transport (Mateyak & Kinzy 2010), while as a putative oncogene, most studies of eEF1A2 have been focussed on cancer. eEF1A2 mediates anti-apoptotic mechanisms, proliferation, and cytoskeleton remodelling, by directly or indirectly interacting with multiple kinases or factors, such as phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt), protein kinase C β (PKC β) and P16^{INK4a} (Amiri *et al.* 2007, Piazzini *et al.* 2010a, Lee *et al.* 2013). Recently, eEF1A1 was also reported to participate in endoplasmic reticulum (ER) stress-induced cell death in Chinese hamster ovary, H9c2 and HepG2 cells (Borradaile *et al.* 2006, Stoianov *et al.* 2015). Nevertheless, eEF1A2's function and its mechanisms of action in glucose homeostasis of skeletal muscle have not yet been investigated.

PKC β and ER stress are two well-known negative modulators of insulin signalling in skeletal muscle. As a member of the serine/threonine-protein kinase family, PKC β is dramatically upregulated in the skeletal muscle of obese and diabetic patients (Itani *et al.* 2000). PKC β -knockout mice exhibit resistance to HFD-induced obesity and show improvement in insulin sensitivity (Standaert *et al.* 1999, Huang *et al.* 2009). eEF1A2 can bind to

phospho-PKC β *in vitro* (Piazzini *et al.* 2010b), indicating a potential association between eEF1A2 and PKC β in skeletal muscle. In addition to hyperactivated PKC β , increased ER stress in the skeletal muscle is another typical characteristic of obese people, patients with T2DM, and HFD-fed mice (Koh *et al.* 2013, Bohnert *et al.* 2018). Knocking out the key ER stress gene, tribble3, in skeletal muscle or inhibiting ER stress via chemical chaperones can protect HFD-fed mice from insulin insensitivity (Ozcan *et al.* 2006, Koh *et al.* 2013). ER stress mediates insulin resistance in myotubes *in vitro* (Deldicque *et al.* 2012), through phosphorylation of inositol-requiring enzyme 1 α (IRE1 α)/c-Jun N-terminal kinase (JNK) (Urano *et al.* 2000). PKC β and ER stress are essential players in the insulin resistance of skeletal muscle. Yet, the upstream modulators of PKC β and ER stress are not fully characterised.

In the present study, we found that *Eef1a2* was downregulated in the skeletal muscle of hyperglycaemic Mongolian gerbils by SSH, which was confirmed in three insulin-resistant animal models. Male hyperglycaemic gerbils and db/db mice both showed aggravated insulin insensitivity after overexpression of eEF1A2 in skeletal muscle. In PA-induced insulin-resistant myotubes, eEF1A2 also impaired insulin sensitivity by enhancing PKC β activation and ER stress.

Materials and methods

Animals

While developing an inbred line of Mongolian gerbils, a spontaneous diabetic branch was identified (Li *et al.* 2016). In this study, 6-month-old diabetic and nondiabetic gerbils, 12-week-old male db/db mice (BKS.Cg-Dock7^m/+Lepr^{db}/Nju) and their control, C57BLKS/JNju mice (Model Animal Research Center of Nanjing University, China), were fed regular murine chow (Ke Ao Xie Li, China). Eight-week-old male C57BL/6J mice (Laboratory Animal Center of the Academy of Military Sciences, China) were given chow or the HFD (60% of total calorie intake) from HFK (China) for 20 weeks. The animals were maintained under standard laboratory conditions with free access to food and water. The study protocol was approved by the Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University (AEEI-2014-082 and AEEI-2017-032). Our study was performed in accordance with the 'Animal Research: Reporting of *In Vivo* Experiments' guidelines.

Construction and analysis of SSH libraries

The quadriceps femoris from two inbred gerbils of the same litter was isolated to build SSH libraries. We grouped gerbils into two categories: (1) gerbils with fasting blood glucose values (10.8 mM) and diabetic pathophysiological lesions and (2) gerbils with normal fasting blood glucose values (3.4 mM) and no pathophysiological lesions. Two subtracted libraries were constructed using the SSH kit. Briefly, after cDNA synthesis, digestion, hybridisation and amplification employing a PCR-Select cDNA Subtraction Kit (TaKaRa, Japan), subtractions were implemented using a five-fold excess amount of driver cDNA to tester cDNA. The products were ligated into T vector (TaKaRa), and DNA sequencing, BLAST and enrichment analysis were performed.

Metabolic phenotyping

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed (Gao *et al.* 2012). For OGTT, the animals were given D-glucose orally at 2 g/kg body weight after 16 h of fasting. For ITT, the mice were injected intraperitoneally with 0.75 IU/kg insulin (Novo Nordisk) after 4 h of fasting. The blood glucose values were measured using a glucometer (SANNUO, China). Fasting serum samples were collected to determine insulin levels using ELISA kits (Millipore), free fatty acid (FFA) levels using the Quantification kit (Abcam) and triglyceride levels using a fully automatic biochemical analyser (Olympus). The homeostatic model assessment for evaluating insulin resistance (HOMA-IR) of the animals was calculated as follows: (fasting insulin (mIU/L) × fasting glucose (mg/dL)/405) (Friedewald *et al.* 1972).

Quantitative RT-PCR (qRT-PCR)

The total RNA was extracted using TRIzol (Thermo Fisher). cDNA was reverse transcribed using FastQuant RT Kit (Tiangen, China). qRT-PCR was conducted to quantify mRNA levels using QuantiTect® SYBR Green PCR kits (Tiangen). The expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Primers are listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article).

Western blotting

Aliquots of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Pall, USA). After blocking, the membranes were incubated with primary antibodies (Supplementary Table 2). After incubation with

secondary antibodies (Cell Signaling), the immunoreactive bands were visualised using SuperSignal chemiluminescent detection system (Thermo Fisher). The anti-eEF1A2 antibody detects both eEF1A1 and eEF1A2. However, eEF1A1 is not expressed in adult skeletal muscle (Knudsen *et al.* 1993, Chambers *et al.* 1998). Therefore, the bands or the immunofluorescence staining detected in the skeletal muscle using the antibody should represent eEF1A2.

Immunofluorescence staining

The quadriceps femoris of fasting mice and myotubes were fixed in paraformaldehyde. After blocking, the slices or cells were incubated with primary antibodies (Supplementary Table 2). Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibodies (Invitrogen) were applied and incubated for 1 h. After staining with Hoechst 33342 (Sigma-Aldrich) and sealing in an anti-fade fluorescence mounting medium (Solarbio), and the images were captured.

Methylation analysis

The DNA was converted using the EZ DNA Methylation Kit (Zymo Research, USA). There was only one CpG island in the promoter region of *Eef1a2* according to our search using EMBOSS CpGplot (Supplementary Fig. 1). The CpG island was amplified by PCR with the following primers: 5'-aggaagagagAGGATTAGTTAGAAGGATTGGTGG-3', containing a 10mer-tag sequence and 5'-agtaatcagactcattataggagaaggctCAAAACCAACAATAAATACCCACAT-3', containing a T7-promoter tag. The PCR conditions were as follows: 95°C for 5 min; 45 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 90 s and 72°C for 5 min. After dephosphorylation of unincorporated dNTPs, the products were cleaved with RNase A and spotted onto SpectroCHIP (Sequenom, USA). The spectra were collected by MassARRAY MALDI-TOF mass spectrometry (Sequenom) and analysed using EpiTYPER v.1.0 software (Sequenom).

Adeno-associated virus vector (AAV) construction, injection, and detection

The AAV-2/9 vector was produced by Hanbio Biotechnology (China). Murine FLAG-tagged-*Eef1a2* cDNA was inserted into the AAV-CMV-MCS-ZsGreen plasmid. The vector was transfected into HEK293T cells along with the pAd-Helper and the pack2/9 packaging plasmids. The viral particles were collected by CsCl gradient centrifugation.

Diabetic gerbils were randomly distributed into four groups (3–4 males or females per group). Meanwhile, a total

of 14 male db/db mice, aged 12 weeks old, were subdivided randomly into two groups. The homology of eEF1A2 amino acid sequences between gerbils and mice was 99.8%; only one amino acid was different (Supplementary Fig. 2) (Li *et al.* 2017). The gerbils and db/db mice were injected with 1.3×10^{11} viral particles of AAV-ZsGreen or murine AAV-eEF1A2.

Magnetic resonance imaging (MRI)

The body composition of the db/db mice injected with AAV was analysed in live animals using PharmaScan 7.0/16 MRI Scanner (Bruker, Germany). Briefly, 24 transverse images of each mouse were acquired, and the absolute quantification of the fat depots was performed using ImageJ software (NIH, USA). Multiplying the area of each region of interest by slice thickness gave the volume. The adipose tissue volume was multiplied by a factor of 0.92 to convert the result into the adipose tissue mass.

Cell culture

The murine skeletal muscle cell line C2C12 (ATCC, USA; RRID: CVCL_0188; sex: female) was incubated in high-glucose (4.5 g/L) Dulbecco's modified Eagle's medium (Gibco) and supplemented with 15% of foetal calf serum (Gibco) in a humidified atmosphere containing 5% of CO₂ at 37°C. When 70% confluence was achieved, the cells were differentiated in 2% horse serum (Gibco) for 5 days. After deprivation, the fully differentiated myotubes were treated with PA for 16 h to set up insulin resistance. Then, the myotubes were treated or not treated with PKC β inhibitor LY333531, ER stress inhibitor 4-phenylbutyric acid (4-PBA), or JNK inhibitor SP600125. After 100 nM insulin stimulation for 15 min, the cells were collected for the subsequent experiments. PA, insulin and the inhibitors were all purchased from Sigma-Aldrich.

Lentiviral vector construction

Murine FLAG-tagged-*Eef1a2* cDNA was cloned into pCDH Expression Lentivector (CD511B-1, System Biosciences, USA). *Eef1a2* short hairpin RNA (shRNA) SH scrambled control (SHC): ATAATATCGGTCTTGAGTTCA, SH1: CCGAGACTTCATCAAGAATAT or SH2: GTCGGGTTCATCAAGAAT were cloned into the pSicoR vector. The expression constructs, psPAX2 and pMd2.G plasmids, were cotransfected into HEK293T cells to produce lentiviral vectors. The viral supernatants were collected by ultracentrifugation. After infection, C2C12

cells were isolated using an FACSCalibur cell sorter (BD Biosciences, USA). The homology between the mRNA coding regions of *Eef1a1* and *Eef1a2* is low in both mice and gerbils. The transfected FLAG-tagged-*Eef1a2* or shRNA was targeted therefore to *Eef1a2*, but not *Eef1a1*.

Glucose uptake analysis

After washes, myotubes were incubated in Krebs–Ringer phosphate buffer (pH 7.4) without glucose supplemented with 0.2% bovine serum albumin for 1 h. After insulin stimulation for 15 min, 37 kBq/ml [³H] 2-deoxy-D-glucose ([³H]-2DG; PerkinElmer) was added to the medium, with incubation for 10 min and the reaction was terminated by addition of 15 μ M cytochalasin B (Sigma-Aldrich). Nonspecific uptake was assessed by pre-incubation with cytochalasin B. After three washes, the cells were lysed. The aliquots were dissolved in 1 mL of a liquid scintillation cocktail, and their radioactivity was counted using a liquid scintillation spectrometer (Perkin Elmer).

Co-immunoprecipitation (Co-IP)

Co-IP was conducted using the Dynabeads® Protein G Immunoprecipitation Kit (Thermo Fisher). Briefly, 10 μ g of anti-FLAG antibodies (Sigma-Aldrich) was incubated with Dynabeads® Protein G for 1 h at room temperature. The Dynabead-antibody complex was added to 1 mg of protein lysates and incubated with rotation at 4°C overnight. After washing, eluting and denaturing, Western blotting was employed to detect relevant bands.

Statistical analysis

The hypergeometric test/Fisher's exact test and the false discovery rate correction method proposed by Benjamini and Hochberg were used in the enrichment analysis (Wu *et al.* 2006). Other data were analysed using *t*-test or one-way ANOVA and Bonferroni *post hoc* test (SPSS 16.0 software, SPSS). *P* < 0.05 was considered to be statistically significant.

Results

eEF1A2 was downregulated in diabetic murine skeletal muscle

To identify DE genes in diabetic skeletal muscle, SSH was initially performed in spontaneous diabetic Mongolian gerbils. Of the 49 genes identified from the DE libraries,

22 genes were upregulated and 27 genes were downregulated in the skeletal muscle of hyperglycaemic gerbils (Supplementary Table 3). Enrichment analysis of human orthologues of DE genes was performed and assessed using KOBAS 3.0. DE genes were significantly enriched in such category as metabolism, non-alcoholic fatty liver disease, aerobic respiration-electron donor II and RNA transport (including *Eef1a2*) (Fig. 1A). In addition, 38 gene sequences were submitted to GenBank as gerbil sequence tags (Supplementary Table 4).

Given that eEF1A2 was downregulated in the skeletal muscle of diabetic gerbils analysed by SSH, three rodent models (diabetic gerbils, db/db and HFD-fed mice) were employed to confirm the findings. All of the models showed excess body weight (Supplementary Fig. 3A, B and C) and severely impaired glucose tolerance (Figs 1B, 2A and B). *Eef1a2* mRNA levels decreased significantly in diabetic skeletal muscle when normalised to β -actin (Figs 1C, 2C and E) or skeletal muscle marker Actinin alpha 2 (*Actn2*) (Figs 1D, 2D and F). eEF1A2 protein levels in skeletal muscle were also found to be markedly downregulated in all three diabetic models (Figs 1E, 2G and H). Immunofluorescence analysis was performed to

determine the expression change and cellular localisation of eEF1A2 and glucose transporter 4 (GLUT4) in skeletal muscle. Strong staining was observed for both eEF1A2 and GLUT4 in chow-fed mice (Fig. 2I), whereas the fluorescence intensity of both eEF1A2 and GLUT4 was significantly lower in HFD-fed mice (Fig. 2J). These results indicated that the expression of eEF1A2 decreased in diabetic murine skeletal muscle.

Eef1a2 promoter methylation was not altered in diabetic mice

To test whether eEF1A2 downregulation in the diabetic skeletal muscle correlated with methylation, the MassARRAY compact system was used to analyse the methylation status of the *Eef1a2* promoter in skeletal muscle or hearts of db/db, their wild-type (WT) control and chow-fed and HFD-fed mice. There were no methylation differences between db/db and WT mice (Fig. 3A and B). Although slightly lower methylation levels were observed in the HFD-fed group compared with the chow-fed group, all of the groups showed hypomethylation (approximately 10%) in the region under study (Fig. 3A, B and C).

Figure 1

eEF1A2 was downregulated in diabetic gerbil skeletal muscle. An enrichment analysis for DE genes from SSH was performed, and $-\log_{10}$ of corrected $P > 1.30103$ denotes corrected $P < 0.05$ (A). OGTT was performed on diabetic Mongolian gerbils and their controls ($n = 2$ male and $n = 2$ female gerbils per group, giving $n = 4$ in total, B). Two days later, the quadriceps femoris from the fasting gerbils was collected and subjected to qRT-PCR analysis. The relative expression levels of *Eef1a2* were normalised to β -actin (C) as an internal control or skeletal muscle marker *Actn2* to exclude the effects of non-skeletal muscle cells (D). eEF1A2 protein levels in gerbils were measured using Western blotting (E). *, ** and *** denote statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.005$, respectively.

Figure 2

eEF1A2 expression decreased significantly in insulin-resistant murine skeletal muscle. OGTT was performed on db/db mice and their WT controls, $n = 4$ per group (A) and chow-fed or HFD-fed mice, $n = 3$ per group (B). Two days later, the quadriceps femoris from fasting db/db mice (C and D), chow-fed and HFD-fed mice (E and F) was collected and subjected to qRT-PCR analysis. The relative expression levels of *Eef1a2* were normalised to β -actin (C and E) or *Actn2* (D and F). eEF1A2 protein levels in db/db mice (G), chow-fed and HFD-fed mice (H) were measured using Western blotting. An immunofluorescence assay was performed on chow-fed (I) and HFD-fed mice (J) by simultaneously labelling with anti-eEF1A2 antibodies (red), anti-GLUT4 antibodies (green) and Hoechst 33342 (blue). The immunofluorescence assay conditions in the two groups were the same. Scale bar = 100 μ m (top) and 25 μ m (bottom). *, ** and *** denote statistical significance at $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively.

Thus, the decreased skeletal muscle eEF1A2 levels in db/db and HFD models were found to be independent of this gene's promoter methylation changes.

To simulate the postprandial expression patterns of eEF1A2 in normal and diabetic mice, fasting mice were simultaneously administered glucose and insulin, followed by skeletal muscle extraction between 0 and 120 min.

In both groups, the skeletal muscle eEF1A2 was upregulated markedly. A 2.43-fold increase of eEF1A2 was observed in chow-fed mice but only a 1.46-fold increase in HFD-fed mice at the 60-min time point, when compared with the 0-min time point (Fig. 3D and E). Therefore, co-treatment with insulin and glucose upregulated eEF1A2 in the skeletal muscle of both normal and insulin-resistant mice.

Figure 3

Eef1a2 promoter methylation was not altered in insulin-resistant mice. After fasting for 16 h, the quadriceps femoris or hearts from db/db mice and their WT controls (A, B) and from chow-fed and HFD-fed mice (A, C) were collected to isolate the DNA. A methylation analysis of the *Eef1a2* promoter in the samples was performed using the MassARRAY compact system, $n = 4$ –16 per group. The colours of the circles (from yellow to blue) represent 0–100% methylation ratios. After fasting for 16 h, 2 g/(kg body weight) glucose and 0.75 IU/(kg body weight) insulin were administered to chow-fed (D) and HFD-fed mice (E) by gavage and intraperitoneal injection, respectively. The quadriceps femoris was collected between 0 and 120 min after administration for Western blotting, $n = 3$ per group. * and ** denote statistical significance at $P < 0.05$ and $P < 0.01$, respectively. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0051>.

eEF1A2 impaired insulin sensitivity in male diabetic gerbils

To assess the effects of eEF1A2 on insulin resistance in the skeletal muscle, male and female diabetic gerbils were subdivided into two groups and the thigh and calf muscles of the bilateral hind limbs were injected with the AAV-ZsGreen empty vector or FLAG-tagged eEF1A2 overexpression vector. Before injection, no differences in body weight and oral glucose tolerance were observed between the two groups of the same sex (Fig. 4C and Supplementary Fig. 4A). Two weeks after injection of AAV, *in vivo* imaging and Western blotting displayed a high efficiency of transfection (Fig. 4A and B). In males, eEF1A2 overexpression in skeletal muscle significantly increased fasting serum glucose, insulin and HOMA-IR levels and further diminished glucose tolerance (Fig. 4D, E, F, G and I). eEF1A2-overexpressing male gerbils exhibited reduced glucose-lowering during ITT compared with empty

AAV-carrying male gerbils (Fig. 4J and L). In contrast, eEF1A2 overexpression in female diabetic skeletal muscle exhibited no significant influence on glucose and insulin levels and glucose or insulin tolerance (Fig. 4D, E, F, H, I, K and L).

eEF1A2 aggravated insulin resistance in male db/db mice

To confirm the impairment of eEF1A2 in insulin sensitivity of male skeletal muscle, the male db/db mice were subdivided randomly into two groups and the thigh and calf muscles of the bilateral hind limbs were injected with the AAV-ZsGreen empty vector or FLAG-tagged eEF1A2 overexpression vector. Before injection, no differences in body weight and glucose tolerance were observed between the groups (Fig. 5C and Supplementary Fig. 4B). Two weeks after injection, *in vivo* imaging and Western blotting identified a high transfection efficiency (Fig. 5A and B).

Figure 4

eEF1A2 overexpression in skeletal muscle impaired insulin sensitivity in male insulin-resistant gerbils. The age-matched gerbils with glucose intolerance were randomly injected with 1.3×10^{11} viral particles of AAV-ZsGreen or AAV-eEF1A2 into the thigh and calf muscles of the bilateral hind limbs (three to four males or females per group). After 2 weeks, the hind-limb skeletal muscle was collected for imaging of ZsGreen by means of *In Vivo* Multispectral System (A) or for analysis of protein expression in the quadriceps femoris by Western blotting (B). Body weight (C), fasting serum glucose levels (D), fasting serum insulin levels (E), HOMA-IR (F), OGTT in the male groups (G), OGTT in the female groups (H), OGTT area under the curve (I), ITT in the male groups (J), ITT in the female groups (K), ITT area under the curve (L) and fasting serum free FFA levels (M) were evaluated. *, ** and *** denote statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.005$, respectively. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0051>.

Compared with the control mice, eEF1A2-overexpressing mice demonstrated significantly increased fasting serum glucose and HOMA-IR levels and aggravated glucose and insulin intolerance (Fig. 5D, F, G, H, I and J). Nevertheless, body weight, serum insulin, FFA, and triglyceride levels of the two groups did not differ (Fig. 5C, E, K and L). In addition, no differences in the fat mass ratios were observed between the two groups (Fig. 5M, N and O). Overall, eEF1A2 overexpression in skeletal muscle aggravated male insulin insensitivity *in vivo*, suggesting that decreased eEF1A2 expression in insulin-resistant skeletal muscle was likely a protective mechanism against insulin resistance.

eEF1A2 was involved in PA-induced insulin resistance in myotubes

Since eEF1A2 impaired insulin sensitivity *in vivo*, the underlying mechanisms were further analysed *in vitro*. The saturated fatty acid PA at the dose of 0.5 or 0.8 mM, which significantly inhibited insulin-stimulated Akt phosphorylation (Supplementary Fig. 5), was used to induce insulin resistance in C2C12 myotubes as previously reported (Powell *et al.* 2004). FLAG-tagged eEF1A2 was overexpressed in C2C12 cells via a lentiviral vector (Fig. 6A and B). Of note, when compared with the control

myotubes, eEF1A2 overexpression exacerbated PA-induced suppression of the insulin-stimulated glucose uptake and membrane GLUT4 levels (Fig. 6C and D) and further inhibited the phosphorylation of Akt, ERK and mTOR (Fig. 6E and F), but showed no significant differences in PI3K and GSK3 β activity (Supplementary Fig. 6A and B). Moreover, the impairment of insulin-stimulated glucose uptake and phosphorylation of Akt by PA exposure were reversed by eEF1A2 knockdown (Fig. 6I and J). Therefore, eEF1A2 was involved in PA-induced insulin resistance in myotubes.

eEF1A2 impaired insulin sensitivity by activating PKC β in insulin-resistant myotubes

Aberrant activation of PKC β is associated with skeletal muscle insulin resistance (Schmitz-Peiffer 2000), but the upstream signals of PKC β are not fully understood. For this reason, we investigated the effects of eEF1A2 on PKC β activation in PA-treated myotubes. We found that the phosphorylation levels of PKC β were significantly elevated following eEF1A2 overexpression in myotubes (Fig. 7A) and diabetic skeletal muscle (Fig. 7B), but were attenuated by eEF1A2 knockdown in myotubes (Fig. 7C). Furthermore, the Co-IP assay revealed that FLAG-eEF1A2 bound to PKC β only in PA-treated

Figure 5

eEF1A2 overexpression in skeletal muscle exacerbated insulin resistance in male db/db mice. Male db/db mice ($n = 7$) with severe glucose intolerance were randomly injected with 1.3×10^{11} viral particles of AAV-ZsGreen or AAV-eEF1A2 into the thigh and calf muscles of bilateral hind limbs. After 2 weeks, the hind-limb skeletal muscle was collected for imaging of ZsGreen on an *In Vivo* Multispectral System (A) or for quantification of protein expression in the quadriceps femoris by Western blotting (B). Body weight (C), fasting serum glucose levels (D), the fasting serum insulin levels (E), HOMA-IR (F), OGTT (G), its area under the curve (H), ITT (I), its area under the curve (J), the fasting serum free fatty acid (FFA) levels (K) and the fasting serum triglyceride levels (L) of the two groups were evaluated. In addition, the fat mass ratios of the AAV-ZsGreen (M) and AAV-eEF1A2 (N) groups were measured using MRI and calculated (O). *, ** and *** denote statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.005$, respectively. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0051>.

myotubes, not in normal myotubes (Fig. 7D). Phospho-PKC β and eEF1A2 co-localised in the cytoplasm of insulin-resistant myotubes (Fig. 7E). Moreover, to test whether PKC β mediates the eEF1A2-inhibited insulin sensitivity, PKC β inhibitor LY333531 and PA were simultaneously incubated with pCDH (empty vector)-carrying or eEF1A2-overexpressing myotubes. As indicated in Fig. 7F, G and H, the addition of LY333531 significantly enhanced insulin-stimulated glucose uptake, membrane GLUT4 levels and phosphorylation of Akt, compared with eEF1A2-overexpressing myotubes. Therefore, eEF1A2 impaired insulin sensitivity by activating PKC β in PA-treated myotubes.

ER stress was involved in eEF1A2-promoted insulin resistance in insulin-resistant myotubes

ER stress is an important contributor to skeletal muscle insulin resistance in obesity and diabetes (Koh *et al.* 2013), and eEF1A1 facilitates ER stress-induced apoptosis *in vitro* (Borradaile *et al.* 2006). Accordingly, we tested whether ER stress was involved in the eEF1A2-promoted insulin intolerance. The qRT-PCR results indicated that the ER stress

marker gene, CCAAT/enhancer-binding protein homologous protein (*Chop*), activating transcription factor 4 (*Atf4*) and oxidoreductin-1 α (*Ero1a*) were significantly upregulated following eEF1A2 overexpression in PA-treated myotubes (Fig. 8A) and the skeletal muscle of db/db mice (Fig. 8B). The disruption of eEF1A2 by RNA interference reduced the ER stress marker gene expression in PA-treated myotubes (Fig. 8C). The amounts of phosphorylated and total IRE1 α and phosphorylated JNK were also increased by eEF1A2 overexpression and were decreased by eEF1A2 knockdown in PA-treated myotubes (Fig. 8D). Further, the ER stress inhibitors, 4-PBA and JNK inhibitor SP600125 were used to test the possibility that eEF1A2 reduced insulin sensitivity by enhancing ER stress in the insulin-resistant myotubes. As indicated in Fig. 8E and F, compared with vehicle and 2mM 4-PBA, 20mM 4-PBA remarkably attenuated the inhibitory effects of eEF1A2 on glucose uptake and phosphorylation of Akt. Similarly, the SP600125 treatment moderately elevated the levels of glucose uptake and Akt phosphorylation in eEF1A2-overexpressing myotubes (Fig. 8G and H). Thus, eEF1A2 aggravated insulin resistance, in part, by promoting ER stress in insulin-resistant skeletal muscle.

Figure 6

eEF1A2 aggravated insulin resistance in myotubes with PA-induced insulin resistance. The C2C12 cells were transfected with an empty vector (pCDH) or the FLAG-tagged-eEF1A2 lentivirus vector (eEF1A2). The infected cells were isolated using an FACSCalibur cell sorter and cultured. The overexpression efficiency was checked by qRT-PCR (A) and Western blotting (B). After differentiation and serum deprivation, the infected myotubes were treated with 0.5 or 0.8 mM PA for 16 h followed by 100 nM of insulin for 15 min. The cells were collected for glucose uptake assays (C) and Western blotting. Membrane protein was isolated to detect protein levels of GLUT4 (D). The myotubes were preincubated with 0.5 or 0.8 mM PA for 16 h and treated with vehicle or 100 nM insulin (Ins) for 15 min. The cells were collected for Western blotting to detect protein levels of p-Akt, Akt, p-mTOR, mTOR, p-ERK and ERK (E-F). The C2C12 cells were transfected with a scramble shRNA-carrying lentivirus (SHC) or eEF1A2 shRNA-carrying lentivirus SH1 or SH2. The efficiency of RNA interference against eEF1A2 was detected by qRT-PCR (G) and Western blotting (H). The myotubes transfected with SHC or SH1 were preincubated with or without 0.8 mM PA for 16 h, and then treated with 100 nM insulin for 15 min. The cells were collected for glucose uptake assays (I) and Western blotting to assess the protein levels of p-Akt and Akt (J). *, ** and *** denote statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.005$, respectively. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0051>.

eEF1A2 inhibited lipogenesis and lipid use in insulin-resistant skeletal muscle

To further uncover the involvement of eEF1A2 in insulin-resistant skeletal muscle, expression levels of the

genes of lipogenesis and lipid lipolysis and oxidation were determined by qRT-PCR. As shown in Fig. 9A and B, the mRNA levels of fatty acid synthesis genes coding for acetyl-CoA carboxylase α (*Acc1*), fatty acid synthase (*Fas*), stearoyl-CoA desaturase 1 (*Scd1*), sterol regulatory

Figure 7

eEF1A2 impaired insulin sensitivity by activating PKCβ in insulin-resistant myotubes. After differentiation and serum deprivation, pCDH-carrying, eEF1A2-overexpressing, and eEF1A2-overexpressing plus 0.1 μM PKC β inhibitor LY333531 (LY)-treated myotubes were preincubated with 0.8 mM PA for 16 h followed by 100 nM of insulin for 15 min. The cells were collected to perform Western blotting to detect amounts of p-PKCβ and PKCβ protein (A). The quadriceps femoris of db/db mice transfected with AAV-ZsGreen or AAV-eEF1A2 for 2 weeks was collected for Western blotting, *n* = 3 per group (B). The myotubes (with SHC or SH1) were preincubated with or without 0.8 mM PA for 16 h, and then treated with 100 nM insulin for 15 min. The cells were collected for Western blotting to measure the amounts of p-PKCβ and PKCβ protein (C). The myotubes (transfected with pCDH or FLAG-tagged eEF1A2) were preincubated with 0.8 mM PA for 16 h, and then stimulated with 100 nM insulin for 15 min. The cells were subjected to a Co-IP assay (D) and simultaneously to an immunofluorescence assay by labelling with anti-FLAG antibodies (red), anti-pPKCβ antibodies (green) and Hoechst 33342 (blue). Scale bar = 75 μm (top) and 25 μm (bottom) (E). The pCDH-transfected, eEF1A2-overexpressing, or eEF1A2-overexpressing plus 0.1 μM LY-treated myotubes were preincubated with 0.8 mM PA for 16 h followed by 100 nM insulin for 15 min. The cells were collected for a glucose uptake assay (F) and Western blotting. Membrane protein was isolated to detect protein levels of GLUT4 (G). Total protein was isolated to detect the amounts of p-Akt and Akt protein (H). *, ** and *** denote statistical significance at *P* < 0.05, *P* < 0.01 and *P* < 0.005, respectively.

element-binding protein 1c (*Srebp1c*) and triglyceride hydrolysis genes coding for lipoprotein lipase (*Lpl*), fatty acid oxidation genes coding for carnitine palmitoyl transferase 1 (*Cpt1*) and energy expenditure gene coding

for uncoupling protein 2 (*Ucp2*), all significantly decreased during eEF1A2 overexpression in PA-treated myotubes. In accordance with the alterations *in vitro*, the eEF1A2 overexpressing skeletal muscle of db/db mice showed

Figure 8

ER stress was involved in eEF1A2-promoted insulin insensitivity in PA-treated myotubes. After differentiation and serum deprivation, the pCDH-transfected and eEF1A2-overexpressing myotubes (A), as well as SHC- and SH1-transfected myotubes (C), were treated with 0.5 or 0.8 mM PA for 16 h followed by 100 nM insulin for 1 h. The cells were collected for qRT-PCR. The quadriceps femoris of the db/db mice transfected with AAV-ZsGreen or AAV-eEF1A2 for 2 weeks was collected for qRT-PCR, $n = 7$ per group (B). The myotubes (transfected with pCDH, FLAG-tagged eEF1A2 and SHC or SH1) were incubated with 0.8 mM PA for 16 h followed by 100 nM insulin for 15 min. The cells were collected for Western blotting (D). The pCDH-carrying, eEF1A2-overexpressing, eEF1A2-overexpressing plus 2 mM ER stress inhibitor 4-PBA (PBA)-treated and eEF1A2-overexpressing plus 20 mM 4-PBA-treated myotubes were preincubated with 0.8 mM PA for 16 h, and then stimulated with 100 nM insulin for 15 min. The cells were collected for a glucose uptake assay (E) and Western blotting (F). The pCDH-carrying, eEF1A2-overexpressing and eEF1A2-overexpressing plus 20 μ M JNK inhibitor SP600125 (SP)-treated myotubes were incubated with 0.8 mM PA for 16 h followed by 100 nM insulin for 15 min. The cells were collected for a glucose uptake assay (G) and Western blotting (H-I). *, ** and *** denote statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.005$, respectively. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0051>.

reduced expressions of *Fas*, *Scd1*, *Srebp1c*, *Lpl* and *Ucp2* (Fig. 9C and D). In addition, the mRNA levels of *Acc1*, *Fas*, *Scd1*, *Srebp1*, *Lpl*, *Cpt1* and *Ucp2* were all increased by the eEF1A2 knockdown in PA-treated myotubes (Fig. 9E and F). These data implied that eEF1A2 inhibited fatty acid synthesis and consumption in insulin-resistant skeletal muscle.

Discussion

Although the roles of eEF1A2 have been extensively studied in tumour, developmental delay, epilepsy and motor neurone degeneration (Abbott *et al.* 2009, Pellegrino *et al.* 2014, Cao *et al.* 2017), a direct association between eEF1A2

and diabetes has not been previously demonstrated. In our study, skeletal muscle eEF1A2 decreased in hyperglycaemic animals, independent of *eEF1A2* promoter methylation. In male insulin-resistant models, the overexpression of eEF1A2 in the skeletal muscle exacerbated hyperglycaemia and glucose intolerance. In insulin-resistant myotubes, eEF1A2 diminished insulin signalling transduction by activating PKC β and ER stress. Overall, eEF1A2 decreased insulin sensitivity via PKC β and ER stress in the skeletal muscle of male insulin-resistant murine models.

Several earlier studies have indicated that diabetes causes a significant decrease in the mRNA translation activity in insulin-sensitive tissues, including skeletal muscle (Kendrick-Jones & Perry 1967, Jefferson *et al.* 1983). The RNA sequencing data also indicate that the expression of translation elongation genes decreases in skeletal muscle of obese people and diabetic patients (Scott *et al.* 2016). Similarly, we found a decrease of eEF1A2 in the fasting state and a smaller increase in the postprandial state in diabetic skeletal muscle. No methylation differences were observed in the *Eef1a2* promoter region between diabetic and nondiabetic tissues, revealing that the altered eEF1A2 expression in diabetic skeletal muscle occurred independent of changes in promoter methylation.

Although eEF1A2 was down-regulated in diabetic skeletal muscle, the link between the decreased expression and insulin resistance is not clear. Our data indicated

that eEF1A2 impaired insulin sensitivity in the skeletal muscle of two male diabetic models (without influencing food intake) (Supplementary Fig. 7) and PA-induced insulin-resistant myotubes. Hence, the decreased eEF1A2 expression in skeletal muscle may be a protective mechanism against the progression of insulin resistance in diabetes. miRNA-663, as one of the reported upstream regulators of eEF1A2, inhibits eEF1A2 expression by direct interactions and is upregulated by an ER stress inducer in cancer cells (Vislovukh *et al.* 2013, Huang *et al.* 2016). Therefore, we hypothesise that eEF1A2 enhances ER stress and that increased miRNA-663 by ER stress directly binds with *Eef1a2* mRNA to induce its silencing in diabetic skeletal muscle, which makes a negative feedback loop to protect against insulin resistance. As one of the most abundant proteins in cells (Newbery *et al.* 2007), eEF1A2 may remain at moderate levels in diabetic skeletal muscle, suggesting that inhibition of eEF1A2 activity in the skeletal muscle may relieve male diabetes symptoms. It is noteworthy that the natural compound, Didemn B an inhibitor of eEF1A in elongation activity, decreased hepatic lipotoxicity, glucose intolerance and food intake in ob/ob mice. However, when compared with the ob/ob mice with the same caloric intake, the compound-treated ob/ob mice did not exhibit improved glucose homeostasis (Hetherington *et al.* 2016), illustrating that inhibiting elongation activity of eEF1A in the whole body regulates

Figure 9

eEF1A2 inhibited lipogenesis and lipid use in insulin-resistant skeletal muscle. After differentiation and serum deprivation, the pCDH-carrying and eEF1A2-overexpressing myotubes were treated with 0.5 or 0.8 mM PA for 16 h followed by 100 nM insulin for 1 h. The cells were collected for qRT-PCR (A and B). The quadriceps femoris of db/db mice transfected with AAV-ZsGreen or AAV-eEF1A2 for 2 weeks was collected to perform qRT-PCR ($n = 7$ per group, C and D). The SHC-transfected or SH1-transfected myotubes were also treated with 0.8 mM PA for 16 h followed by 100 nM insulin for 1 h and were collected for qRT-PCR analysis (E and F). **Legend:** acetyl-CoA carboxylase α (*Acc1*), fatty acid synthase (*Fas*), stearoyl-CoA desaturase 1,2 (*Scd1,2*), sterol regulatory element-binding protein 1c (*Srebp1c*), lipoprotein lipase (*Lpl*), carnitine palmitoyl transferase 1 (*Cpt1*) and uncoupling protein 2 (*Ucp2*). * or different letters denote statistical significance at $P < 0.05$; ** and *** denote statistical significance at $P < 0.01$ and $P < 0.005$, respectively. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0051>.

glucose metabolism by reducing food intake, but not by directly ameliorating insulin resistance. Taken together, we conclude that eEF1A2 was involved in insulin resistance in skeletal muscle of male rodents.

Unlike male gerbils, eEF1A2 overexpression in female gerbils had no significant effects on insulin resistance, revealing that the role of eEF1A2 in insulin sensitivity displayed sex differences. The sex of the C2C12 cell line is female. However, the eEF1A2 overexpression *in vitro* could still significantly reduce insulin signalling transduction in insulin-resistant states. Thus, we speculate that sex hormones may result in sex differences of eEF1A2 actions. Female sex hormones, especially oestrogen, are beneficial to glucose and energy homeostasis. Female mice lacking oestrogen receptor alpha (ER α) or aromatase, which converts androgens to oestrogens, both develop obesity with decreased energy expenditure (Geer & Shen 2009). In the skeletal muscle, both ER α null and oophorectomy remarkably reduce GLUT4 expression and membrane translocation and glycogen synthesis, by decreasing insulin-stimulated Akt phosphorylation (Rincon *et al.* 1996, Barros *et al.* 2006). We showed that eEF1A2 also functioned in insulin-stimulated Akt phosphorylation and GLUT4 translocation in skeletal muscle. Thus, oestrogen is likely to play a more dominant role in insulin signalling transduction and alleviates eEF1A2's actions in skeletal muscle. On the other hand, anti-oxidative stress and anti-inflammatory effects of oestrogen in insulin-responsive tissues may, in part, attenuate the whole-body impairment of glucose homeostasis induced by eEF1A2 in female animals (Geer & Shen 2009).

Considering the suppression of insulin sensitivity by eEF1A2, it is necessary to discover the mechanisms behind this action. eEF1A2 was recently found to bind and co-localise with phospho-PKC β in differentiating C2C12 cells (Piazzi *et al.* 2010b). Our data indicated that PKC β and eEF1A2 also interacted directly in the cytoplasm of PA-treated myotubes. The PKC β inhibitor reversed the suppressive effects of eEF1A2 on insulin sensitivity. Thus, we demonstrated that eEF1A2 aggravated insulin resistance, in part, by activating PKC β in insulin-resistant myotubes. Besides, PKC β did not bind with eEF1A2 in normal myotubes, revealing that eEF1A2 might play different roles in different metabolic states. In addition to PKC β , ER stress was elevated by eEF1A2. The disruption of eEF1A1 does not alter PA-induced ER stress marker glucose-regulated protein 78 (GRP78) expression, but it reduces ER stress-induced cell death *in vitro* (Talapatra *et al.* 2002, Borradaile *et al.* 2006). Didemnin B cannot alleviate liver ER stress of ob/ob mice, compared with

the control group with the same food consumption (Hetherington *et al.* 2016). Considering that the liver does not express eEF1A2 (Chambers *et al.* 1998), it is likely that eEF1A1 affects ER stress-induced apoptosis, rather than lipotoxic ER stress itself. In contrast, Didemnin B abolishes PA-induced GRP78 up-regulation in HepG2 cells (Stoianov *et al.* 2015), which express both eEF1A1 and eEF1A2 (Grassi *et al.* 2007). In our study, eEF1A2 enhanced ER stress. The suppression of ER stress, in part, relieved the impairment of insulin signalling by eEF1A2. Thus, ER stress was involved in eEF1A2-promoted insulin insensitivity of PA-treated myotubes. This effect may, in part, be dependent on the translational function, which warrants further investigations by adding Didemnin B or by disrupting other translation elongation elements. Moreover, eEF1A2 may also promote PA-induced ER stress by inactivating Akt signalling (Hyoda *et al.* 2006), and chronic ER stress causes Akt inactivation (Appenzeller-Herzog & Hall 2012), which is likely to establish positive feedback of ER stress.

Lipid accumulation impairs skeletal muscle insulin sensitivity. Inhibiting lipogenesis and delivery gene expression by eEF1A2 seems beneficial to insulin action (Kim *et al.* 2001, Postic & Girard 2008), whereas the decreased CPT1 and UCP2 in the skeletal muscle attenuate fatty acid oxidation and aggravate insulin resistance (Li *et al.* 2000, Bruce *et al.* 2009). Therefore, eEF1A2 is anticipated to play a paradoxical role in lipid metabolism-altered insulin action. The mechanism by which eEF1A2 downregulated these genes remains unclear, but it seems indirect. The increased expressions of *Lpl*, *Cpt1*, *Ucp2*, proliferator-activated receptor α (*Ppara*) and *Pparg* coactivator-1 (*Pgc1a*) and augmented lipid oxidation are observed in ATF4-null mice (Wang *et al.* 2010). PPAR α and PGC1 α upregulate lipogenic gene expression (Kersten *et al.* 2000, Lin *et al.* 2005). We speculate that eEF1A2 inhibits lipogenesis and utilisation gene expression by up-regulating ATF4.

In conclusion, we demonstrated that eEF1A2 impaired insulin sensitivity in male insulin-resistant skeletal muscle via PKC β activation and ER stress. Our study identifies the role of eEF1A2 in glucose metabolism in animals, enhances our understanding of the mechanisms of insulin resistance in skeletal muscle, and contributes to the theoretical foundation for T2DM treatment.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0051>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Abbas W, Kumar A & Herbein G 2015 The eEF1A proteins: at the crossroads of oncogenesis, apoptosis, and viral infections. *Frontiers in Oncology* **5** 75. (<https://doi.org/10.3389/fonc.2015.00075>)
- Abbott CM, Newbery HJ, Squires CE, Brownstein D, Griffiths LA & Soares DC 2009 eEF1A2 and neuronal degeneration. *Biochemical Society Transactions* **37** 1293–1297. (<https://doi.org/10.1042/BST0371293>)
- Amiri A, Noei F, Jeganathan S, Kulkarni G, Pinke DE & Lee JM 2007 eEF1A2 activates Akt and stimulates Akt-dependent actin remodeling, invasion and migration. *Oncogene* **26** 3027–3040. (<https://doi.org/10.1038/sj.onc.1210101>)
- Appenzeller-Herzog C & Hall MN 2012 Bidirectional crosstalk between endoplasmic reticulum stress and mTOR signaling. *Trends in Cell Biology* **22** 274–282. (<https://doi.org/10.1016/j.tcb.2012.02.006>)
- Barros RP, Machado UF, Warner M & Gustafsson JA 2006 Muscle GLUT4 regulation by estrogen receptors ERbeta and ERalpha. *PNAS* **103** 1605–1608. (<https://doi.org/10.1073/pnas.0510391103>)
- Bohnert KR, McMillan JD & Kumar A 2018 Emerging roles of ER stress and unfolded protein response pathways in skeletal muscle health and disease. *Journal of Cellular Physiology* **233** 67–78. (<https://doi.org/10.1002/jcp.25852>)
- Borradaile NM, Buhman KK, Listenberger LL, Magee CJ, Morimoto ET, Ory DS & Schaffer JE 2006 A critical role for eukaryotic elongation factor 1A-1 in lipotoxic cell death. *Molecular Biology of the Cell* **17** 770–778. (<https://doi.org/10.1091/mbc.e05-08-0742>)
- Bruce CR, Hoy AJ, Turner N, Watt MJ, Allen TL, Carpenter K, Cooney GJ, Febbraio MA & Kraegen EW 2009 Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet-induced insulin resistance. *Diabetes* **58** 550–558. (<https://doi.org/10.2337/db08-1078>)
- Cao S, Smith LL, Padilla-Lopez SR, Guida BS, Blume E, Shi J, Morton SU, Brownstein CA, Beggs AH, Krueger MC, *et al.* 2017 Homozygous eEF1A2 mutation causes dilated cardiomyopathy, failure to thrive, global developmental delay, epilepsy and early death. *Human Molecular Genetics* **26** 3545–3552. (<https://doi.org/10.1093/hmg/ddx239>)
- Chambers DM, Peters J & Abbott CM 1998 The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1alpha, encoded by the Eef1a2 gene. *PNAS* **95** 4463–4468. (<https://doi.org/10.1073/pnas.95.8.4463>)
- DeFronzo RA & Tripathy D 2009 Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care* **32** (Suppl 2) S157–S163. (<https://doi.org/10.2337/dc09-S302>)
- DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J & Felber JP 1981 The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30** 1000–1007. (<https://doi.org/10.2337/diab.30.12.1000>)
- Deldicque L, Hespel P & Francaux M 2012 Endoplasmic reticulum stress in skeletal muscle: origin and metabolic consequences. *Exercise and Sport Sciences Reviews* **40** 43–49. (<https://doi.org/10.1097/JES.0b013e3182355e8c>)
- Friedewald WT, Levy RI & Fredrickson DS 1972 Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry* **18** 499–502.
- Gao J, He J, Shi X, Stefanovic-Racic M, Xu M, O'Doherty RM, Garcia-Ocana A & Xie W 2012 Sex-specific effect of estrogen sulfotransferase on mouse models of type 2 diabetes. *Diabetes* **61** 1543–1551. (<https://doi.org/10.2337/db11-1152>)
- Geer EB & Shen W 2009 Gender differences in insulin resistance, body composition, and energy balance. *Gender Medicine* **6** (Suppl 1) 60–75. (<https://doi.org/10.1016/j.genm.2009.02.002>)
- Grassi G, Scaggiante B, Farra R, Dapas B, Agostini F, Baiz D, Rosso N & Tiribelli C 2007 The expression levels of the translational factors eEF1A 1/2 correlate with cell growth but not apoptosis in hepatocellular carcinoma cell lines with different differentiation grade. *Biochimie* **89** 1544–1552. (<https://doi.org/10.1016/j.biochi.2007.07.007>)
- Hetherington AM, Sawyez CG, Sutherland BG, Robson DL, Arya R, Kelly K, Jacobs RL & Borradaile NM 2016 Treatment with didemnin B, an elongation factor 1A inhibitor, improves hepatic lipotoxicity in obese mice. *Physiological Reports* **4** e12963. (<https://doi.org/10.14814/phy2.12963>)
- Huang W, Bansode R, Mehta M & Mehta KD 2009 Loss of protein kinase Cbeta function protects mice against diet-induced obesity and development of hepatic steatosis and insulin resistance. *Hepatology* **49** 1525–1536. (<https://doi.org/10.1002/hep.22815>)
- Huang Y, Liu J, Fan L, Wang F, Yu H, Wei W & Sun G 2016 miR-663 overexpression induced by endoplasmic reticulum stress modulates hepatocellular carcinoma cell apoptosis via transforming growth factor beta 1. *Oncotargets and Therapy* **9** 1623–1633. (<https://doi.org/10.2147/OTT.S96902>)
- Hyoda K, Hosoi T, Horie N, Okuma Y, Ozawa K & Nomura Y 2006 PI3K-Akt inactivation induced CHOP expression in endoplasmic reticulum-stressed cells. *Biochemical and Biophysical Research Communications* **340** 286–290. (<https://doi.org/10.1016/j.bbrc.2005.12.007>)
- Itani SI, Zhou Q, Pories WJ, MacDonald KG & Dohm GL 2000 Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. *Diabetes* **49** 1353–1358. (<https://doi.org/10.2337/diabetes.49.8.1353>)
- Jefferson LS, Liao WS, Peavy DE, Miller TB, Appel MC & Taylor JM 1983 Diabetes-induced alterations in liver protein synthesis. Changes in the relative abundance of mRNAs for albumin and other plasma proteins. *Journal of Biological Chemistry* **258** 1369–1375.
- Kahns S, Lund A, Kristensen P, Knudsen CR, Clark BF, Cavallius J & Merrick WC 1998 The elongation factor 1 A-2 isoform from rabbit: cloning of the cDNA and characterization of the protein. *Nucleic Acids Research* **26** 1884–1890. (<https://doi.org/10.1093/nar/26.8.1884>)
- Kendrick-Jones J & Perry SV 1967 Protein synthesis and enzyme response to contractile activity in skeletal muscle. *Nature* **213** 406–408. (<https://doi.org/10.1038/213406a0>)
- Kersten S, Desvergne B & Wahli W 2000 Roles of PPARs in health and disease. *Nature* **405** 421–424. (<https://doi.org/10.1038/35013000>)
- Kim JK, Fillmore JJ, Chen Y, Yu C, Moore IK, Pypaert M, Lutz EP, Kako Y, Velez-Carrasco W, Goldberg IJ, *et al.* 2001 Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *PNAS* **98** 7522–7527. (<https://doi.org/10.1073/pnas.121164498>)
- Knudsen SM, Frydenberg J, Clark BF & Leffers H 1993 Tissue-dependent variation in the expression of elongation factor-1 alpha isoforms: isolation and characterization of a cDNA encoding a novel variant of human elongation-factor 1 alpha. *European Journal of Biochemistry* **215** 549–554. (<https://doi.org/10.1111/j.1432-1033.1993.tb18064.x>)

- Koh HJ, Toyoda T, Didesch MM, Lee MY, Sleeman MW, Kulkarni RN, Musi N, Hirshman MF & Goodyear LJ 2013 Tribbles 3 mediates endoplasmic reticulum stress-induced insulin resistance in skeletal muscle. *Nature Communications* **4** 1871. (<https://doi.org/10.1038/ncomms2851>)
- Lee MH, Choi BY, Cho YY, Lee SY, Huang Z, Kundu JK, Kim MO, Kim DJ, Bode AM, Surh YJ, *et al.* 2013 Tumor suppressor p16(INK4a) inhibits cancer cell growth by downregulating eEF1A2 through a direct interaction. *Journal of Cell Science* **126** 1744–1752. (<https://doi.org/10.1242/jcs.113613>)
- Li B, Nolte LA, Ju JS, Han DH, Coleman T, Holloszy JO & Semenkovich CF 2000 Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. *Nature Medicine* **6** 1115–1120. (<https://doi.org/10.1038/80450>)
- Li X, Lu J, Wang Y, Huo X, Li Z, Zhang S, Li C, Guo M, Du X & Chen Z 2016 Establishment and characterization of a newly established diabetic gerbil Line. *PLoS ONE* **11** e0159420. (<https://doi.org/10.1371/journal.pone.0159420>)
- Li YN, Guo M, Du XY, Li CL, Huo XY, Lu J, Lv JY, Liu X & Chen ZW 2017 Cloning and homology analysis of Eef1a2 of Mongolian gerbils. *Laboratory Animal Science* **34** 20–25 (in Chinese).
- Lin J, Handschin C & Spiegelman BM 2005 Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metabolism* **1** 361–370. (<https://doi.org/10.1016/j.cmet.2005.05.004>)
- Mateyak MK & Kinzy TG 2010 eEF1A: thinking outside the ribosome. *Journal of Biological Chemistry* **285** 21209–21213. (<https://doi.org/10.1074/jbc.R110.113795>)
- Newbery HJ, Loh DH, O'Donoghue JE, Tomlinson VA, Chau YY, Boyd JA, Bergmann JH, Brownstein D & Abbott CM 2007 Translation elongation factor eEF1A2 is essential for post-weaning survival in mice. *Journal of Biological Chemistry* **282** 28951–28959. (<https://doi.org/10.1074/jbc.M703962200>)
- Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ & Hotamisligil GS 2006 Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* **313** 1137–1140. (<https://doi.org/10.1126/science.1128294>)
- Pellegrino R, Calvisi DF, Neumann O, Kolluru V, Wesely J, Chen X, Wang C, Wuestefeld T, Ladu S, Elgohary N, *et al.* 2014 EEF1A2 inactivates p53 by way of PI3K/AKT/mTOR-dependent stabilization of MDM4 in hepatocellular carcinoma. *Hepatology* **59** 1886–1899. (<https://doi.org/10.1002/hep.26954>)
- Piazzi M, Bavelloni A, Faenza I, Blalock W, Urbani A, D'Aguanno S, Fiume R, Ramazzotti G, Maraldi NM & Cocco L 2010a eEF1A phosphorylation in the nucleus of insulin-stimulated C2C12 myoblasts53 is a novel substrate for protein kinase C beta1. *Molecular and Cellular Proteomics* **9** 2719–2728. (<https://doi.org/10.1074/mcp.M110.003152>)
- Piazzi M, Bavelloni A, Faenza I, Blalock W, Urbani A, D'Aguanno S, Fiume R, Ramazzotti G, Maraldi NM & Cocco L 2010b eEF1A phosphorylation in the nucleus of insulin-stimulated C2C12 myoblasts(5)(3) is a novel substrate for protein kinase C beta1. *Molecular and Cellular Proteomics* **9** 2719–2728. (<https://doi.org/10.1074/mcp.M110.003152>)
- Postic C & Girard J 2008 Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *Journal of Clinical Investigation* **118** 829–838. (<https://doi.org/10.1172/JCI34275>)
- Powell DJ, Turban S, Gray A, Hajduch E & Hundal HS 2004 Intracellular ceramide synthesis and protein kinase C ζ activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. *Biochemical Journal* **382** 619–629. (<https://doi.org/10.1042/BJ20040139>)
- Rincon J, Holmang A, Wahlstrom EO, Lonnroth P, Bjornorp P, Zierath JR & Wallberg-Henriksson H 1996 Mechanisms behind insulin resistance in rat skeletal muscle after oophorectomy and additional testosterone treatment. *Diabetes* **45** 615–621. (<https://doi.org/10.2337/diab.45.5.615>)
- Schmitz-Peiffer C 2000 Signalling aspects of insulin resistance in skeletal muscle: mechanisms induced by lipid oversupply. *Cellular Signalling* **12** 583–594. ([https://doi.org/10.1016/S0898-6568\(00\)00110-8](https://doi.org/10.1016/S0898-6568(00)00110-8))
- Scott LJ, Erdos MR, Huyghe JR, Welch RP, Beck AT, Wolford BN, Chines PS, Didion JP, Narisu N, Stringham HM, *et al.* 2016 The genetic regulatory signature of type 2 diabetes in human skeletal muscle. *Nature Communications* **7** 11764. (<https://doi.org/10.1038/ncomms11764>)
- Soares DC & Abbott CM 2013 Highly homologous eEF1A1 and eEF1A2 exhibit differential post-translational modification with significant enrichment around localised sites of sequence variation. *Biology Direct* **8** 29. (<https://doi.org/10.1186/1745-6150-8-29>)
- Standaert ML, Bandyopadhyay G, Galloway L, Soto J, Ono Y, Kikkawa U, Farese RV & Leitges M 1999 Effects of knockout of the protein kinase C beta gene on glucose transport and glucose homeostasis. *Endocrinology* **140** 4470–4477. (<https://doi.org/10.1210/endo.140.10.7073>)
- Stoianov AM, Robson DL, Hetherington AM, Sawyez CG & Borradaile NM 2015 Elongation factor 1A-1 is a mediator of hepatocyte lipotoxicity partly through its canonical function in protein synthesis. *PLoS ONE* **10** e0131269. (<https://doi.org/10.1371/journal.pone.0131269>)
- Talapatra S, Wagner JD & Thompson CB 2002 Elongation factor-1 alpha is a selective regulator of growth factor withdrawal and ER stress-induced apoptosis. *Cell Death and Differentiation* **9** 856–861. (<https://doi.org/10.1038/sj.cdd.4401078>)
- Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP & Ron D 2000 Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287** 664–666. (<https://doi.org/10.1126/science.287.5453.664>)
- Vislovukh A, Kratassiouk G, Porto E, Gralievskaya N, Beldiman C, Pinna G, El'skaya A, Harel-Bellan A, Negrutskii B & Groisman I 2013 Proto-oncogenic isoform A2 of eukaryotic translation elongation factor eEF1 is a target of miR-663 and miR-744. *British Journal of Cancer* **108** 2304–2311. (<https://doi.org/10.1038/bjc.2013.243>)
- Wang C, Huang Z, Du Y, Cheng Y, Chen S & Guo F 2010 ATF4 regulates lipid metabolism and thermogenesis. *Cell Research* **20** 174–184. (<https://doi.org/10.1038/cr.2010.4>)
- Wu J, Mao X, Cai T, Luo J & Wei L 2006 KOBAS server: a web-based platform for automated annotation and pathway identification. *Nucleic Acids Research* **34** W720–W724. (<https://doi.org/10.1093/nar/gkl167>)

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