EMBO reports

scientific report

Transformation of eEF1Bδ into heat-shock response transcription factor by alternative splicing

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Protein translation factors have crucial roles in a variety of stress responses. Here, we show that eukaryotic elongation factor 1Bδ (eEF1Bδ) changes its structure and function from a translation factor into a heat-shock response transcription factor by alternative splicing. The long isoform of eEF1Bδ (eEF1BδL) is localized in the nucleus and induces heat-shock element (HSE)-containing genes in cooperation with heat-shock transcription factor 1 (HSF1). Moreover, the amino-terminal domain of eEF1BδL binds to NF-E2-related factor 2 (Nrf2) and induces stress response haem oxygenase 1 (HO1). Specific inhibition of eEF1BδL with small-interfering RNA completely inhibits Nrf2-dependent HO1 induction. In addition, eEF1BδL directly binds to HSE oligo DNA *in vitro* and associates with the HSE consensus in the HO1 promoter region *in vivo*. Thus, the transcriptional role of eEF1BδL could provide new insights into the molecular mechanism of stress responses.

Keywords: eEF1B; heat shock; splicing

EMBO *reports* (2011) **12**, 673–681. doi:10.1038/embor.2011.82

INTRODUCTION

The adaptive biological responses underlying resistance to various stressors, including thermal and oxidative stress, seem to be important for eukaryotic behaviour, lifespan and diseases (Morimoto, 2008; Prahlad & Morimoto, 2008; Saunders *et al*, 2009). These stress-response pathways are regulated by master transcription factors, such as heat-shock factor 1 (HSF1) and NF-E2-related factor 2 (Nrf2; Morimoto, 1998; Motohashi & Yamamoto, 2004). Most heat-shock proteins (HSPs) are molecular chaperones that were originally defined according to their increased expression in response to cellular insults such as elevated temperature and oxidative stress, through these transcription factors (Jacquier-Sarlin & Polla, 1996; Morimoto, 1998). Chaperones have crucial roles in these stress responses and stress-related diseases (Morimoto, 1998, 2008; Prahlad & Morimoto, 2008).

Translational repression is a well-characterized mechanism of adaptation to a variety of stresses; therefore, protein translation factors are important in stress responses and human diseases (Holcik & Sonenberg, 2005; Tettweiler *et al*, 2005; Scheper *et al*, 2007; Anderson *et al*, 2009). Protein translation in eukaryotes requires a set of non-ribosomal proteins known as elongation factors (eEFs; Merrick & Nyborg, 2000; Andersen *et al*, 2003). The factors involved in amino acyl-transfer RNA recruitment onto the ribosome are eEF1A and eEF1B, whereas ribosomal translocation requires eEF2. eEF1B catalyses the exchange of guanosine 5′-diphosphate bound to the G-protein eEF1A in the elongation cycle. Thus, eEF1B functions as a guanine nucleotide exchange factor (GEF) for eEF1A (Merrick & Nyborg, 2000; Le Sourd *et al*, 2006). In vertebrates, the eEF1B complex is composed of four subunits: catalytic eEF1Bα, eEF1Bβ, eEF1Bδ and structural eEF1Bγ (Merrick & Nyborg, 2000; Andersen *et al*, 2003; Le Sourd *et al*, 2006). However, the molecular mechanisms used by these subunits for protein translation, and the other cellular functions of each subunit remain to be established.

In this study, we attempt to identify the functional roles of the eEF1B complex in the stress response in mammalian cells. We find that eEF1Bδ has a long isoform (eEF1BδL) that is highly expressed in the brain and testis. eEF1BδL is localized in the nucleus and facilitates transcription of heat-shock element (HSE)-containing genes in cooperation with HSF1. These results show that tissue-specific alternative splicing changes a translation factor to an HSE-dependent transcription factor.

RESULTS AND DISCUSSION

eEF1BδL is a brain- and testis-specific isoform

To investigate how the eEF1B complex contributes to stress responses, we examined the tissue distribution of eEF1Bδ by using eEF1Bδ antibody, and found high expression levels of the long isoform of eEF1Bδ (eEF1BδL) protein in the cerebrum, cerebellum and testis (Fig 1A). Several splicing variants of eEF1Bδ were detected around 30–40 kDa and have been previously reported to

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Received 24 November 2010; revised 13 March 2011; accepted 7 April 2011; published online 20 May 2011

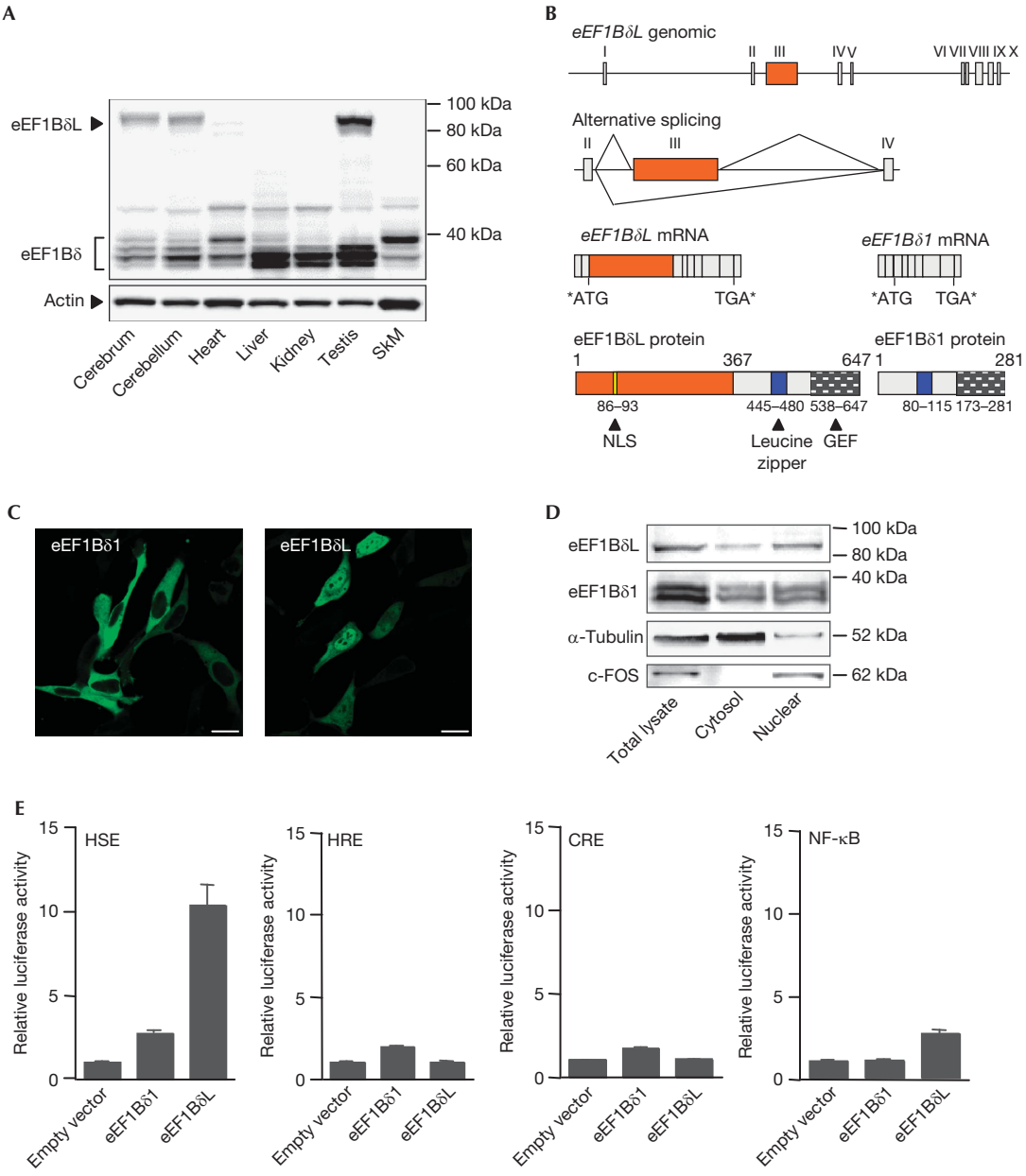


Fig 1 | Characteristics of eEF1BδL. (**A**) Immunoblot analysis of eEF1Bδ isoforms was performed on total lysates from adult mouse tissues with eEF1Bδ antibody. (**B**) Schematic illustration of the *eEF1Bδ* gene and protein. Short- or long-isoform eEF1Bδ mRNA is expressed depending on whether exon III is skipped. Numbers of amino acids are shown. (**C**) HeLa cells were transfected with expression plasmids encoding GFP–eEF1Bδ1 or eEF1BδL fusion protein. GFP fluorescence was analysed by confocal microscopy. Scale bar, 20 μm. (**D**) Subcellular localization of endogenous eEF1Bδ1 and eEF1BδL in mouse hippocampal neurons with eEF1Bδ antibody. (**E**) HEK293 cells were co-transfected with empty vector, expression plasmids encoding Flag-tagged-eEF1Bδ1, or eEF1BδL protein and reporter plasmids as indicated. Luciferase activity was measured by the dual-luciferase reporter assay. Data represent means ± s.e.m. ($n = 4$). CRE, cAMP-responsive element; eEF1Bδ, eukaryotic elongation factor 1Bδ; eEF1BδL, long isoform of eEF1Bδ; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; HRE, hypoxia-responsive element; HSE, heat-shock element; mRNA, messenger RNA; NF-κB, nuclear factor-κB; NLS, nuclear localization signal.

be translation factors (Le Sourd *et al*, 2006). In agreement with these results, high levels of eEF1BδL messenger RNA (mRNA) were detected in the brain and testis, whereas eEF1Bδ1 mRNA was ubiquitously expressed (supplementary Fig S1A online). In the brain, eEF1BδL was found to be expressed from embryonic day (E) 15 to the adult stage (supplementary Fig S1B online). Two isoforms were generated by alternative splicing events among exons II, III and IV (Fig 1B). The short isoform eEF1Bδ1 is ubiquitously

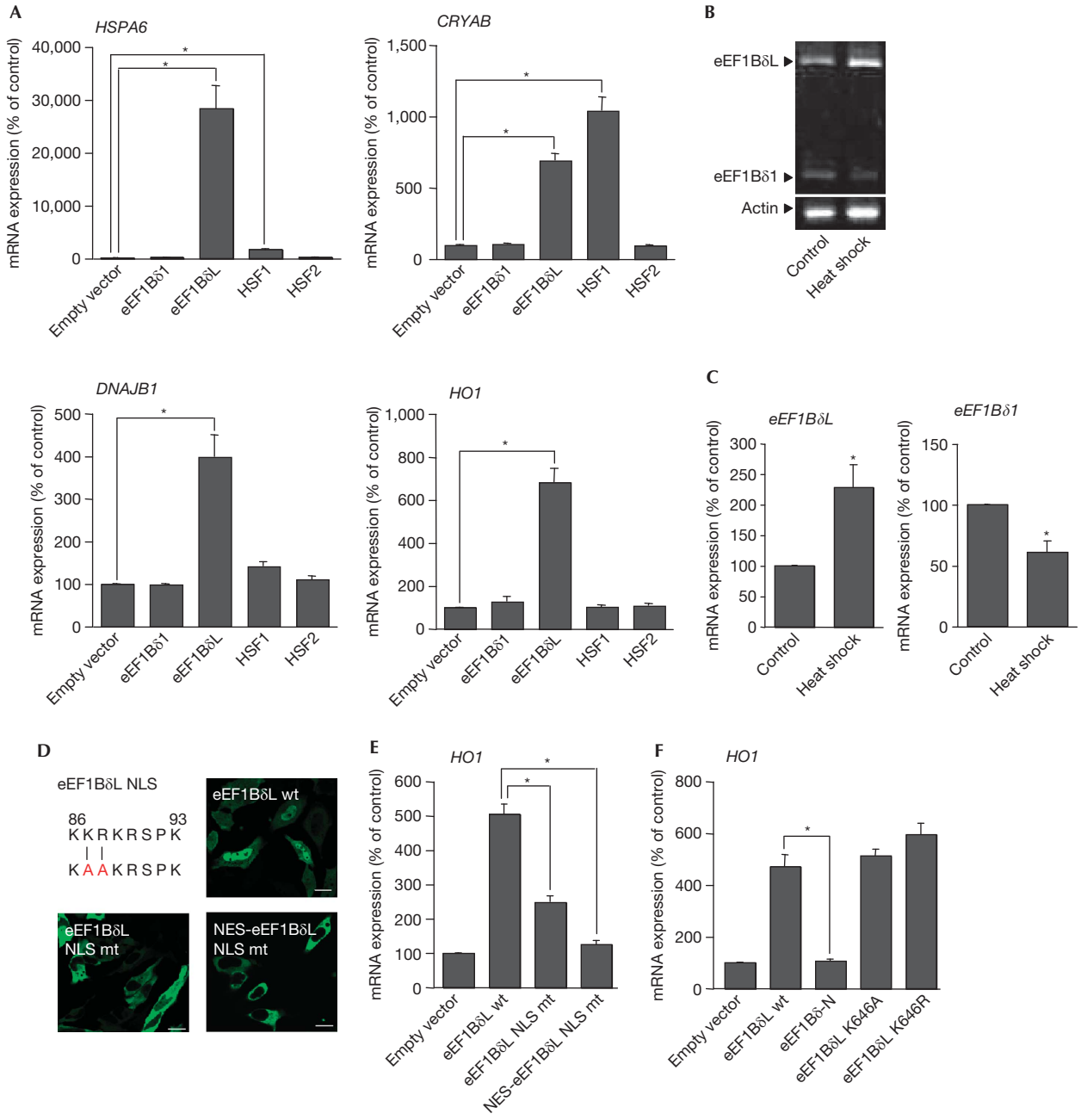


Fig 2 | eEF1BδL regulates induction of heat-shock-responsive genes. (**A**) HEK293 cells were transfected with empty vector, expression plasmids encoding Flag-tagged-eEF1Bδ1, eEF1BδL protein, HSF1 or HSF2. RNA was extracted and gene expression was analysed by quantitative RT–PCR. (**B**) Regulated alternative splicing in control and heat-shock-treated neurons was analysed by RT–PCR, using a primer to specifically monitor splicing in the *eEF1Bδ* gene with a representative gel image. Heat-shock treatment was performed at 42 °C for 1.5 h, followed by recovery period of 18 h at 37 °C. (**C**) Quantification of eEF1Bδ1 and eEF1BδL mRNA levels in (**B**) is presented in a graph (**C**). (**D**) Upper panel: amino-acid sequence alignment for the NLS motif (amino acids 86–93) in eEF1BδL protein. Mutated amino acids are highlighted (red). HEK293 cells were transfected with expression plasmids encoding GFP–eEF1BδL, eEF1BδL NLS mutant, or NES–eEF1BδL NLS mutant fusion protein. GFP fluorescence was analysed by confocal microscopy. Scale bar, 20 μm. (**E**) Quantitative RT–PCR analysis of HO1 mRNA in HEK293 cells transfected with empty vector, expression plasmids encoding Flag-tagged-eEF1BδL, eEF1BδL NLS mutant or NES–eEF1BδL NLS mutant protein. (**F**) Quantitative RT–PCR analysis of HO1 mRNA in HEK293 cells transfected with empty vector, expression plasmids encoding Flag-tagged-eEF1BδL wild-type, eEF1Bδ-N, eEF1BδL K646A or K646R mutant protein. Data represent means ± s.e.m. *t*-test, $*P<0.05$ compared with corresponding control (**A**, $n=5$; **C**, $n=7$; **E,F**, $n=4$). eEF1Bδ, eukaryotic elongation factor 1Bδ; eEF1BδL, long isoform of eEF1Bδ; GFP, green fluorescent protein; HO1, haem oxygenase 1; HSF, heat-shock transcription factor; mRNA, messenger RNA; mt, mutant; NES, nuclear export signal; NLS, nuclear localization signal; RT–PCR, reverse transcription-PCR; wt, wild type.

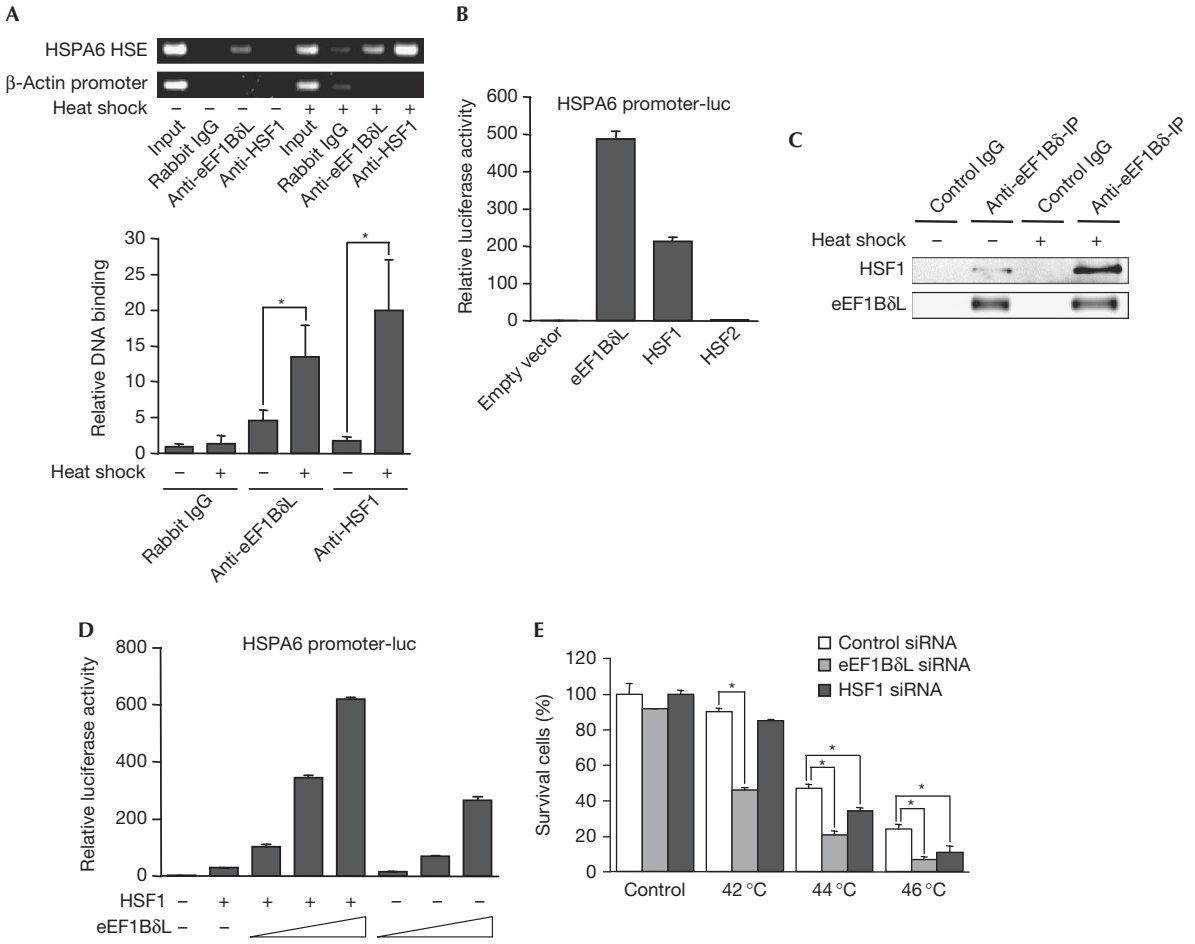


Fig 3 | eEF1BδL binds to HSF1 and associates with HSPA6 promoter. (**A**) CHIP assay was performed on endogenous eEF1BδL protein in HEK293 cells treated with or without heat shock at 42 °C for 1 h. The heat-shock element (HSE) fragment of HSPA6 promoter was detected by PCR amplification from immunoprecipitates, as indicated. β-Actin promoter was used as a control. Quantification of the CHIP analysis by quantitative PCR is shown in the graph (bottom). (**B**) HEK293 cells were co-transfected with empty vector, expression plasmids encoding Flag-tagged-eEF1BδL, HSF1 or HSF2 protein and reporter plasmid driven by the HSPA6 promoter, and luciferase activity was measured. (**C**) A co-immunoprecipitation assay on endogenous eEF1BδL and HSF1 protein was performed in HEK293 cells treated with or without heat shock at 42 °C for 1 h. Immunoprecipitates with eEF1Bδ antibody were blotted with antibodies as indicated. (**D**) HEK293 cells were co-transfected with 50 ng empty vector or 50 ng expression plasmids encoding Flag-tagged-HSF1 protein and with either empty vector or increasing amount of expression plasmids encoding Flag-tagged-eEF1BδL protein (50, 100 or 200 ng), and HSPA6 promoter-driven reporter activity was measured. (**E**) HeLa cells treated with 5 nM eEF1BδL or HSF1 siRNA were incubated at 42, 44 or 46 °C for 4 h and cell viability was measured. Data represent means ± s.e.m. *t*-test, $*P<0.05$ compared with corresponding control (**A**, $n=6$; **B,D**, $n=3$; **E**, $n=4$). CHIP, chromatin immunoprecipitation; eEF1Bδ, eukaryotic elongation factor 1Bδ; eEF1BδL, long isoform of eEF1Bδ; HSF, heat-shock transcription factor; IgG, immunoglobulin G; IP, immunoprecipitation; siRNA, small interfering RNA.

transcribed by skipping exon III, whereas the long isoform eEF1BδL contains the 1130-nucleotide exon III and a start codon (AUG) that extends in-frame into the subsequent eEF1Bδ1 sequence. Exon III encodes a 367-amino-acid amino-terminus in the eEF1BδL protein, containing a putative nuclear localization signal (NLS) at amino acids 86–93. The carboxy-terminal region, which is the same as in eEF1Bδ1, contains a leucine zipper and a GEF.

To examine subcellular localization, green fluorescent protein–eEF1Bδ1 or eEF1BδL fusion protein was transfected into HeLa cells. eEF1Bδ1 was localized in the cytoplasm and excluded from the nucleus (Fig 1C). By contrast, eEF1BδL was localized in the cytoplasm and the nucleus (Fig 1C). Localization of endogenous eEF1BδL in mouse neurons was tested by subcellular fractionation, followed by immunoblot analysis using eEF1Bδ antibody, which recognizes residues 347–647. Both endogenous eEF1Bδ1 and eEF1BδL proteins were detected in the cytoplasm and nuclear fraction (Fig 1D). eEF1BδL was more abundant in the nuclear fraction than eEF1Bδ1 (Fig 1D).

eEF1BδL induces expression of HSE-containing gene

We investigated the nuclear function of eEF1BδL with various luciferase reporters. When overexpressed in HEK293 cells, eEF1BδL, but not eEF1Bδ1, induced HSE-containing reporter

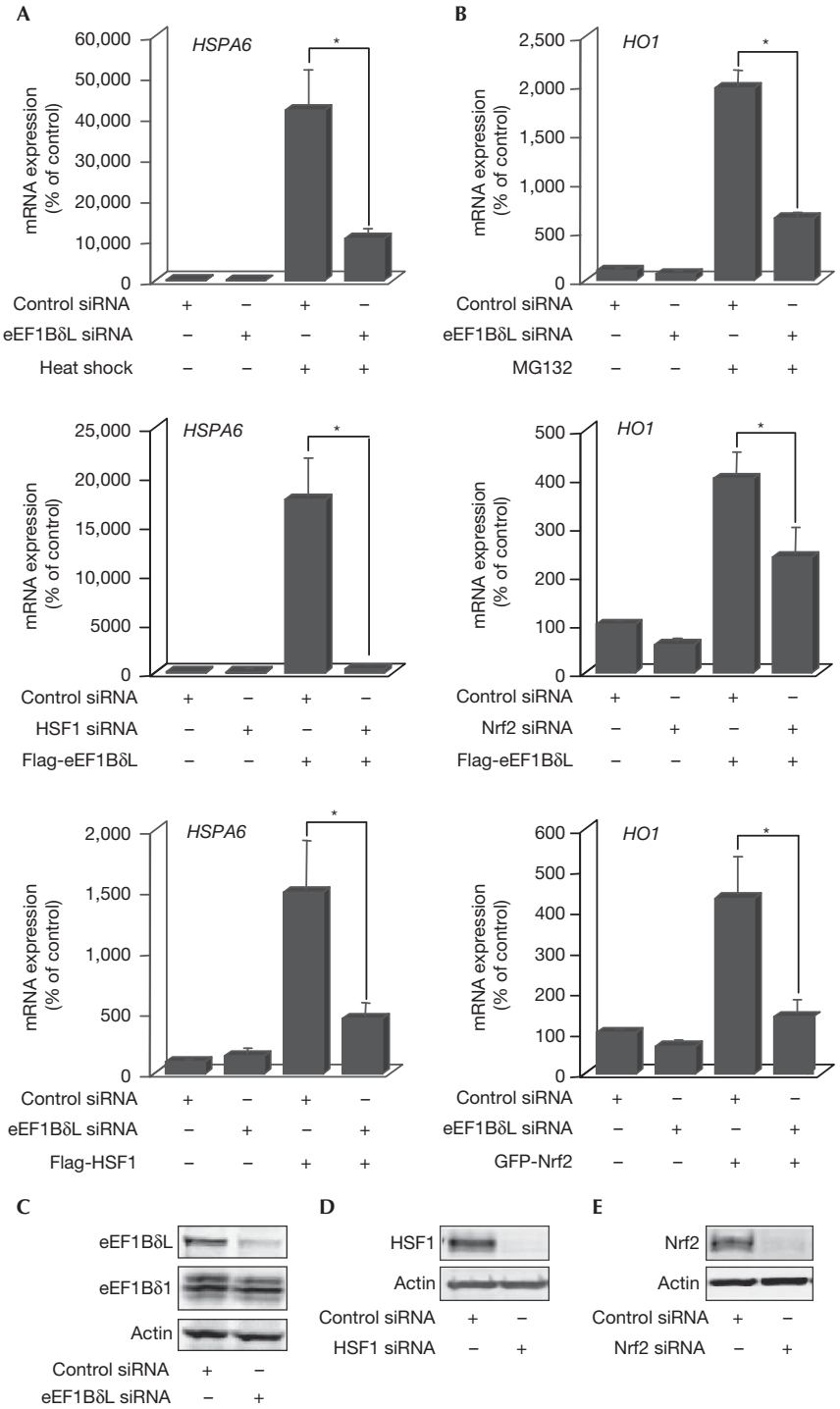
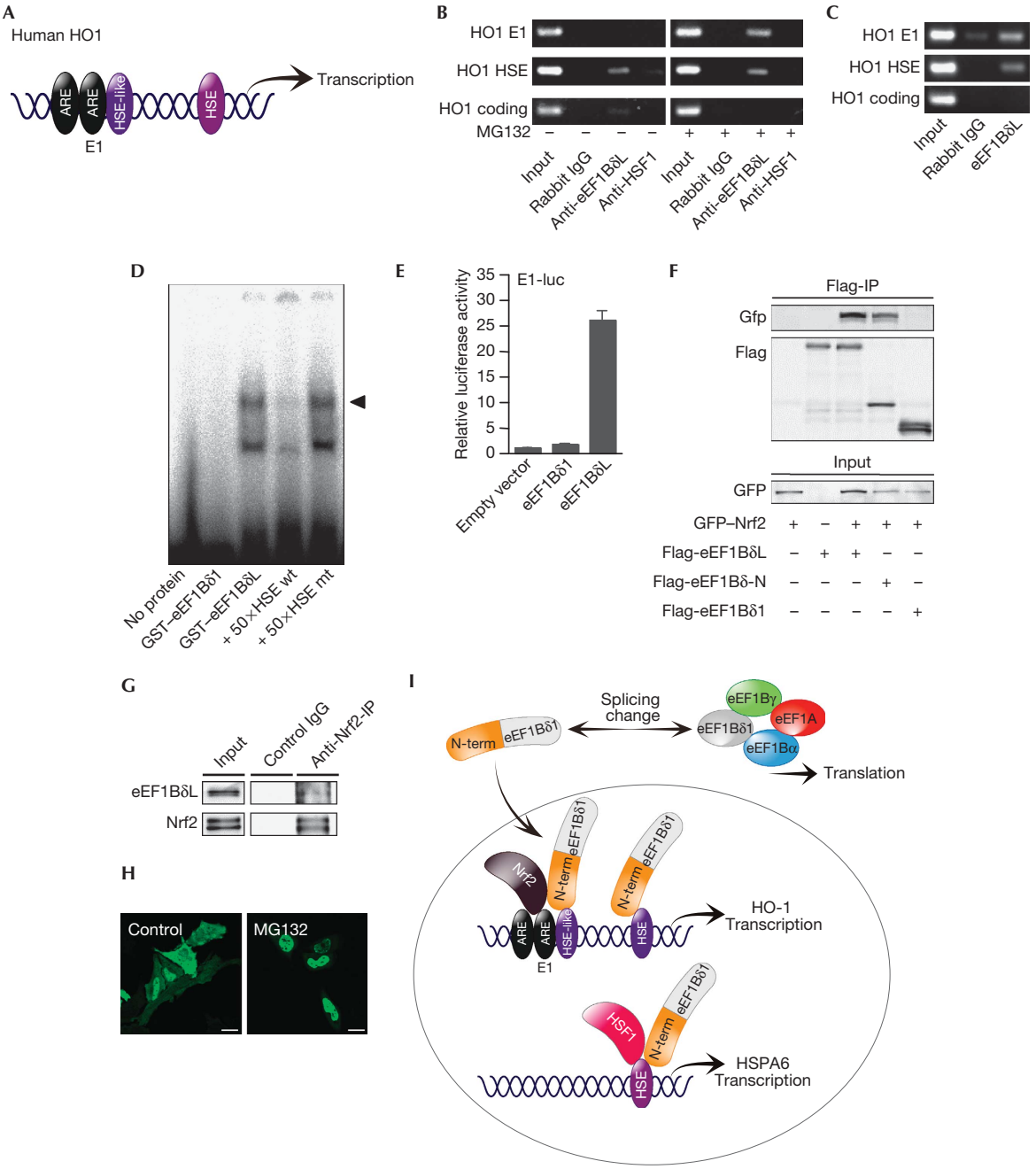


Fig 4 | eEF1BδL is required for stress-induced gene expression through HSF1 and Nrf2. (**A**) Upper panel: effects of eEF1BδL knockdown on HSPA6 mRNA expression induced by heat-shock treatment at 42 °C for 1 h, followed by recovery period of 3 h at 37 °C. Middle panel: effect of HSF1 knockdown on HSPA6 expression induced by overexpression of Flag-eEF1BδL. Lower panel: effect of eEF1BδL knockdown on HSPA6 expression induced by overexpression of Flag-HSF1. (**B**) Upper panel: effects of eEF1BδL knockdown on HO1 mRNA expression induced by 10 μM MG132 treatment for 6 h. Middle panel: , effects of Nrf2 knockdown on HO1 expression induced by overexpression of Flag-eEF1BδL. Lower panel: effect of eEF1BδL knockdown on HO1 expression induced by overexpression of GFP–Nrf2. Data represent means ± s.e.m. *t*-test, $*P<0.05$ compared with corresponding control (**A,B**, $n=4$). (**C–E**) Immunoblot analysis of endogenous eEF1BδL and eEF1Bδ1 (**C**), HSF1 (**D**) and Nrf2 (**E**) protein levels in HEK293 cells transfected with 50 nM eEF1BδL siRNA, 5 nM HSF1 or 5 nM Nrf2 SMARTpool siRNA as indicated. eEF1Bδ, eukaryotic elongation factor 1Bδ; eEF1BδL, long isoform of eEF1Bδ; GFP, green florescence protein; HO1, haem oxygenase 1; HSF1, heat-shock transcription factor 1; mRNA, messenger RNA; Nrf2, NF-E2-related factor 2; siRNA, small-interfering RNA.



activity (Fig 1E). By contrast, eEF1BδL had minimal effects on other promoters containing the consensus hypoxia-responsive element, cAMP-responsive element or nuclear factor-κB binding element, thus suggesting that eEF1BδL might function as a modulator of HSE-containing genes. Among the eEF1B complex, *eEF1BδL* was the only gene able to activate the HSE reporter (supplementary Fig S2A online). Although eEF1A is a co-activator of HSF1 (Shamovsky *et al*, 2006), HSE-dependent transcriptional activity was not observed in cells overexpressing eEF1A (supplementary Fig S2A,B online). We next examined the expression of genes induced by eEF1BδL. Total RNA was collected from HEK293 cells transfected with expression plasmids encoding Flag-tagged-eEF1Bδ1 or eEF1BδL protein and was analysed using microarray. eEF1BδL induced several HSE-containing genes (supplementary Fig S2C online), which was validated by quantitative reverse transcription (RT)–PCR (supplementary Fig S2D online). To compare the effect of eEF1BδL with HSF1 and HSF2, we examined functionally well-known genes including *HSPA6*, *DNAJB1*, *CRYAB* and *haem oxygenase 1* (*HO1*) by using quantitative RT–PCR in transfected HEK293 cells. Consistent and marked induction by eEF1BδL was seen in the indicated genes (Fig 2A). HSF1 induced the *HSPA6* and *CRYAB* genes, as previously reported (Head *et al*, 1996; Trinklein *et al*, 2004), but not *DNAJB1* and *HO1*, whereas HSF2 did not increase the

◀ **Fig 5** | eEF1BδL directly binds to heat-shock element consensus in HO1 promoter. (**A**) Schematic representation of human HO1 promoter. (**B**) CHIP assay was performed on endogenous eEF1BδL and HSF1 protein in HEK293 cells treated with or without 10 μM MG132 for 6 h. The E1 or HSE fragment of HO1 promoter was detected by PCR amplification from immunoprecipitates, as indicated. HO1 coding region was used as control. (**C**) CHIP assay was performed on endogenous eEF1BδL protein in mouse testis. (**D**) An electrophoretic mobility shift assay was performed using HSE consensus oligo in the presence of GST–eEF1Bδ1 or eEF1BδL recombinant protein. A 50-fold molar excess of the wild-type HSE oligo or the mutated HSE oligo to the binding reaction was also added, as indicated. HSE–eEF1BδL binding complexes are marked with arrows. (**E**) HEK293 cells were co-transfected with empty vector, expression plasmids encoding Flag-tagged-eEF1Bδ1 or eEF1BδL protein and reporter plasmid driven by E1 of the HO1 promoter, and luciferase activity was measured. Data represent mean ± s.e.m. ($n = 4$). (**F**) A co-immunoprecipitation assay of Flag-eEF1BδL and GFP–Nrf2 protein was performed in transfected HEK293 cells treated with 2 μM MG132 for 12 h. Upper and middle panels: immunoprecipitates of eEF1Bδ-L, -N or -1 with anti-Flag beads were blotted with antibodies as indicated. Lower panel: whole-cell lysates were blotted with GFP antibody. (**G**) A co-immunoprecipitation assay on endogenous eEF1BδL and Nrf2 protein was performed in HEK293 cells treated with 2 μM MG132 for 12 h. Immunoprecipitates with Nrf2 antibody were blotted with antibodies as indicated. The input levels of eEF1BδL and Nrf2 are indicated. (**H**) HeLa cells were transfected with expression plasmids encoding GFP–eEF1BδL fusion protein. 24 h after transfection, cells were treated with 10 μM MG132 for 3 h, GFP fluorescence was then analysed by confocal microscopy. Scale bar, 20 μm. (**I**) eEF1Bδ1 forms a complex with eEF1Bα and eEF1Bγ. Their complex formation supports the canonical function of GTP/GDP exchange on eEF1A protein and has a crucial role in translation fidelity in the cytoplasm. eEF1BδL protein is produced by a splicing change, and functions as a transcription factor. This protein is recruited to the promoter of the HO1 or *HSPA6* gene and facilitates transcription in cooperation with Nrf2 or HSF1. ARE, antioxidant-responsive element; CHIP, chromatin immunoprecipitation; E1, enhancer 1; eEF1Bδ, eukaryotic elongation factor 1Bδ; eEF1BδL, long isoform of eEF1Bδ; GFP, green florescence protein; GST, glutathione-*S*-transferase; HO1, haem oxygenase 1; HSE, heat-shock element; HSF1, heat-shock transcription factor 1; IgG, immunoglobulin G; IP, immunoprecipitation; mt, mutant; Nrf2, NF-E2-related factor 2; siRNA, small-interfering RNA; wt, wild type.

expression of these genes. The levels of eEF1Bδ1, eEF1BδL and HSF1 expression in transfected cells were almost equal (supplementary Fig S3A online), but HSF2 expression was slightly lower. It has been reported that HSF2 is regulated by the ubiquitin-proteasome pathway (Mathew *et al*, 1998), so HSF2 might be rapidly degraded after protein expression. Thus, it was suggested that HSF1 and eEF1BδL selectively induce these genes. It was confirmed that endogenous HSF1-protein level and association with HSE were not affected by eEF1BδL overexpression (supplementary Fig S3B,C online), indicating that induction of these genes by eEF1BδL is not due to the activation of HSF1. We sought to determine whether the splicing change in the *eEF1Bδ* gene occurs due to heat stress. By RT–PCR analysis, it was shown that levels of the eEF1BδL isoform significantly increased and the short isoform decreased in response to heat stress (Fig 2B,C). This effect was reflected by a concomitant increase in eEF1BδL-protein level (supplementary Fig S3D online).

Next, to confirm that eEF1BδL does not induce these HSE-containing genes through translational regulation of HSE activator proteins, we mutated crucial basic residues for NLS to alanine in eEF1BδL (Fig 2D). Consistent with our proposal, eEF1BδL NLS-mutant seemed to be excluded from the nucleus (Fig 2D). This mutant has an intact C-terminal domain and translational activity, but cannot translocate into the nucleus and associate with HSE. Neither this mutant or the nuclear export signal-fused mutant induced the *HO1* gene, compared with wild-type eEF1BδL (Fig 2E). In addition, mutation of Lys 646 in the GEF domain of eEF1BδL, which is essential for translation elongation in the eEF1B complex (Andersen *et al*, 2001), did not affect HO1 mRNA expression (Fig 2F). These results indicate that induction of the *HO1* gene by eEF1BδL is not caused by translation regulation in the cytoplasm. The N-terminal domain of eEF1BδL did not induce HO1 mRNA expression (Fig 2F). These results also indicate that the C-terminal domain (eEF1Bδ1) of eEF1BδL might be involved in regulation of *HO1* gene induction. It was confirmed that these eEF1BδL mutants were located in the nucleus (supplementary Fig S4 online).

eEF1BδL and HSF1 co-exist in the HSPA6 promoter

To examine whether eEF1BδL physically associates with the HSE in the HSPA6 promoter region, a chromatin immunoprecipitation (CHIP) assay was performed on endogenous eEF1BδL in HEK293 cells. We performed an immunoblot analysis of eEF1BδL on cultured cells used in this study; both Hela and HEK293 cells expressed eEF1BδL protein (supplementary Fig S1C online). *HSPA6* is a heat-shock-inducible gene and has several HSEs in the proximal region on this promoter (Voellmy *et al*, 1985; Leung *et al*, 1990; Wang *et al*, 2000). eEF1BδL and HSF1 interact with HSE in the HSPA6 promoter region, and this interaction increased in heat-shock conditions (Fig 3A). Moreover, eEF1BδL efficiently induced HSPA6 promoter-driven reporter activity (Fig 3B). These results indicate that eEF1BδL can induce *HSPA6* gene transcription through association with HSE region in its promoter. To examine the protein–protein interaction of eEF1BδL with HSF1, we performed an immunoprecipitation study. An interaction was apparent between endogenous eEF1BδL and HSF1, and this was facilitated by heat-shock treatment (Fig 3C). In a reciprocal immunoprecipitation assay, eEF1BδL was present in the HSF1 immunoprecipitates (supplementary Fig S5A online). To explore the role of eEF1BδL in HSF1-mediated gene transcription, we examined the effects of eEF1BδL and HSF1 co-transfection on HSPA6 promoter-driven transcription. Co-transfection with increasing amounts of eEF1BδL enhanced HSF1-induced reporter activity (Fig 3D). This suggests that eEF1BδL induces transcription of *HSPA6* genes in cooperation with HSF1. Next, to examine whether eEF1BδL is functionally important for cell survival during heat stress, we inhibited the expression of endogenous eEF1BδL by small-interfering RNA (siRNA) treatment. At 5 nM siRNA, HeLa cells were less viable by eEF1BδL-specific siRNA during incubation at 42, 44 and 46 °C for 4 h than control siRNA-treated cells (Fig 3E). HSF1 knockdown had a similar effect at 44 and 46 °C (Fig 3E). At 20 nM siRNA, HSF1 knockdown also affected viability at 42 °C (supplementary Fig S5B online). This effect of eEF1BδL-specific siRNA was confirmed in cultured hippocampal neurons (supplementary Fig S5C,D online).

eEF1BδL regulates HSE-responsive gene induction

We further examined whether eEF1BδL is required for *HSPA6* gene induction by heat stress in HEK293 cells. *HSPA6* gene induction was inhibited following heat-shock treatment by knockdown of eEF1BδL, using eEF1BδL-specific siRNA targeting sequence within exon III (Fig 4A, upper panel). Knockdown by another siRNA targeting a different part of the mRNA sequence of eEF1BδL also inhibited *HSPA6* gene induction following heat-shock treatment (supplementary Fig S6A,B online). In addition, HSF1 knockdown completely inhibited *HSPA6* gene induction by eEF1BδL overexpression (Fig 4A, middle panel). Furthermore, eEF1BδL seems to be involved in the induction of heat-shock response (*HSR*) genes by HSF1, because *HSPA6* gene induction by HSF1 overexpression was inhibited by knockdown of eEF1BδL (Fig 4A, lower panel). We further examined whether eEF1BδL is required for target *HO1* gene induction by proteotoxic stress using the proteasome inhibitor MG132 (Wu *et al*, 2004). eEF1BδL knockdown significantly inhibited *HO1* induction following treatment with MG132 (Fig 4B, upper panel). It has been shown that heterodimers of Nrf2 are a key transcription factor for *HO1* gene induction (Inamdar *et al*, 1996; Itoh *et al*, 1997). Furthermore, HSF1 overexpression did not induce *HO1* gene expression (Fig 2A), and *HSPA6* gene induction by MG132 treatment was completely inhibited by HSF1 knockdown, whereas *HO1* gene induction was partly inhibited (supplementary Fig S6C online). Therefore, we tested the importance of Nrf2 for *HO1* gene induction by eEF1BδL in HEK293 cells transfected with Nrf2 siRNA; the effects of eEF1BδL overexpression were suppressed (Fig 4B, middle panel). To examine whether Nrf2 needs eEF1BδL for *HO1* induction, HEK293 cells were transfected with eEF1BδL-specific siRNA, followed by Nrf2 overexpression. Induction of *HO1* gene by Nrf2 was inhibited by knockdown of eEF1BδL (Fig 4B, lower panel). This showed that eEF1BδL is an essential protein for Nrf2-dependent *HO1* gene induction. We confirmed the effects of eEF1BδL siRNA on endogenous eEF1BδL expression and the absence of an effect on eEF1Bδ1 expression by immunoblot analysis (Fig 4C). Fig 4D,E also show validation of the effects of HSF1 and Nrf2 siRNA, respectively. In supplementary Fig S6D online, we assessed whether eEF1BδL knockdown affects total protein synthesis. eEF1BδL siRNA did not change the bulk translation rate in control or heat-shock conditions.

eEF1BδL binds to the HSE in the HO1 promoter

We then attempted to characterize the molecular mechanisms of *HO1* gene transcription by examining the inducible enhancer E1, which is involved in the response to several HO1 inducers (Ryter *et al*, 2006). The *HO1* gene has a proximal HSE immediately upstream from the transcription start site, and distal HSE and antioxidant-responsive elements (AREs) are present within the E1 region (Fig 5A; Alam & Cook, 2006). To examine whether eEF1BδL binds to E1 and/or HSE in the HO1 promoter region, a CHIP assay was performed. Endogenous eEF1BδL, but not HSF1, binds to the E1 and proximal HSE regions under MG132 treatment (Fig 5B). The primer for proximal HSE regions detects HSE in the −384 to −365 region of the HO1 promoter (Okinaga *et al*, 1996). In addition, eEF1BδL associates with the E1 and HSE regions in mouse testis (Fig 5C). These data were supported by CHIP assay, in which eEF1BδL or Nrf2 was overexpressed in HEK293 cells (supplementary Fig S7A online). An electrophoretic mobility shift assay was performed—using recombinant eEF1BδL protein expressed in *Escherichia coli* as the glutathione-*S*-transferase fusion protein (supplementary Fig S7B online)—to further confirm the direct eEF1BδL–HSE interaction. eEF1BδL, but not eEF1Bδ1, directly binds to HSE oligo DNA, as indicated by the electrophoretic mobility shift (arrow; Fig 5D). Its binding was in competition with excess unlabelled HSE oligo, but not with the mutated HSE oligo (Fig 5D). In addition, eEF1BδL markedly induced E1-luc activity (Fig 5E). However, eEF1BδL did not bind to ARE oligo DNA and did not induce ARE reporter activity (supplementary Fig S7C,D online). These results indicate that the N-terminal eEF1BδL domain contains an HSE-specific DNA-binding region. The E1 region has two ARE regions, which are also known to be stress-responsive elements (Ryter *et al*, 2006). Nrf2 and small Maf proteins are associated with this region and induce the *HO1* gene (Inamdar *et al*, 1996; Itoh *et al*, 1997). To determine the relationship between Nrf2 and eEF1BδL, we performed a co-immunoprecipitation assay of eEF1BδL and Nrf2. The results showed that the N-terminal domain of eEF1BδL specifically interacts with Nrf2 (Fig 5F). Endogenous eEF1BδL also interacts with Nrf2, examined in HEK293 cells (Fig 5G). This interaction was mediated by the 122–244 amino-acid region of eEF1BδL (supplementary Fig S8A online). In a reciprocal immunoprecipitation assay, eEF1BδL was present in the Nrf2 immunoprecipitates (supplementary Fig S8B online). We next assessed whether translocation of eEF1BδL was affected in the stress condition. MG132 treatment triggered the nuclear translocation of eEF1BδL (Fig 5H).

eEF1BδL is a HSR-transcription factor

Our study demonstrates that the *eEF1Bδ* gene encodes translation and HSE-dependent transcription factors, and coordinates these functions by tissue-specific alternative splicing (Fig 5I). This finding could provide new insights into protein biogenesis and chaperone induction by stressors. The way in which HSF1 selectively binds and is regulated at HSE in target promoters is an area of interest, but remains poorly understood (Trinklein *et al*, 2004; Morimoto, 2008). The finding that eEF1BδL regulates HSE-containing gene transcription with HSF1 and Nrf2 confirms the selectivity of *HSR* genes and demonstrates a new molecular mechanism for the relationship between thermal and oxidative stresses.

METHODS

Plasmids and siRNAs. Mammalian expression vectors for eEF1Bδ1, eEF1BδL, HSF1, HSF2 and Nrf2 were based on p3XFLAG-Myc-CMV-24 or pEGFP-C1. Site-directed mutagenesis was performed for the eEF1BδL NLS mutant, K646A and K646R mutant using a Quickchange kit (Stratagene, Santa Clara, CA, USA) according to the manufacturer's instructions. Double-stranded siRNA for human eEF1BδL (target sequence 5′-CUGG-CUCAGCAAGCCUGCCUA-3′) was synthesized by QIAGEN (Valencia, CA, USA). SMARTpool siRNAs targeting human Nrf2 and HSF1 were obtained from Dharmacon (Lafayette, CO, USA).
Cell culture and transfection. HeLa cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C with 5% CO_2. Primary cultured neurons were obtained from the hippocampus of approximately 16- to 18-embryonic-day fetal C57BL6/J mice and maintained in neurobasal medium with 2% B-27 supplement (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO_2. Transfections were

performed using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's instructions. For siRNA knockdown, siRNAs were transfected into cells using DharmaFECT transfection reagents (Dharmacon), according to the manufacturer's instructions. Except in Fig 3E, heat-shock treatment was performed at 42 °C. In Fig 3E, heat-shock treatment was performed at 42, 44 and 46 °C.

RT–PCR analysis. For analysis of the eEF1Bδ splicing product, total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed with oligo dT primer and the Superscript III first-strand synthesis system (Invitrogen), according to the manufacturer's instructions. Splicing products were amplified by PCR (35 cycles) with the following primers: 5′-TGAGCTCGCAGTTC CAGGTTTTGG-3′ and 5′-TCGAAGGTTCTGGTTCTCCAC-3′. The primers produced a 1,483-bp product for eEF1BδL and a 338-bp product for eEF1Bδ1.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank T. Ogawa and M. Kobayashi for experimental support and J. Alam for the pSX2 (pE1-luc) plasmid and critical reading of this manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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