Neuroscience Letters 612 (2016) 199–203

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research paper

Unfolded protein response in hypothalamic cultures of wild-type and ATF6α-knockout mice

Wenjun Lu [a], Daisuke Hagiwara [a,*], Yoshiaki Morishita [a,*], Masayoshi Tochiya [a], Yoshinori Azuma [a], Hidetaka Suga [a], Motomitsu Goto [a], Ryoichi Banno [a], Yoshihisa Sugimura [a], Seiichi Oyadomari [b], Kazutoshi Mori [c], Hiroshi Arima [a,*]

[a] Department of Endocrinology and Diabetes, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan
[b] Institute for Genome Research, University of Tokushima, Tokushima 770-8503, Japan
[c] Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

HIGHLIGHTS

- Thapsigargin (TG) activated all the UPR arms similarly in mouse hypothalamic cultures.
- TG-induced upregulation of BiP and CHOP was attenuated in ATF6α$^{-/-}$ mouse hypothalamus.
- TG-induced upregulation of ERAD components was also attenuated in ATF6α$^{-/-}$ mouse hypothalamus.

ARTICLE INFO

Article history:
Received 20 October 2015
Received in revised form
16 November 2015
Accepted 15 December 2015
Available online 18 December 2015

Keywords:
Activating transcription factor 6α (ATF6α)
Hypothalamus
Endoplasmic reticulum (ER) stress
Unfolded protein response (UPR)
Endoplasmic reticulum-associated degradation (ERAD)

ABSTRACT

Recent studies suggest that endoplasmic reticulum (ER) stress in the hypothalamus could affect systemic homeostatic regulation in areas such as energy and water balance. Activating transcription factor 6α (ATF6α) is an ER stress transducer which increases the expression of ER chaperones and ER-associated degradation (ERAD) components under ER stress. In the present study, we examined the regulation of the unfolding protein response (UPR) in mouse hypothalamic cultures of wild-type (WT) and ATF6α$^{-/-}$ mice. Thapsigargin (TG), an ER stressor, significantly increased the mRNA expression of immunoglobulin heavy chain binding protein (BiP), spliced X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and ERAD components, in hypothalamic cultures of WT mice with the same threshold (0.1 μM) and similar time courses. On the other hand, TG-induced upregulation of BiP and CHOP as well as most ERAD-related genes, but not spliced XBP1 or ATF4, was attenuated in ATF6α$^{-/-}$ mice compared with WT mice. Our data suggest that all the UPR arms are activated similarly in the mouse hypothalamus under ER stress conditions, where ATF6α regulates the expression of ER chaperones, CHOP, and ERAD components.

1. Introduction

In the endoplasmic reticulum (ER), newly synthesized secretory and transmembrane proteins are folded and assembled. This process can be affected under various stress conditions. When the load of unfolded and misfolded proteins surpasses the folding capacity of the ER, unfolded proteins accumulate in the ER lumen, consequently leading to ER stress. In order to cope with ER stress and maintain homeostasis of the protein quality control system, the unfolded protein response (UPR) occurs through three distinct types of ER stress transducers localized in the ER membrane [14]: inositol requiring enzyme 1 (IRE1) [6,17], protein kinase RNA-like ER kinase (PERK) [12], and activating

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; IRE1, inositol requiring enzyme 1; PERK, protein kinase RNA-like ER kinase; ATF6α, activating transcription factor 6α; XBP1, X-box binding protein 1; eIF2α, eukaryotic translation initiation factor 2α; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; BiP, immunoglobulin heavy chain binding protein; ERAD, ER-associated degradation; ERAC, ER-associated compartment; FNDI, familial neurohypophysial diabetes insipidus; TG, thapsigargin; EDEM1, ER degradation-enhancing α–mannosidase-like protein 1; MEF, mouse embryonic fibroblast.

* Corresponding authors. Fax: +81 52 744 2206.
E-mail addresses: d-hagiwara@med.nagoya-u.ac.jp (D. Hagiwara), yoshi214@med.nagoya-u.ac.jp (Y. Morishita), arima105@med.nagoya-u.ac.jp (H. Arima).

http://dx.doi.org/10.1016/j.neulet.2015.12.031
0304-3940/

transcription factor 6α (ATF6α) [13]. Activation of IRE1 results in splicing mRNA of the X-box binding protein 1 (XBP1), which upregulates UPR gene transcription [26]. The active form of PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), resulting in the suppression of global protein synthesis and a subsequent reduction in the workload of the ER [11]. eIF2α also facilitates the upregulation of activating transcription factor 4 (ATF4) and thereby induces expression of C/EBP homologous protein (CHOP) [10]. On the other hand, ATF6α is translocated from the ER to the Golgi apparatus and cleaved by Golgi-resident proteases under ER stress conditions [5,25]. A cleaved form of ATF6α acts as a transcriptional factor and upregulates the expression of ER chaperones including immunoglobulin heavy chain binding protein (BiP) and ER-associated degradation (ERAD) components, in order to enhance the capacity of protein folding and degradation mechanisms [13,15,23,28].

The hypothalamus is responsible for various systemic homeostatic mechanisms such as energy and water balance, circadian rhythm, sleep-awake cycle, thermoregulation, and the sympathetic and parasympathetic nervous system. Recent studies including those from our laboratory have provided evidence that ER stress in the hypothalamus is related to some pathophysiological conditions: a high fat diet inducing leptin resistance and obesity increased phosphorylation of PERK and IRE1 in the hypothalamus [19]; the absence of ATF6α led to disruption of the segregation of misfolded proteins into a sub-compartment of ER (ERAC: ER-associated compartment) of vasopressin neurons in the hypothalamus in a mouse model for familial neurohypophysial diabetes insipidus (FNDI) [3]. These data suggest that UPR in the hypothalamus plays a crucial role in homeostasis and pathophysiology. However, the detailed regulation of UPR in the hypothalamus under ER stress is not yet clear.

In the present study, we first examined the regulation of UPR in the hypothalamus in wild-type (WT) mice using hypothalamic organotypic cultures, which maintain intrinsic properties [1,8,9,18]. We also examined the role of ATF6α in the hypothalamus by comparing the UPR between WT and ATF6α$^{-/-}$ mice.

2. Materials and methods

2.1. Animals

ATF6α$^{-/-}$ mice [23] were produced by intercrossing male and female heterozygotes, and their WT littermates were used as control mice. Mice were maintained under controlled conditions (23.0 ± 0.5 °C, lights on from 9:00 AM to 9:00 PM). All procedures were approved by the Animal Experimentation Committee of the Nagoya University Graduate School of Medicine, and performed in accordance with its institutional guidelines for animal care and use.

2.2. Hypothalamic organotypic culture

Seven-day-old WT and ATF6α$^{-/-}$ mice were decapitated, and cultures of three hypothalamic slices containing the arcuate nucleus, ventromedial hypothalamic nucleus, supraoptic nucleus, and paraventricular nucleus were performed as described previously [9]. Explants were incubated with thapsigargin (TG, Calbiochem, San Diego, CA, USA), while control slices were incubated with vehicle (0.1% dimethylsulfoxide; Sigma–Aldrich, St. Louis, MO, USA). Total duration of cultures was 48 h in all explants.

2.3. Quantitative real-time RT-PCR

Total RNA extractions from mouse hypothalamic slice explants and quantitative real-time RT-PCR analyses were performed as described previously [9]. The primer sequences are summarized in Table S1.

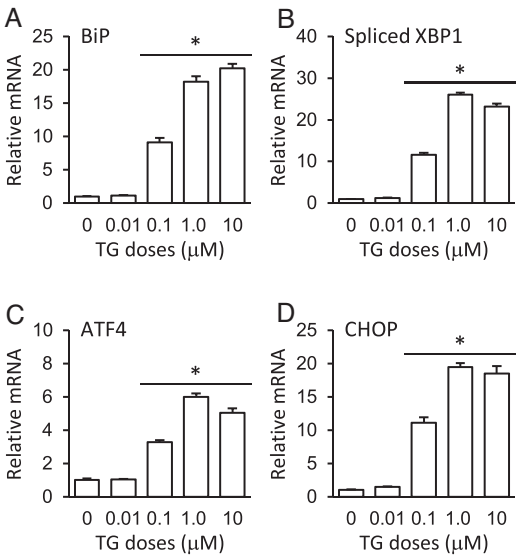

Fig. 1. Dose response experiments of TG on BiP, spliced XBP1, ATF4, and CHOP mRNA expression in hypothalamic cultures of WT mice. Quantitative real-time RT-PCR analysis for BiP (A), spliced XBP1 (B), ATF4 (C), and CHOP mRNA (D) in hypothalamic slice explants of WT mice incubated with vehicle and 0.01–10 μM TG for 12 h. Results are expressed as means ± SE (n = 5–8). $^{*}P < 0.05$ compared with vehicle.

2.4. Statistical analysis

Statistical significance of differences between groups was analyzed by one- or two-way ANOVA followed by Bonferroni's test as appropriate. Results are expressed as means ± SE, and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Dose response experiments of TG on UPR in hypothalamic cultures of WT mice

Incubation of hypothalamic explants of WT mice with 0.1–10 μM TG, but not 0.01 μM TG, significantly increased the expression levels of BiP, spliced XBP1, ATF4, and CHOP mRNA (Fig. 1), as well as ERAD-related genes (HRD1, EDEM1, HERP1, and Derlin-1, 2, 3) (Fig. 2) at 12 h.

3.2. Time course experiments of TG on UPR in hypothalamic cultures of WT mice

Incubation of hypothalamic explants of WT mice with 1 μM TG significantly increased the expression levels of BiP, spliced XBP1, ATF4, and CHOP mRNA (Fig. 3), as well as ERAD-related genes (HRD1, EDEM1, HERP1, and Derlin-1, 2, 3) (Fig. 4) at all time points (6, 12, 24 h) examined.

3.3. Time course experiments of TG on UPR in hypothalamic cultures of ATF6α$^{-/-}$ mice

There were no significant differences in basal expression levels of UPR-related genes examined between genotypes except Derlin-3, which had a significantly lower expression in ATF6α$^{-/-}$ mice (Figs. 3 and 4). TG-induced upregulation of BiP mRNA was significantly attenuated in ATF6α$^{-/-}$ mice (Fig. 3A). On the other hand, expression levels of spliced XBP1 and ATF4 were significantly

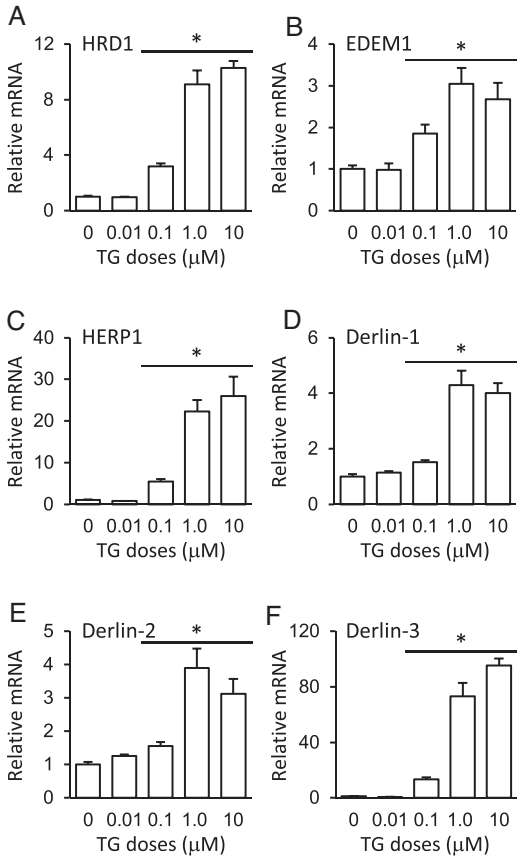

Fig. 2. Dose response experiments of TG on ERAD-related gene expression in hypothalamic cultures of WT mice. Quantitative real-time RT-PCR analysis for HRD1 (A), EDEM1 (B), HERP1 (C), Derlin-1 (D), Derlin-2 (E), and Derlin-3 (F) in hypothalamic slice explants of WT mice incubated with vehicle and 0.01–10 μM TG for 12 h. Results are expressed as means ± SE (n = 5–8). $^{*}P < 0.05$ compared with vehicle.

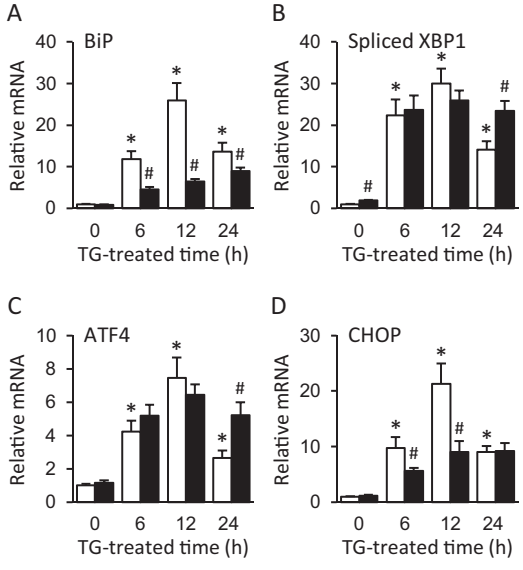

Fig. 3. Time course experiments of TG on BiP, spliced XBP1, ATF4, and CHOP in hypothalamic cultures of WT and ATF6α$^{-/-}$ mice. Quantitative real-time RT-PCR analysis for BiP (A), spliced XBP1 (B), ATF4 (C), and CHOP mRNA (D) in hypothalamic slice explants of WT (open bar) and ATF6α$^{-/-}$ mice (closed bar) incubated with vehicle and 1 μM TG for 6, 12, and 24 h. Results are expressed as means ± SE (n = 6–8). $^{*}P < 0.05$ compared with vehicle in WT mice and $^{\#}P < 0.05$ compared between genotypes at each corresponding incubation time.

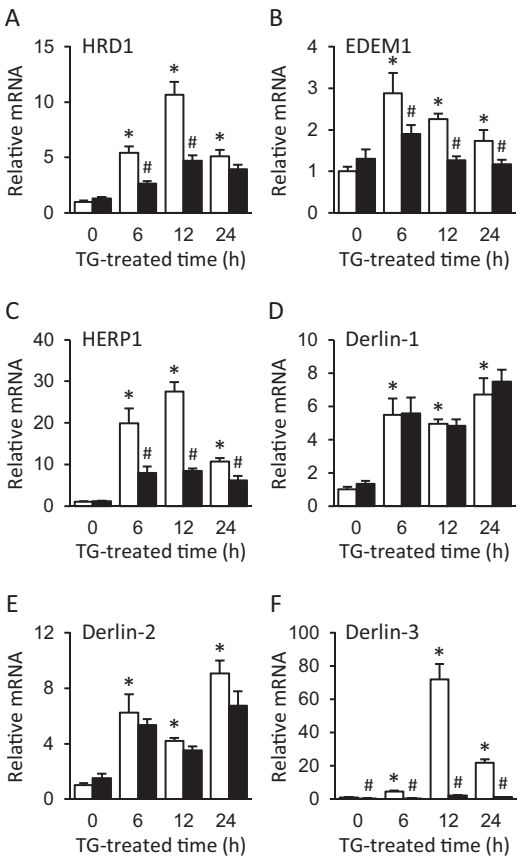

Fig. 4. Time course experiments of TG on ERAD-related gene expression in hypothalamic cultures of WT and ATF6α$^{-/-}$ mice. Quantitative real-time RT-PCR analysis for HRD1 (A), EDEM1 (B), HERP1 (C), Derlin-1 (D), Derlin-2 (E), and Derlin-3 (F) in hypothalamic slice explants of WT (open bar) and ATF6α$^{-/-}$ mice (closed bar) incubated with vehicle and 1 μM TG for 6, 12, and 24 h. Results are expressed as means ± SE (n = 6–8). $^{*}P < 0.05$ compared with vehicle in WT mice and $^{\#}P < 0.05$ compared between genotypes at each corresponding incubation time.

higher at 24 h in ATF6α$^{-/-}$ mice compared with WT mice (Fig. 3B and C). Of note, the upregulation of CHOP mRNA was significantly attenuated in ATF6α$^{-/-}$ mice (Fig. 3D). TG-induced upregulation of ERAD-related genes was significantly attenuated in ATF6α$^{-/-}$ mice compared with WT mice with the exception of Derlin-1 and 2, which had similar expression levels between genotypes (Fig. 4).

4. Discussion

In the present study, we investigated the regulation of UPR-related gene expression in mouse hypothalamic organotypic cultures treated with an ER stress inducer TG. Our data showed that UPR-related genes examined were all upregulated similarly in hypothalamic cultures of WT mice. We also showed that TG-induced upregulation of BiP, CHOP, and most ERAD-related genes was attenuated in the hypothalamus of ATF6α$^{-/-}$ mice compared with WT mice.

It is worth noting that time-courses and dose-responses were similar among all the UPR-related genes examined in the hypothalamus of WT mice. UPR is composed of both pro-survival and pro-apoptotic pathways, and our data demonstrated that pro-survival genes such as BiP [7] and pro-apoptotic genes such as CHOP [11] were upregulated with the same threshold and similar time-courses in mouse hypothalamic organotypic cultures. Similar results were also reported in mouse embryonic fibroblasts (MEFs) [20]. These data suggest that both pro-survival and pro-apoptotic

genes are induced at the same time in response to ER stress, at least in acute conditions.

There is evidence to indicate that BiP expression is regulated by ATF6α [23]. Consistent with this, our data showed that BiP mRNA expression induced by TG was attenuated in hypothalamic cultures of ATF6α$^{-/-}$ mice. The upregulation of spliced XBP1 and ATF4 at later time points could be interpreted as compensated UPR in the absence of ATF6α, as reported previously [21–24]. On the other hand, while CHOP is supposed to be downstream of PERK, our data showed that the upregulation induced by TG was attenuated in ATF6α$^{-/-}$ mice, indicating that CHOP is at least in part regulated by ATF6α under ER stress in hypothalamus, as suggested in previous studies using cell lines [2,16,27].

The upregulation of most ERAD-related genes examined were attenuated in hypothalamic cultures of ATF6α$^{-/-}$ mice, as in ATF6α$^{-/-}$ MEFs [2]. These data suggest that ATF6α plays a pivotal role in ERAD in the hypothalamus. In particular, the expression of Derlin-3 in ATF6α$^{-/-}$ mice was significantly decreased compared with that in WT mice even under basal condition, and ER stress-induced upregulation was almost completely suppressed in ATF6α$^{-/-}$ mice. On the other hand, there were no differences in expression levels of Derlin-1 and 2 between WT and ATF6α$^{-/-}$ mice. These data suggest that among the Derlin family, only Derlin-3 is downstream of ATF6α, as was reported previously in MEFs [2] and heart [4].

ER stress is induced by accumulation of misfolded/unfolded proteins in the ER lumen, and ER chaperones as well as ERAD function to reduce ER stress as the protein quality control system. In a previous study, we demonstrated that misfolded proteins were accumulated in ERAC of vasopressin neurons in the hypothalamus of FNDI mice, and that the formation of ERAC was hampered in the absence of ATF6α [3]. As upregulation of BiP mRNA expression in the vasopressin neurons was attenuated in ATF6α$^{-/-}$ mice, we speculated that ER chaperones including BiP might play a major role in the formation of ERAC [3]. Now that the upregulation of most ERAD-related genes was attenuated in the hypothalamus of ATF6α$^{-/-}$ mice, it is likely that ERAD might also contribute to the maintenance of ERAC under ER stress conditions.

In the present study, we examined the UPR signaling in hypothalamic slice explants. It remains unclear whether the UPR in other brain regions is regulated similarly as in the hypothalamus. Furthermore, it also remains to be elucidated whether there are any differences in the UPR signaling among individual hypothalamic nuclei. To analyze the UPR signaling in other brain regions as well as individual hypothalamic nuclei is an important direction in future.

In conclusion, our data revealed that all the UPR arms (ATF6α, IRE1, and PERK) are activated similarly, and that ATF6α regulates the expression of ER chaperones, CHOP, and ERAD components under ER stress in the mouse hypothalamus.

Acknowledgments

We thank Michiko Yamada and Mayumi Katagiri for their helpful technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2015.12.031.

References

[1] K. Adachi, M. Goto, T. Onoue, T. Tsunekawa, M. Shibata, S. Hagimoto, Y. Ito, R. Banno, H. Suga, Y. Sugimura, Y. Oiso, H. Arima, Mitogen-activated protein kinase phosphatase 1 negatively regulates MAPK signaling in mouse hypothalamus, Neurosci. Lett. 569 (2014) 49–54.
[2] Y. Adachi, K. Yamamoto, T. Okada, H. Yoshida, A. Harada, K. Mori, ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum, Cell Struct. Funct. 33 (2008) 75–89.
[3] Y. Azuma, D. Hagiwara, W. Lu, Y. Morishita, H. Suga, M. Goto, R. Banno, Y. Sugimura, S. Oyadomari, K. Mori, A. Shiota, N. Asai, M. Takahashi, Y. Oiso, H. Arima, Activating transcription factor 6α is required for the vasopressin neuron system to maintain water balance under dehydration in male mice, Endocrinology 155 (2014) 4905–4914.
[4] P.J. Belmont, W.J. Chen, M.N. San Pedro, D.J. Thuerauf, N. Gellings Lowe, N. Gude, B. Hilton, R. Wolkowicz, M.A. Sussman, C.C. Glembotski, Roles for endoplasmic reticulum-associated degradation and the novel endoplasmic reticulum stress response gene Derlin-3 in the ischemic heart, Circ. Res. 106 (2010) 307–316.
[5] X. Chen, J. Shen, R. Prywes, The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi, J. Biol. Chem. 277 (2002) 13045–13052.
[6] J.S. Cox, C.E. Shamu, P. Walter, Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase, Cell 73 (1993) 1197–1206.
[7] Y. Fu, J. Li, A.S. Lee, GRP78/BiP inhibits endoplasmic reticulum BIK and protects human breast cancer cells against estrogen starvation-induced apoptosis, Cancer Res. 67 (2007) 3734–3740.
[8] S. Hagimoto, H. Arima, K. Adachi, Y. Ito, H. Suga, Y. Sugimura, M. Goto, R. Banno, Y. Oiso, Expression of neuropeptide Y and agouti-related protein mRNA stimulated by glucocorticoids is attenuated via NF-κB p65 under ER stress in mouse hypothalamic cultures, Neurosci. Lett. 553 (2013) 165–169.
[9] D. Hagiwara, H. Arima, Y. Morishita, L. Wenjun, Y. Azuma, Y. Ito, H. Suga, M. Goto, R. Banno, Y. Sugimura, A. Shiota, N. Asai, M. Takahashi, Y. Oiso, Arginine vasopressin neuronal loss results from autophagy-associated cell death in a mouse model for familial neurohypophysial diabetes insipidus, Cell Death Dis. 5 (2014) e1148.
[10] H.P. Harding, I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, D. Ron, Regulated translation initiation controls stress-induced gene expression in mammalian cells, Mol. Cell 6 (2000) 1099–1108.
[11] H.P. Harding, Y. Zhang, A. Bertolotti, H. Zeng, D. Ron, Perk is essential for translational regulation and cell survival during the unfolded protein response, Mol. Cell 5 (2000) 897–904.
[12] H.P. Harding, Y. Zhang, D. Ron, Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase, Nature 397 (1999) 271–274.
[13] K. Haze, H. Yoshida, H. Yanagi, T. Yura, K. Mori, Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress, Mol. Biol. Cell 10 (1999) 3787–3799.
[14] C. Hetz, The unfolded protein response: controlling cell fate decisions under ER stress and beyond, Nat. Rev. Mol. Cell Biol. 13 (2012) 89–102.
[15] K. Lee, W. Tirasophon, X. Shen, M. Michalak, R. Prywes, T. Okada, H. Yoshida, K. Mori, R.J. Kaufman, IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response, Genes Dev. 16 (2002) 452–466.
[16] Y. Ma, J.W. Brewer, J.A. Diehl, L.M. Hendershot, Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response, J. Mol. Biol. 318 (2002) 1351–1365.
[17] K. Mori, W. Ma, M.J. Gething, J. Sambrook, A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus, Cell 74 (1993) 743–756.
[18] Y. Morishita, H. Arima, M. Hiroi, M. Hayashi, D. Hagiwara, N. Asai, N. Ozaki, Y. Sugimura, H. Nagasaki, A. Shiota, M. Takahashi, Y. Oiso, Poly(A) tail length of neurohypophysial hormones is shortened under endoplasmic reticulum stress, Endocrinology 152 (2011) 4846–4855.
[19] L. Ozcan, A.S. Ergin, A. Lu, J. Chung, S. Sarkar, D. Nie, M.G. Myers, U. Ozcan, Endoplasmic reticulum stress plays a central role in development of leptin resistance, Cell Metab. 9 (2009) 35–51.
[20] D.T. Rutkowski, S.M. Arnold, C.N. Miller, J. Wu, J. Li, K.M. Gunnison, K. Mori, A.A. Sadighi Akha, D. Raden, R.J. Kaufman, Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins, PLoS Biol. 4 (2006) e374.
[21] M. Usui, S. Yamaguchi, Y. Tanji, R. Tominaga, Y. Ishigaki, M. Fukumoto, H. Katagiri, K. Mori, Y. Oka, H. Ishihara, Atf6α-null mice are glucose intolerant due to pancreatic β-cell failure on a high-fat diet but partially resistant to diet-induced insulin resistance, Metabolism 61 (2012) 1118–1128.
[22] J. Wu, D.T. Rutkowski, M. Dubois, J. Swathirajan, T. Saunders, J. Wang, B. Song, G.D. Yau, R.J. Kaufman, ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress, Dev. Cell 13 (2007) 351–364.
[23] K. Yamamoto, T. Sato, T. Matsui, M. Sato, T. Okada, H. Yoshida, A. Harada, K. Mori, Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1, Dev. Cell 13 (2007) 365–376.
[24] K. Yamamoto, K. Takahara, S. Oyadomari, T. Okada, T. Sato, A. Harada, K. Mori, Induction of liver steatosis and lipid droplet formation in ATF6alpha-knockout mice burdened with pharmacological endoplasmic reticulum stress, Mol. Biol. Cell 21 (2010) 2975–2986.

[25] J. Ye, R.B. Rawson, R. Komuro, X. Chen, U.P. Davé, R. Prywes, M.S. Brown, J.L. Goldstein, ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs, Mol. Cell 6 (2000) 1355–1364.
[26] H. Yoshida, T. Matsui, A. Yamamoto, T. Okada, K. Mori, XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor, Cell 107 (2001) 881–891.
[27] H. Yoshida, T. Okada, K. Haze, H. Yanagi, T. Yura, M. Negishi, K. Mori, ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response, Mol. Cell Biol. 20 (2000) 6755–6767.
[28] H. Yoshida, T. Okada, K. Haze, H. Yanagi, T. Yura, M. Negishi, K. Mori, Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response, Mol. Cell Biol. 21 (2001) 1239–1248.