

Repression of Global Protein Synthesis by Eif1a-Like Genes That Are Expressed Specifically in the Two-Cell Embryos and the Transient Zscan4-Positive State of Embryonic Stem Cells

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Abstract

Mouse embryonic stem (ES) cells are prototypical stem cells that remain undifferentiated in culture for long periods, yet maintain the ability to differentiate into essentially all cell types. Previously, we have reported that ES cells oscillate between two distinct states, which can be distinguished by the transient expression of Zscan4 genes originally identified for its specific expression in mouse two-cell stage embryos. Here, we report that the nascent protein synthesis is globally repressed in the Zscan4-positive state of ES cells, which is mediated by the transient expression of newly identified eukaryotic translation initiation factor 1A (Eif1a)-like genes. Eif1a-like genes, clustered on Chromosome 12, show the high sequence similarity to the Eifa1 and consist of 10 genes (Eif1al1–Eif1al10) and 9 pseudogenes (Eif1al-ps1–Eif1al-ps9). The analysis of the expressed sequence tag database showed that Eif1a-like genes are expressed mostly in the two-cell stage mouse embryos. Microarray analyses and quantitative real-time polymerase chain reaction analyses show that Eif1a-like genes are expressed specifically in the Zscan4-positive state of ES cells. These results indicate a novel mechanism to repress protein synthesis by Eif1a-like genes and a unique mode of protein synthesis regulation in ES cells, which undergo a transient and reversible repression of global protein synthesis in the Zscan4-positive state.

Key words: Eif1a-like genes; global translational repression; embryonic stem cell; two-cell stage embryo; Zscan4

1. Introduction

Zinc finger and SCAN domain-containing 4 (*Zscan4*) genes are originally identified as genes that are specifically expressed in the two-cell stage of mouse pre-implantation embryos (Falco *et al.*, 2007). The two-cell stage is critical for mouse development, as this is the time when the dramatic epigenetic changes occur¹ concomitantly with the transcriptional activation of the maternally derived genome and paternally derived genome for the first time (called zygotic

genome activation, ZGA).^{2,3} Although human genome contains only one copy of *ZSCAN4*, mouse genome contains nine copies of genes (*Zscan4a*, *Zscan4b*, *Zscan4c*, *Zscan4d*, *Zscan4e*, *Zscan4f*, and three pseudogenes).⁴ *Zscan4d* is expressed predominantly in the two-cell stage mouse embryos, whereas *Zscan4c* is expressed predominantly in the mouse embryonic stem (ES) cells.⁴ We here collectively call them *Zscan4*.

The expression of *Zscan4* in mouse ES cells is transient and reversible, resulting in the unique expression pattern with expression in only 1–5% of ES cells at

one time in culture.^{4–6} Further analysis reveals that ES cells oscillate between the Zscan4-negative (Zscan4⁻) state and Zscan4-positive (Zscan4⁺) state, in which Zscan4 functions to extend telomeres and to enhance genomic stability.⁶ A recent study shows that this transient state is also marked with the expression of endogenous retroviruses, such as MuERV-L, which are also highly expressed in the two-cell stage mouse embryos.⁷ Additionally, Zscan4 has also been shown to further enhance the efficiency of generating mouse induced pluripotent stem cells and to increase their quality.^{8,9}

Through the global expression analysis, we have noticed that a eukaryotic translation initiation factor 1A (Eif1a) is highly up-regulated in the Zscan4⁺ state of ES cells compared with the Zscan4⁻ state.¹⁰ Studies using a yeast model have shown that, together with Eif1, Eif1a plays an important role in the identification of translation initiation codon in eukaryotes.^{11,12} According to the Mouse Genome Informatics database¹³ at The Jackson Laboratory, *Eif1a* was originally identified as a mouse eukaryotic elongation factor *Tu* in erythroleukemic cells.¹⁴ It was also identified as a translation initiation factor *eIF-4C*, a gene expressed transiently in the two-cell stage of the pre-implantation mouse embryos.¹⁵ Although *Eif1a* has been used as a specific marker for ZGA in the two-cell stage embryos,¹⁶ its function in either ZGA or two-cell embryos is not clear.

In this study, we have identified a cluster of *Eif1a*-like genes on mouse Chromosome 12 and revealed that, not *Eif1a* on Chromosome 18, but *Eif1a*-like genes are expressed specifically in the two-cell stage mouse embryos and in the Zscan4⁺ state of mouse ES cells. We also found that the nascent protein synthesis is globally repressed in the Zscan4⁺ state of ES cells, which can be explained by the specific expression of *Eif1a*-like genes in these cells, as we have also shown that the over-expression of *Eif1a*-like genes can indeed repress the global protein synthesis. Together, the findings from this study demonstrate a new mechanism by which global protein translation can be regulated in the ES cells.

2. Materials and methods

2.1. Mouse embryonic stem cell culture and transfection

Mouse ES cell lines, V6.5, derived from F1 hybrid mouse strain C57BL/6 × 129/Sv (Thermo Scientific, Rockford, IL, USA), and MC1, derived from 129S6/SvEvTac mouse strain (Transgenic Core Laboratory, Johns Hopkins University School of Medicine, Baltimore, MD, USA), were used in this study. ES cells were maintained on 0.1% gelatin-coated culture plates in complete ES cell media consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Grand Island, NY, USA) supplemented with

15% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), 1000 U/ml leukaemia inhibitory factor (LIF; ESGRO, Chemicon Millipore, Billerica, MA, USA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM GlutaMAX, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (all media supplements were from Gibco Invitrogen unless otherwise stated). ES cell culture medium was changed daily, and cells were passaged every 3 days. ES cells were transfected using Effectene (Qiagen, Valencia, CA, USA) following the manufacturer's protocol.

2.2. Vectors and DNA Constructs

3 × FLAG tag were added to the 5-prime end of mouse *Eif1a* (NM_010120.5), *Eif1a16* (U106343), and *Eif1a19* (U285359) and cloned into the pPyCAG-BstXI-IP vector (a generous gift from Dr Hitoshi Niwa, RIKEN Center for Developmental Biology, Japan, http://www.cdb.riken.jp/pcs/protocol/vector/vector_top.html) at *XhoI* and *NotI* sites.

2.3. Nascent protein synthesis labelling

Click-iT L-azidohomoalanine (AHA) Alexa Fluor 488 Protein Synthesis Assay Kit (Invitrogen Life Sciences) was used to label newly synthesized protein following the manufacturer's protocol. Cells were plated on 12-well plates and allowed to adhere overnight. Cells were cultured in methionine-free complete ES cell media for 30 min, labelled in AHA-containing methionine-free complete ES cell media for 1 h, and subsequently fixed for immunofluorescence studies or protein extracted using RIPA lysis buffer (Thermo Scientific). Tetramethylrhodamine (TAMRA) detection of AHA-labelled protein samples were performed using the Click-IT TAMRA Protein Analysis Detection Kit (Invitrogen Life Sciences) following the manufacturer's protocol.

2.4. Immunofluorescence

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 20 min and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) for 15 min at room temperature. Cells were blocked for 30 min at room temperature in PBS containing 10% FBS, 10% goat serum (Gibco Invitrogen), and 10% donkey serum (Sigma-Aldrich). The cells were incubated in primary antibodies overnight at 4°C followed by secondary antibodies for 2 h at room temperature and lastly stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Roche, Indianapolis, IN, USA). Custom-made polyclonal rabbit anti-Zscan4 antibodies (GenScript, Piscataway, NJ, USA) were generated against the C-terminal

epitope Zscan4 as described previously.⁶ Anti-FLAG M2 (F1804; Sigma), mouse anti-Zscan4 (Abnova, Taipei, Taiwan), anti-Eif1a (Abcam, Cambridge, MA, USA), anti-eIF2 α phosphoserine 51 (Abcam), anti-histone H3 phosphoserine 10 (Abcam), anti-actin (Sigma), and fluorescent Alexa Fluor secondary (Invitrogen) antibodies were purchased. Fluorescence was visualized and captured using a Nikon Eclipse TE300 inverted microscope (Nikon, Melville, NY, USA).

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot analysis

Protein samples were prepared by lysing mouse ES cells in RIPA buffer (Thermo Scientific) following manufacturer's protocol. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific). 20 μ g of protein samples were loaded onto NuPAGE 4–12% Bis-Tris gel (Novex Life Technologies, Carlsbad, CA) and ran in MOPS sodium dodecyl sulphate running buffer (Invitrogen) at 200 V for 50 min. Protein transfer was performed using the Bio-Rad transfer system at 80 V for 3 h at 4°C onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK). Membranes were blocked in Tris-buffered saline (BioSource, Chevy Chase, MD, USA) containing 0.05% Tween 20 (Bio-Rad) and 5% milk. Primary antibodies were incubated at 4°C overnight. Enhanced chemi-luminescence solutions (Pierce Thermo Scientific) were applied to the membranes and were exposed against Kodak X-Omat AR2 film (Perkin Elmer).

2.6. Quantitative real-time PCR

RNA samples were isolated from cells using the RNeasy mini kit (Qiagen). One microgram of total RNA was used for cDNA synthesis using the high capacity cDNA reverse transcription kit (Invitrogen). Quantitative polymerase chain reaction (PCR) analysis was performed in triplicates with the SYBR green master mix and run on 7900HT system (Applied Biosystems). All procedures above were performed following the respective manufacturer's protocols. Primers used for PCR reactions were: wild type *Eif1a* forward, 5'-GGCCATCGTGAGCTGAAGTA-3'; reverse, 5'-AAGTGCAGCCGTACACATCT-3'; *Eif1a16* forward, 5'-CAACACAATTGGCATCTTGACG-3'; reverse, 5'-GCATTGACAAAACACACTCATGC-3'; *Eif1a19* forward, 5'-CAATGACAGGTAGTTGTTAAGAGCA-3'; reverse, 5'-GAAAA TGGCTTAACACAAAGGACA-3'; *Gapdh* forward, 5'-CCCAATGTGTCCGTCGTG-3'; reverse, 5'-GCCTGCTTCA CCACCTTCT-3'. All measurements were normalized to *Gapdh* loading control and relative expression of *Eif1a*, and variant expression were compared with untreated control samples using the $\Delta\Delta C_t$ method.¹⁷

3. Results

3.1. Nascent protein synthesis is globally repressed in the Zscan4⁺ state of mouse ES cells

Zscan4, a specific marker for two-cell embryo, has been shown in previous studies to be transiently expressed in mouse ES cells.^{4,6} Mouse ES cells oscillate between Zscan4⁺ and Zscan4⁻ states. Eif1a, a translation initiation factor, is found to be co-upregulated with Zscan4 in two-cell embryo^{18–20} and was also found to be one of the top up-regulated genes in cell enriched for Zscan4 (*pZscan4*-Emerald).¹⁰ In earlier studies, we have also noticed that, at the RNA levels, the expression of *Zscan4* is often accompanied with the expression of *Eif1a*.²¹ Because Eif1a is a known translation initiation factor, we hypothesized that the high expression of Eif1a in the Zscan4⁺ state may play a role in the protein synthesis in these cells.

To test this notion, we first examined the nascent protein synthesis in mouse ES cells using the AHA assay. To our surprise, the dramatic repression of global protein synthesis was observed in ~95% of Zscan4-positive cells when compared with surrounding Zscan4-negative ES cells (Fig. 1Ai–iii). To further validate this result, we established a stable mouse ES cell line in which the puromycin-resistant gene is driven by the Zscan4c promoter, thus allowing the enrichment of cells in the Zscan4⁺ state by adding puromycin (Fig. 1B). The AHA assay on these cells confirmed the global repression of protein synthesis in the cells enriched by the Zscan4⁺ state (Fig. 1C). As expected, levels of proteins detected by antibodies against Eif1a increased in ~85% of the Zscan4-enriched ES cells, despite the repression of global protein synthesis (Fig. 1Aiv–vi). The immunohistochemistry result was further supported by the western blot analysis of ES cells enriched with the Zscan4⁺ state (Fig. 1C).

It is known that the high expression of proteins could cause the repression of global protein synthesis by the stress-induced response in cells.²² To test this possibility, we carried out immunostaining of ES cells with antibodies against Zscan4 and phosphorylated Eif2 α at serine 51 (a marker for stress-induced global protein synthesis repression).^{23,24} Co-expression of Zscan4 and phospho-Eif2 α (Ser51) was not observed, indicating that the global protein repression observed in endogenously Zscan4-enriched ES cells was not due to stress-induced response (Fig. 2A). Another condition in which cells are known to undergo global protein synthesis repression is during mitosis (M phase) of the cell cycle.^{25,26} To check whether the Zscan4⁺ cells are in the G2/M phase, ES cells were fixed and immunostained with antibodies against Zscan4 and histone H3 phosphoserine 10 (H3S10ph; a marker of the late G₂ and M phase).^{27–29} Cells in late G₂ and prophase cells

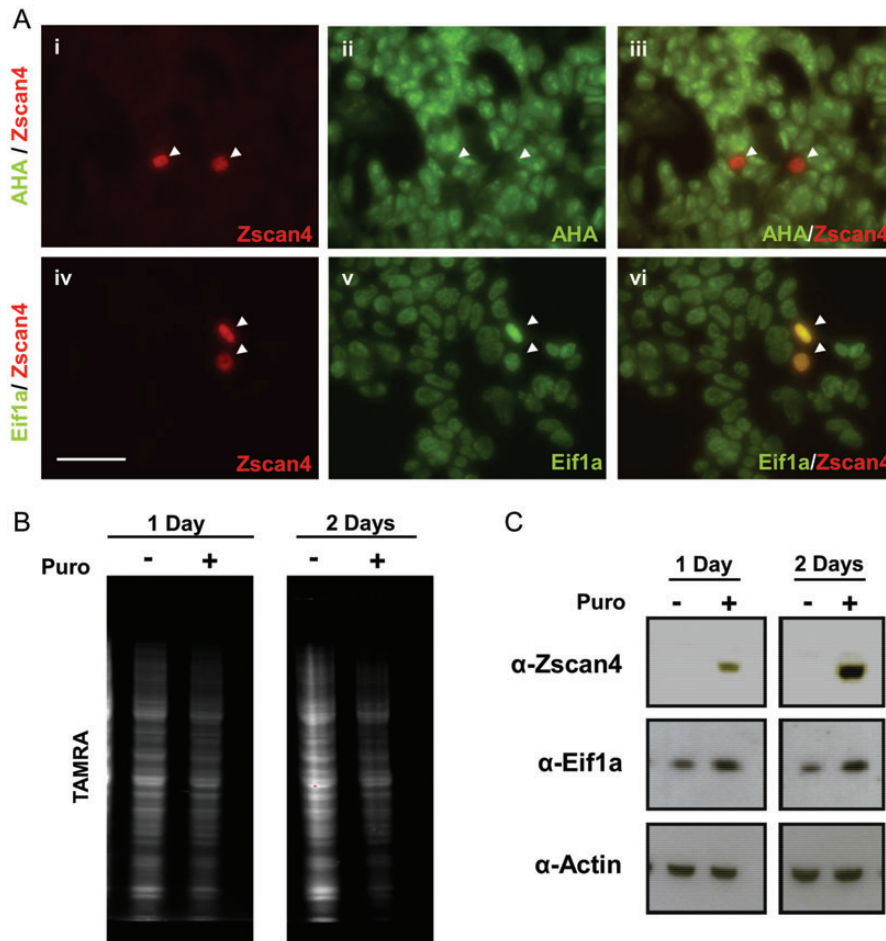


Figure 1. Global nascent protein synthesis is repressed in *Zscan4*⁺ state cells and has elevated levels of Eif1a. (A) To determine global protein synthesis, mouse ES cells were cultured in methionine-free ES cell media for 30 min, labelled with AHA, a methionine analogue, for 1 h and visualized with Alexa Fluor 488 alkyne through Click-iT reaction and co-stained with *Zscan4* antibody. *Zscan4*⁺ ES cells (i, arrowhead) showed dramatic repression in AHA staining (ii and iii). Eif1a levels were determined in *Zscan4*⁺ cells by co-staining with the respective antibodies. *Zscan4*⁺ cells (iv) showed enhanced Eif1a expression (v and vi). Pictures were taken at $\times 40$ magnification using a Nikon Eclipse TE300 microscope (scale bar indicates 50 μ m). (B) *pZscan4*-puro mouse ES cells were treated with (+) or without (-) 1.5 mg/ml puromycin for 1 or 2 days. Cells were labelled with AHA as described above and protein harvested using RIPA buffer containing protease inhibitors. AHA-labelled samples were then detected using TAMRA alkyne via Click-iT reaction. Samples were run on a 4–12% Bis-Tris SDS-PAGE acrylamide gel, and TAMRA was visualized under UV illuminance. (C) Western blot analysis was performed on the same gel (C) to determine the levels of *Zscan4* and Eif1a. Protein from the acrylamide gel was transferred onto the polyvinylidene difluoride (PVDF) membrane and blotted with antibodies against *Zscan4*, Eif1a, and beta-actin.

showed more speckled staining of H3S10ph, whereas cells undergoing metaphase had more intense and compacted staining H3S10ph. Consistent with previous studies cells in the metaphase showed a decrease in protein synthesis as measured by AHA labelling (Fig. 2Bi–iv). However, co-staining of H3S10ph and *Zscan4* antibodies was not observed, indicating that *Zscan4*⁺ state cells repressed protein synthesis through a different mechanism. Together, these data demonstrate that the protein synthesis repression observed in *Zscan4*⁺ state cells does not occur through stress-induced or mitosis-induced mechanisms and suggests an alternative mechanism that is responsible for global protein synthesis repression observed in these cells.

3.2. Identification of Eif1a-like genes that are predominantly expressed in two-cell embryos and the *Zscan4*⁺ state of ES cells

Results thus far suggest that Eif1a plays a role in repressing global protein synthesis, which would be a novel and unexpected function of Eif1a, if confirmed. To gain more insights into the Eif1a gene, we looked into all oligonucleotide probes used to target different regions of the *Eif1a* gene in the DNA microarray.³⁰ Interestingly, we found that not all probes against *Eif1a* showed an increase in expression in *Zscan4*⁺ state ES cells compared with the *Zscan4*⁻ state. Probes that targeted a part of the protein-coding region showed a 10.5-fold increase in the *Zscan4*⁺ state of ES cells compared with the *Zscan4*⁻ state, whereas

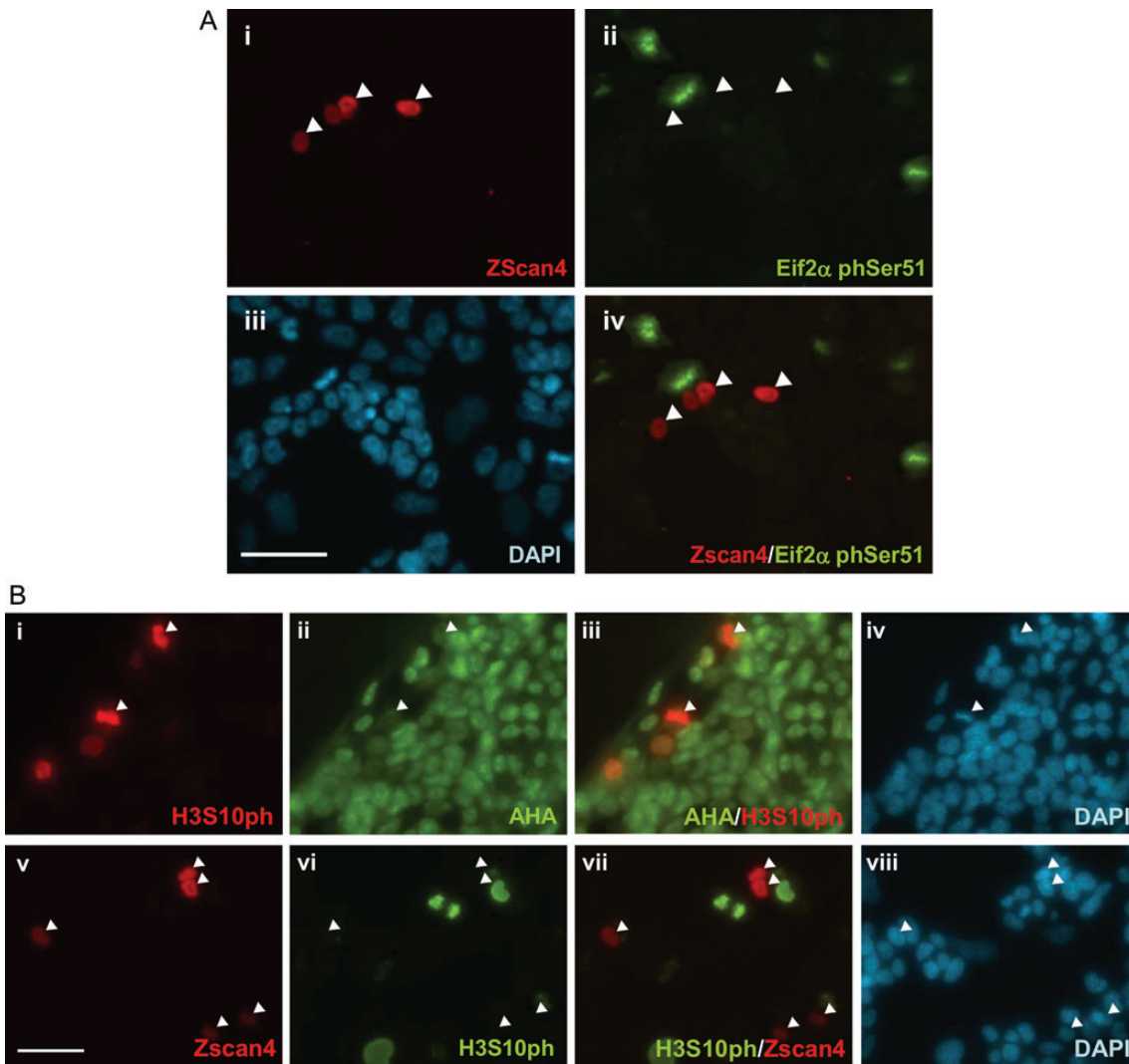


Figure 2. Global nascent protein synthesis observed in *Zscan4*⁺ state cells not due to stress-induced response or cells being blocked in the mitosis stage of cell cycle. (A) Mouse ES cells were co-immunostained with antibodies against *Zscan4* (i) and *Eif2α* phosphoserine 51 antibodies (ii) and visualized using Alexa Fluor 568 and 488, respectively. DNA was visualized by 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining (iii). White arrows indicate the position of *Zscan4*⁺ state cells. Pictures were taken at $\times 40$ magnification using a Nikon Eclipse TE300 microscope (scale bar indicates 50 μ m). White arrows indicate *Zscan4*-positive cells. (B) To determine global protein synthesis, mouse ES cells were cultured in methionine-free ES cell media for 30 min, labelled with AHA, a methionine analogue, for 1 h and visualized with Alexa Fluor 488 alkyne through Click-iT reaction (i) and co-stained with histone H3 phosphoserine 10 (H3S10ph) antibody (ii). DNA was visualized by DAPI staining (iv and viii). White arrows indicate the position of H3S10ph stained cells in i–iv. To investigate whether cells under *Zscan4*⁺ state are blocked in the mitotic phase of the cell cycle, mouse ES cells were co-immunostained with *Zscan4* (v) and H3S10ph (vi) antibodies. Pictures were taken at $\times 40$ magnification using a Nikon Eclipse TE300 microscope (scale bar indicates 50 μ m).

probes that targeted the 3'-untranslated region (UTR) or 5'-UTR of *Eif1a* did not show a change in expression (see more detailed analysis below). This suggested the presence of more than one *Eif1a* genes.

Thorough analysis of NIA Mouse Gene Index³¹ and Mouse Genome Sequence (9 and 10 mm)³² identified *Eif1a* (Chromosome 18), *Eif1ax* (Chromosome X), and a cluster of 19 *Eif1a*-like genes on mouse Chromosome 12. Based on their protein-coding capacity, we noted 10 *Eif1a*-like genes (named here *Eif1a11*–*Eif1a10*) and nine pseudogenes (named here *Eif1a-ps1*–*Eif1a-ps9*; Fig. 3A). The *Eif1a*-like genes could be

distinguished from each other by their mRNA sequences (Fig. 3B) and protein sequences (Fig. 4A) when the sequences were aligned using the Clustal OMEGA program.^{33,34} However, the protein sequences between the coding region of *Eif1a*³⁵ (Chromosome 18) and the *Eif1a*-like genes were highly conserved, especially in the N-terminal half of the protein sequences (Supplementary Fig. S1), which is the region to which the *Eif1a* antibody (ab38976, Abcam) we used was raised against. Considering the predominant expression of *Eif1a*-like genes in the *Zscan4*⁺ state of ES cells, the results shown in Fig. 1Aiv–vi and C required re-

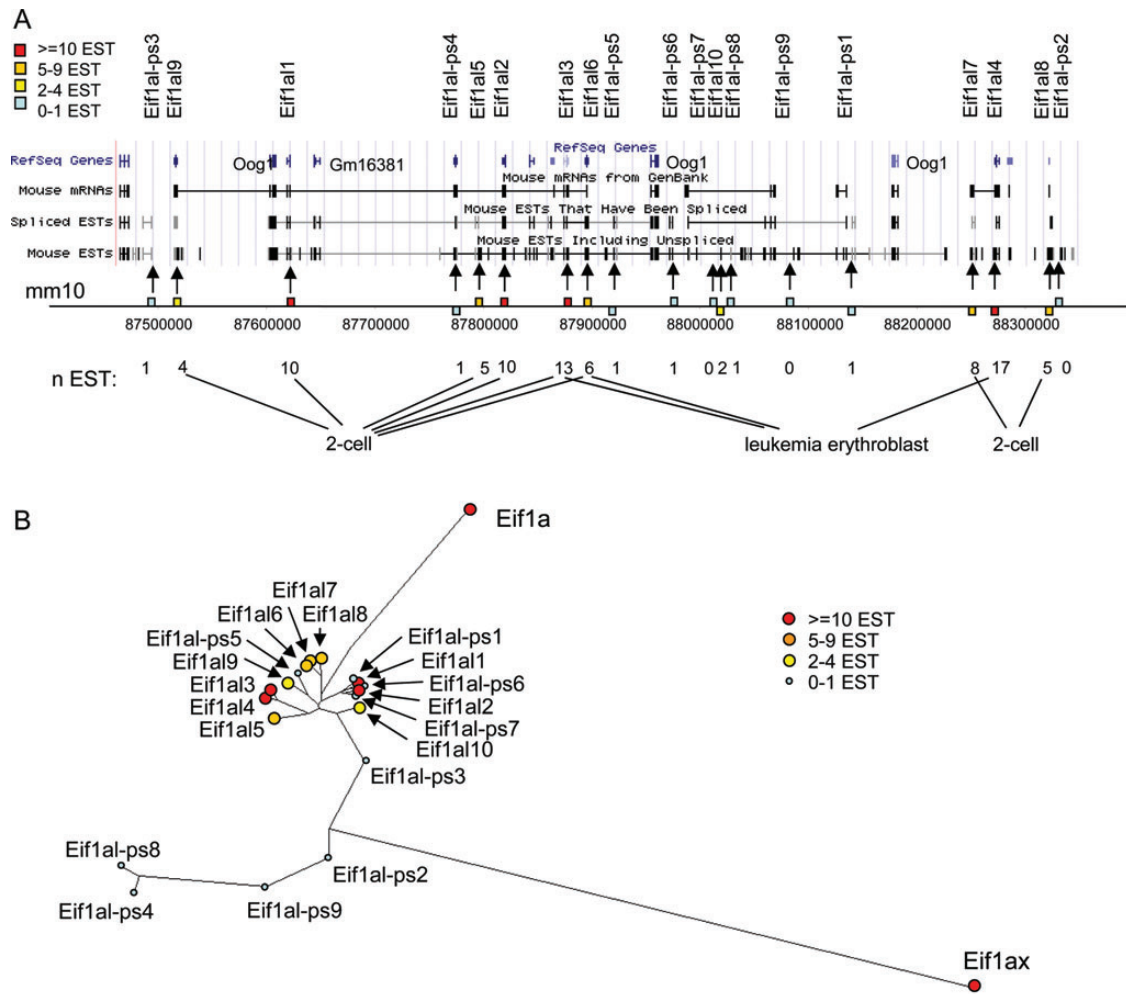


Figure 3. A cluster of *Eif1 a*-like genes is newly identified. (A) Schematic diagram of Chromosome 12 *Eif1 a*-like genes. *Eif1 a*-like genes were initially found by transcript assembly from the EST database (NIA Mouse Gene Index, <http://lgsun.grc.nia.nih.gov/geneindex/>).³¹ Subsequently, the full set of these genes was discovered by aligning known sequences (*Eif1 a*, *Eif1 ax*, and *Eif1 a* like) to the genome (10 mm) using the BLAT software (<http://genome.ucsc.edu/cgi-bin/hgBlat>), and then displayed within the UCSC Genome Browser. Genome targets were extracted and repeatedly aligned until no new alignment was found. Forward-strand genes are shown by coloured boxes above the genome line, and reverse-strand genes are shown below the line. The colour of each box corresponds to the number of ESTs that matched these genes in the UCSC Genome Browser. (B) Dendrogram of similarity between mRNA sequences of *Eif1 a*, *Eif1 ax*, and *Eif1 a*-like genes. Multiple sequence alignment was done by CLUSTALW (<http://www.genome.jp/tools/clustalw>), and the dendrogram was plotted using TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). The colour of each circle corresponds to the number of ESTs that matched to genes in the UCSC Genome Browser.

interpretation, as the enhanced immunofluorescence signal detected by the *Eif1 a* antibody in cells enriched with the *Zscan4*⁺ state was of *Eif1 a*-like genes and not *Eif1 a*.

The analysis of expressed sequence tag (EST) frequency in the NIA Mouse Gene Index³¹ showed that *Eif1 a*-like genes were expressed mostly in two-cell embryos and to some extent in leukaemia erythroblasts (Fig. 3A; Supplementary Table S1). With the current genomic information available, *Eif1 a*-like genes clusters were not identified in human or rat genomes. However, due to the highly repetitive nature of this region, there is potential for identification of this cluster in future genome assemblies. Currently, this

Eif1 a-like gene cluster can only be confirmed in the mouse. Interestingly, 5 of 19 genes and pseudogenes from this mouse Chromosome 12 cluster (*Eif1 a*-ps3, *Eif1 a*9, *Eif1 a*2, *Eif1 a*-ps6, and *Eif1 a*-ps7) were found to have synteny to human Chromosome 1 pseudogene EIF1AXP1 (ENSG00000236698) using the Multi-VISTA browser.³⁶

Next, we looked at the microarray data with the new annotations of the 60-mer probes for *Eif1 a* and *Eif1 a*-like genes. Thus, the probe that targeted a protein-coding region can detect both *Eif1 a* and *Eif1 a*-like genes, showing that a 10.5-fold increase in the *Zscan4*⁺ state of ES cells compared with the *Zscan4*⁻ state, whereas probes that targeted the 3'-UTR of

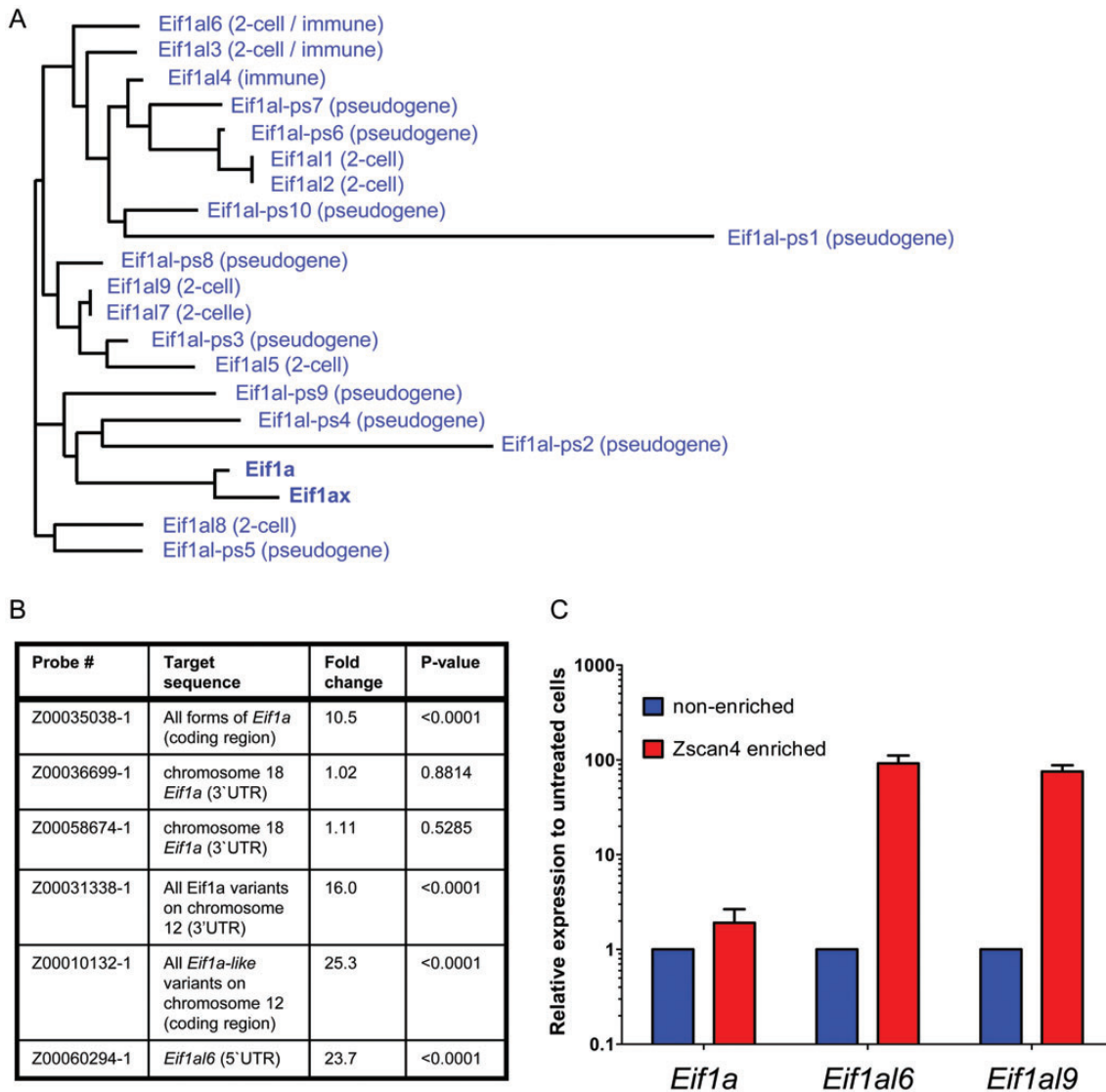


Figure 4. mRNA levels of *Eif1a*-like genes on Chromosome 12 are highly up-regulated, but not *Eif1a* in *Zscan4*⁺ state mouse ES cells. (A) The phylogeny tree of *Eif1a*-like gene protein sequence alignment was performed using Clustal OMEGA (EMBL-EBI). (B) Microarray data¹⁰ were performed on samples isolated from FACS-enriched *pZscan4*-Emerald mouse ES cells and compared with non-enriched cells. Fold changes in *Eif1a* and *Eif1a*-like gene mRNA levels were determined using NIA Array Analysis programme. *P*-values were determined using analysis of variance. (C) Quantitative real-time PCR analysis of *Eif1a* and *Eif1a*-like genes (*Eif1a6* and *Eif1a9*): mRNA levels in *pZscan4*-EGFP-puro mouse ES cells enriched for *Zscan4*⁺ state cells by puromycin selection for 2 days. Results were normalized to *Gapdh* and made relative to the non-enriched sample for each primer set (mean \pm SEM; non-enriched $n = 4$, *Zscan4*⁺ state enriched $n = 2$).

Eif1a, detecting only *Eif1a*, did not show a change in expression (Fig. 4B). More specifically for *Eif1a*-like genes, microarray probes against a conserved region on 3'-UTR (16.1-fold increase, P -value <0.0001), coding region (25.4-fold increase, P -value <0.0001), and 5'-UTR of *Eif1a6* (23.8-fold increase, P -value <0.0001) all showed an increase in the *Zscan4*⁺ state of ES cells compared with the *Zscan4*⁻ state (Fig. 4B).

To further validate the microarray results, we carried out that quantitative real-time PCR on RNA samples extracted from cells enriches with the *Zscan4*⁺ state by puromycin selection of the *pZscan4*-puromycin cells using PCR primers designed specifically against a

non-conserved region of the 3'-UTR of *Eif1a* versus *Eif1a6* (U106343) and *Eif1a9* (U285359). Primers against the specific *Eif1a*-like genes may also detect other *Eif1a*-like genes, because this region in the 3'-UTR among the *Eif1a*-like genes are highly similar to, but are different from the *Eif1a* sequence. The results showed that the two *Eif1a*-like genes tested (*Eif1a6* and *Eif1a9*) had nearly a 100-fold increase in RNA levels in cells enriched with the *Zscan4*⁺ state compared with non-puromycin-selected cells (Fig. 4C). The expression of *Eif1a* was only slightly enhanced in cells enriched with the *Zscan4*⁺ state (~1.5-fold increase).

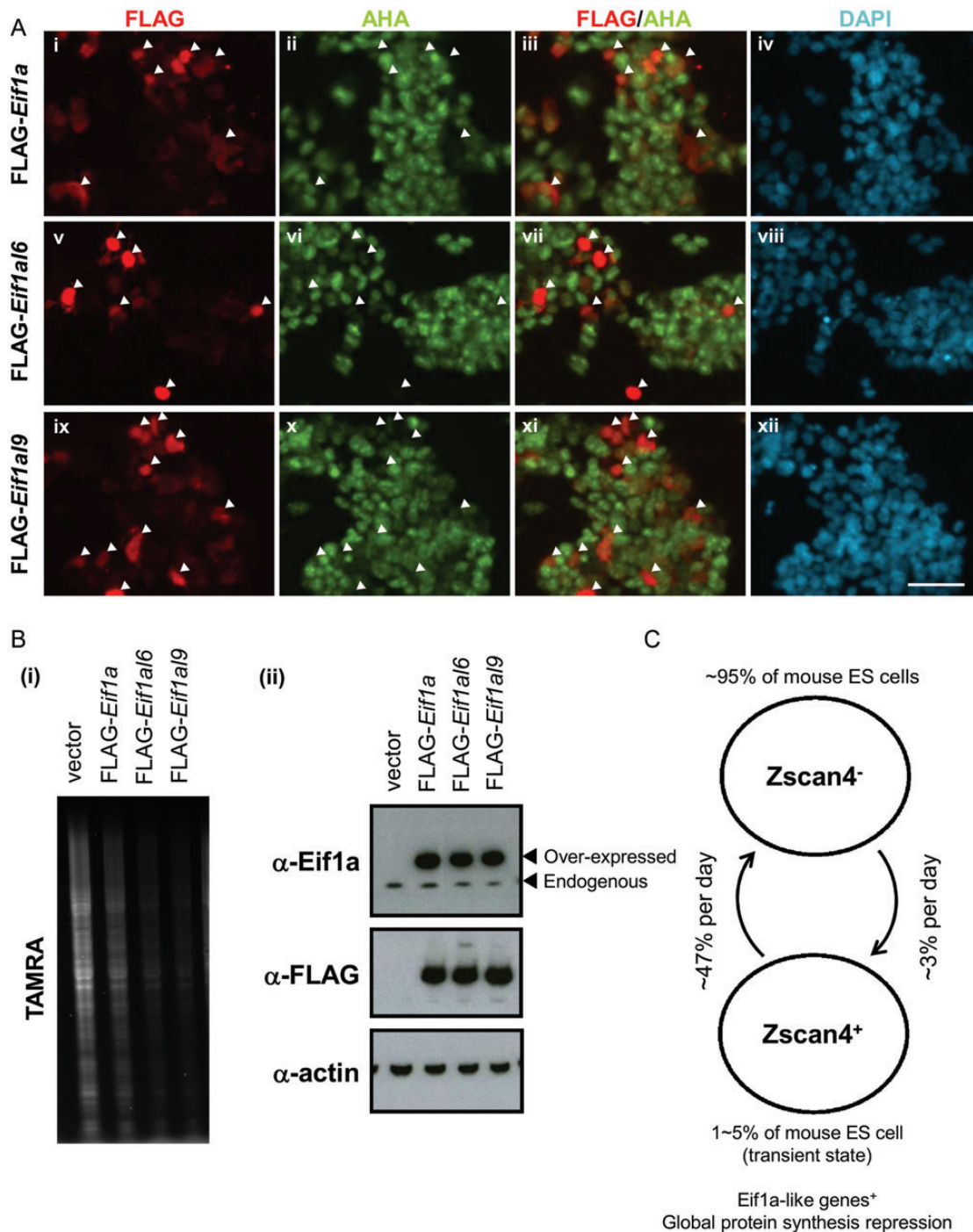


Figure 5. Over-expression of Eif1 a-like genes represses global nascent protein translation. (A) mESC were transiently transfected with FLAG-tagged *Eif1a* (i–iv) or FLAG-*Eif1a*-like genes, *Eif1a16* (v–viii) or *Eif1a19* (ix–xii), constructs for 18 h before selection with puromycin for 24 h. Cells were then cultured in methionine-free ES cell media for 30 min and labelled with AHA for 1 h. AHA-labelled cells were visualized with Alexa Fluor 488 alkyne through Click-iT reaction (ii, vi, and x) and co-stained with FLAG antibody (i, v, and ix). DNA was visualized by DAPI staining (iv, viii, and xii). Pictures were taken at $\times 40$ magnification using a Nikon Eclipse TE300 microscope (scale bar indicates 50 μ m). White arrows indicated cells with high levels of FLAG-*Eif1a* or FLAG-*Eif1a*-like genes over-expression. (B) Cells were labelled with AHA as described above and protein harvested using RIPA buffer containing protease inhibitors. AHA-labelled samples were then detected using TAMRA alkyne via Click-iT reaction (i). Samples were run on a 4–12% Bis-Tris SDS–PAGE acrylamide gel, transferred on the PVDF membrane, and blotted with antibodies against Zscan4, Eif1a and beta-actin (ii). (C) The model of global protein synthesis repression in Zscan4⁺ and Zscan4⁻ states in mouse ES cells. Diagram modified from Zalzman *et al.*⁶

Together, these results demonstrated that *Eif1a*-like genes, not *Eif1a*, were highly expressed in two-cell stage pre-implantation embryos and in the *Zscan4*⁺ state of ES cells.

3.3. Over-expression of *Eif1a*-like genes can repress global protein synthesis

To further determine the mechanism for global protein synthesis repression in the *Zscan4*⁺ state of ES cells, we investigated whether over-expression of *Eif1a*-like genes can repress global protein synthesis. We generated FLAG-tagged constructs of *Eif1a16* (FLAG-*Eif1a16*) and *Eif1a19* (FLAG-*Eif1a19*) and transfected them into ES cells, followed by the analysis of the nascent protein synthesis determined via AHA labelling and immunofluorescence. Both cells transfected with either FLAG-*Eif1a16* or FLAG-*Eif1a19* showed reduction in AHA labelling, whereas cells transfected with the FLAG-*Eif1a* did not always show a consistent reduction in AHA labelling (Fig. 5A). Furthermore, using the TAMRA secondary antibody to detect the AHA-labelled proteins on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel of samples extracted from cells transfected with *Eif1a*-like genes, FLAG-*Eif1a*-like cells showed a clear reduction in AHA labelling, when compared with cells transfected with either vector alone or FLAG-*Eif1a* (Fig. 5Bi).

The western blot analysis of the SDS-PAGE gel used for the TAMRA antibody detection above showed that the levels of FLAG-*Eif1a*-like proteins and FLAG-*Eif1a* proteins were similar to detected by *Eif1a* and FLAG antibodies (Fig. 5Bii). This indicates that the protein synthesis repression observed was not due to differences in expression levels of the various constructs. Taken together, these results showed that over-expression of *Eif1a*-like genes were able to repress protein synthesis globally and demonstrated a mechanism by which global protein synthesis was repressed in the *Zscan4*⁺ state of ES cells.

4. Discussion

We report here a novel mechanism to repress global protein synthesis in mouse ES cells. As shown previously, mouse ES cells oscillate between the *Zscan4*⁻ and *Zscan4*⁺ states: the *Zscan4*⁻ state turns into the *Zscan4*⁺ state at a rate of ~3% per day, whereas the *Zscan4*⁺ state turns into the *Zscan4*⁻ state at a rate of ~47% per day (Fig. 5C).⁶ The *Zscan4*⁺ state is transient and relatively short; however, while ES cells are in the *Zscan4*⁺ state, several dramatic changes occur, including the up-regulation of meiosis-specific genes, a rapid extension of telomeres by a telomerase-independent recombination-based mechanism.⁶ The

current work now adds the global repression of nascent protein synthesis as one of the unique feature of the transient *Zscan4*⁺ state. These results indicate that ES cells undergo the short period of the repressed protein synthesis in the standard cell culture condition.

The current study also provides further evidence for the commonality between the *Zscan4*⁺ state of mouse ES cells and the two-cell stage mouse embryos. The expression of *Zscan4*, which is originally identified as genes expressed specifically in two-cell embryos,⁴ has indicated such commonality.⁶ Further evidence for such commonality has been provided recently: this transient state is also marked with the expression of endogenous retroviruses, such as MuERV-L, which are also expressed specifically in mouse two-cell stage embryos.⁷ Finding the unique expression of *Eif1a*-like genes both in the two-cell mouse embryos and in the *Zscan4*⁺ state of ES cells provides further connection between them. Considering the global repression of protein synthesis in the *Zscan4*⁺ state of ES cells, it would be interesting to know whether there is a novel mechanism for protein synthesis, or protein synthesis repression, during the ZGA in the development of pre-implantation embryo.

High sequence homology of *Eif1a*-like genes to *Eif1a* suggests that only the few amino acid differences to the *Eif1a*-like genes are responsible for global protein repression. Thus, it is highly likely that *Eif1a*-like genes function as a naturally occurring dominant-negative factor, which interferes with the function of *Eif1a* as the ubiquitous factor important for the translation machinery in eukaryotes. This may indicate the possible use of *Eif1a*-like genes as potent repressors of the global protein synthesis in basic and clinical applications.

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