Protective effects of UFL1 against endoplasmic reticulum stress-induced autophagy in bovine mammary epithelial cells

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Abstract

Ubiquitin-fold modifier 1 (UFM1)-specific ligase 1 (UFL1) is an important component of the UFM1 conjugation system, which is required for various cellular processes including protein translation, apoptosis, autophagy, and signal transduction. However, both, the expression of UFL1 in mammary cells and its role in endoplasmic reticulum (ER) stress in bovine mammary epithelial cells (BMECs) remain to be fully elucidated. Here, we characterized the potential roles of UFL1 in BMECs. Amino acid sequence comparison indicated that bovine *UFL1* shares a high level of sequence identity with the *UFL1* of other ruminant species. Notably, UFL1 expression in BMECs was increased by endoplasmic reticulum (ER) stress induced by treatment with tunicamycin (TM). ER stress-related gene expression was further increased in UFL1 knockdown cells upon TM treatment. Moreover, UFL1 overexpression inhibited TM-stimulated ER stress and alleviated ER stress-induced autophagy. Together, our results indicated that UFL1 is a novel ER stress-responsive protein in BMECs. Thus, our study provides a basis for further research into ER stress-related processes in bovine mammary tissues and potential targets for alleviating ER stress in these cells.

Keywords UFL1 · Bovine mammary epithelial cells · ER stress · Autophagy

Introduction

Ubiquitin-fold modifier 1 (UFM1)-specific ligase 1 (UFL1, also known as KIAA0776, NLBP, and Maxer) is an enzyme that is a part of the UFM1 conjugation system of ubiquitin-like proteins (Ubls), which also includes UFM1, Uba5, UFC1, UFL1, UfSP1, UfSP2, Ufbp1, and CDK5RAP3 (Veen and Ploegh 2012; Daniel and Liebau 2014; Zhang et al. 2015). UFL1 is expressed in the lung, testis, breast, liver, and pituitary (Tatsumi et al. 2010; Lemaire et al. 2011; Daniel and Liebau 2014). UFL1 plays a fundamental role in various cells and tissues by promoting UFM1 ubiquitylation or “ufmylation” of target proteins and thus

affects various cellular process such as DNA repair, protein translation, cell viability, cell cycle progression, signal transduction, apoptosis, autophagy, and immune and antiviral responses (Hershko and Ciechanover 1991; Mizushima et al. 2007; Cort et al. 2010; Komatsu et al. 2014; Ishimura et al. 2016). UFL1 recognizes and promotes the ufmylation of the UFM1 target ASC1 and thereby protects healthy breast tissue against tumorigenesis caused by estrogen receptor signaling (Yoo et al. 2014). Similarly, UFL1 has a role in cardiac morphology, with adult UFL1 knockout (ko) mice exhibiting a significantly decreased cardiac weight (per body weight) as well as left ventricular chamber dilation, wall thinning, and cardiac dysfunction. Thus, UFL1 is suggested to mediate multiple biological functions in various cellular processes.

The endoplasmic reticulum (ER) is a subcellular organelle that critically mediates various processes required for normal protein homeostasis, including protein folding, maturation, and secretion. Various physiological and pathological factors disrupt protein homeostasis by causing unfolded proteins to accumulate, leading to ER stress (Ron and Walter 2007). Tunicamycin (TM) was recently demonstrated to induce ER stress both in vivo and in vitro (Jung et al. 2015; Zhu et al. 2017). Once induced, ER stress can trigger autophagy via different pathways, including that mediated by UFL1.

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Notably, the hematopoietic stem cells in UFL1-deficient mice exhibited both ER stress and activation of the unfolded protein response (Zhang et al. 2015). UFL1 small interfering RNA (siRNA)-mediated silencing is sufficient to significantly increase ER stress-induced apoptosis in pancreatic beta cells (Lemaire et al. 2011). These studies suggest that UFL1 plays critical roles in the process of ER stress.

Mammary epithelial cells form an essential component of the mammary glands that synthesize milk to rear offspring. However, the biological function of UFL1 in bovine mammary epithelial cell (BMEC) ER stress remains unclear. Therefore, the present study aimed to characterize UFL1 localization and assess the impact of disrupting or augmenting UFL1 production on ER stress in BMECs. The generated results will provide a foundation for future research aiming to provide effective strategies to reduce ER stress in BMECs.

Materials and methods

Cell culture and treatment

All experiments were approved in accordance with the manual of Institutional Animal Care and the Use of Laboratory Animals (SYXK2011-0036) published by the Committee of Nanjing Agricultural University and were prepared in accordance with the guidelines of the Care and Use of Laboratory Animals. BMECs were harvested from Chinese Holstein cows in a local slaughterhouse as previously described (Sun et al. 2015). BMEC cultures were prepared in accordance with a previous study (Leibowitz et al. 2013). Briefly, cells were incubated in HyClone Dulbecco's Modified Eagle's Medium (DMEM) Ham's F12 (DMEMF-12) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml of antibiotic (penicillin and streptomycin; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C and 5% CO₂. TM was diluted in dimethyl sulfoxide (5 mg/ml). All cell cultures were serum starved before TM treatment. Cells were grown to 70 to 80% confluence, before each in vitro experiment was done.

Immunohistochemistry

The location of UFL1 protein in bovine mammary was detected by immunohistochemistry. The mammary tissue was fixed in 4% paraformaldehyde buffer (PH 7.4) for 24 h, then were embedded in paraffin blocks. Sections were cut at 4 µm and were dewaxed via xylene followed by a gradual series of ethanol. The sections were incubated with anti-UFL1 in a humidified chamber at 4 °C overnight. Next, the sections were incubated with goat anti-rabbit IgG kit (SA1023/SA2002; Boster Biological Technology). Diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, MO, USA) with 0.01% H₂O₂ was used for the visualization of the UFL1

protein. The sections were stained with hematoxylin and covered with VectaMount. All images were captured by a Nikon YS100 microscope (Nikon) with a digital camera (Nikon; Tokyo, Japan). All antibody details are provided in Table 1.

Immunofluorescence

Immunofluorescence was performed according to a previous study (Sun et al. 2015). Briefly, cells were fixed in 4% paraformaldehyde buffer (PH 7.4) at 37 °C for 30 min and then permeabilized with 0.1% Triton X-100 for 15 min. Cells were blocked in 5% BSA (A4737; Sigma-Aldrich, USA) at room temperature for 1 h and incubated in anti-UFL1 at 4 °C overnight. After incubation, cells were washed in PBS and then incubated with Alexa Fluor 647 conjugated goat anti-rabbit for 1 h at room temperature. Micrographs were captured on a Zeiss LSM700 META microscope (Carl Zeiss, Oberkochen, Germany). All antibody details are provided in Table 1.

CCK-8 analysis

Cell viability was tested by a CCK-8 assay according to the manufacturer's recommendations. Cells were seeded in 96-well plates and treated with 5 µg/ml TM at 37 °C and 5% CO₂. After treatment, 10 µl/well of CCK-8 (Beyotime Biotechnology, Haimen, China) was added into each well for 4 h. The 96-well plates were put in a spectrophotometer (Thermo, USA) and examined at 450 µm. Experiments were repeated 3 times.

Flow cytometry analysis

The ratio of apoptosis was analyzed according to manufacturer's suggestion (FITC Annexin V Apoptosis Detection Kit I, BD, USA). Cells were stained with propidium iodide and annexin V at room temperature for 20 min. Cells were collected for measurement of ratio of apoptosis by a flow cytometer (FCM) with a FACSCalibur (BD Biosciences, Bedford, MA). Data was analyzed by FlowJo 10 (Stanford University, USA). This work was supported by the National Experimental Teaching Center for Animal Science of Nanjing Agricultural University.

RT-PCR analysis for mRNA expression

The BMECs were homogenized in a TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA with PrimeScript reverse transcriptase reagent kit (Perfect real time; TaKaRa, Dalian, China). PCR conditions included one cycle at 95 °C for 30s, annealing at 95 °C for 5 s, and 60 °C for 30s for 40 cycles. Each sample was tested in triplicate, and the data

Table 1 Information of antibodies

Antibodies	Cat no.	Company	Dilution of immunohistochemistry	Dilution of immunofluorescence	Dilution of western blot
UFL1	26087-1-AP	Proteintech group	1:200	1:200	1:1000
LC3	14600-1-AP	Proteintech group	_____	_____	1:1000
p62	18420-1-AP	Proteintech group	_____	_____	1:1000
GAPDH	10494-1-AP	Proteintech group	_____	_____	1:4000
Goat-IgG rabbit	10285-1-AP	Proteintech group	_____	_____	1:4000
Alexa Fluor 647 conjugated Goat Anti-Rabbit IgG (H + L)	AS060	ABclonal	_____	1:200	_____

were analyzed using the $2^{-\Delta\Delta C}$ method. All primers were designed by Primer 5.0 software and are presented in Table 2.

Western blot analysis

Cells were lysed in a RIPA lysis buffer (P0013B, Beyotime Biotechnology, Haimen, China) and phenylmethylsulfonyl fluoride (ST506, Beyotime Biotechnology, Haimen, China) mix buffer (RIPA lysis buffer: phenylmethylsulfonyl fluoride = 1000:1). Protein concentrations were determined by a BCA protein assay kit (P009, Beyotime Biotechnology, Haimen, China). Samples (40 μ g) were separated by 10% SDS polyacrylamide gel and then transferred onto PVDF membranes (FFP39, Beyotime Biotechnology, Haimen, China). The membranes were blocked by 5% fat-free milk in TBST (with 20% Tween, 20-mM Tris-buffered saline) for 1 h at room temperature and then incubated for 12 h at 4 °C with primary antibodies (UFL1, LC3, and p62). After the membranes were washed in free TBST for 3 times, and then incubated with secondary antibody for 2 h at room temperature. The membranes were washed in free TBST for 3 times and visualized by an ECL reagent (Amersham Life Science, Arlington

Heights, USA). Photographs were captured by Luminescent Image Analyzer LAS4000 (Fujifilm, Tokyo, Japan). The chemiluminescence intensity of immunoblot signals were calculated by ImageJ software (National Institutes of Health, Bethesda, MD, USA). All antibody details are provided in Table 1.

Cell transfection

UFL1 siRNA, UFL1 cDNA plasmid, and empty vector were purchased from GenePharma (Shanghai, China). Sequences for cow UFL1 siRNA were sense, 5'-GCAGCAGA AGCUUGUGAUATT-3'; antisense, 5'-UAUCACAA GCUUCUGCUGCTT-3'. Transfection of siRNA and cDNA plasmid was performed with a Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions.

Statistical analysis

All results were obtained in triplicate and data were presented as a mean $\pm$ standard error of the mean. Statistical analyses

Table 2 Primer sets for quantitative real-time PCR

Gene	GenBank number	Primer	Sequence of nucleotide (5'-3')
<i>UFL1</i>	NM_001080276.2	F	AACCTCTTCGGCCTCTGGAA
		R	GAGCAGCCTGGGTATCATCT
<i>ATF6</i>	XM_003581916.3	F	CCTGTTTGCTGAACCTGGCT
		R	CGAAAGTGGCTGAGGTTCTG
<i>CHOP</i>	NM_001078163.1	F	CAAGCAACGCATGA AGGAGA
		R	GCGCTCGATTCTCTGTTTGA
<i>GRP78</i>	NM_001075148.1	F	GTTCGGAAGGACAACAGAGC
		R	GAGTCAGGGTCTCCGAGAAG
<i>GAPDH</i>	NM_001034034.2	F	AAGGTCGGAGTGAAC
		R	CGTTCTCTGCCTTGACTGTG

were performed by Student's *t* test and one-way ANOVA followed by Tukey's test and determined using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). *P* values < 0.05 were defined as significant differences.

Results

UFL1 gene sequence analysis

An amino acid sequence comparison showed that the bovine *UFL1* sequence was highly homologous (97.3%, 96.9%, and 92.4%, respectively) with that of *Capra hircus* (goat), *Ovis aries* (sheep), and *Sus scrofa* (pig), moderately homologous (88.5% and 88.4%, respectively) with that of *Mus musculus* (mouse) and *Rattus rattus* (rat), and shared limited homology (62% and 33.1%, respectively) with the *Danio rerio* (zebrafish) and *Drosophila melanogaster* (fruit fly) *UFL1* sequences (Table 3). These results confirm that bovine and ruminant *UFL1* show high sequence identity and that the *UFL1* protein sequences are highly conserved among the ruminant species.

UFL1 protein localization in bovine mammary tissues

Immunohistochemistry and immunofluorescence were conducted to clarify the localization of *UFL1* in the bovine mammary. As shown in Fig. 1a, *UFL1* was mainly distributed in mammary luminal cells. Furthermore, strong and weak *UFL1* immunolabelling patterns were detected in the cytoplasm and nuclei of BMECs, respectively (Fig. 1b).

Table 3 Pairwise comparison of *UFL1* between cow and other 13 species

Species	Symbol	Identity (%)
Cow (<i>Bos taurus</i>)	<i>UFL1</i>	
vs. Human	<i>UFL1</i>	92.1%
vs. mulatta	<i>UFL1</i>	91.9%
vs. <i>Canis lupus familiaris</i>	<i>UFL1</i>	91.6%
vs. <i>Mus musculus</i>	<i>UFL1</i>	88.3%
vs. <i>Rattus norvegicus</i>	<i>UFL1</i>	92.4%
vs. <i>Sus scrofa</i>	<i>UFL1</i>	92.4%
vs. <i>Equus caballus</i>	<i>UFL1</i>	93.0%
vs. <i>Ovis aries</i>	<i>UFL1</i>	96.9%
vs. <i>Capra hircus</i>	<i>UFL1</i>	97.7%
vs. <i>Gallus gallus</i>	<i>UFL1</i>	74.5%
vs. <i>Xenopus tropicalis</i>	<i>UFL1</i>	67.7%
vs. <i>Danio rerio</i>	<i>UFL1</i>	62.7%
vs. <i>Drosophila melanogaster</i>	<i>UFL1</i>	33.2%

ER stress stimulates increased *UFL1* expression

We found that TM induced ER stress at a concentration of 5 µg/ml, as evidenced by the increased expression of ER stress markers, *ATF6*, *CHOP*, and *GRP78* (Fig. 2a–c; *P* < 0.05). Treatment with 5 µg/ml TM also significantly induced higher apoptosis compared with the control group (Fig. 2e, f; *P* < 0.05). The results showed that the production of the autophagy marker LC3 was significantly increased, while p62 was significantly decreased in the TM-treated mammary cells compared with the control group (Fig. 2g–i; *P* < 0.05). Together, these results indicated that TM induces ER stress and activates autophagy at a concentration of 5 µg/ml; therefore, this concentration was selected for all the subsequent experiments.

Next, western blot and quantitative (q) PCR methods were used to characterize *UFL1* expression patterns in normal and ER stress BMECs. The results of this analysis showed that the 4 h treatment with TM caused a significant increase in both *UFL1* mRNA and protein levels compared with that observed in the control cells (Fig. 3a, b; *P* < 0.05). This indicates that ER stress induces *UFL1* expression.

Abrogating *UFL1* expression aggravates TM-induced ER stress

BMECs were transfected with a siRNA construct targeting *UFL1* to silence *UFL1* expression and investigate the role of *UFL1* in ER stress. We first verified that this approach significantly reduced *UFL1* protein production and then used it for the subsequent experiments (Fig. 4a, b; *P* < 0.05).

Cells transfected with *UFL1* siRNA (siUFL1) and treated with TM expressed more *ATF6*, *CHOP*, and *GRP78* than the control cells. Moreover, TM-treated cells also increased the expression of *ATF6*, *CHOP*, and *GRP78* (Fig. 4c–e; *P* < 0.05), which indicated that knocking down *UFL1* increased ER stress. In contrast, we observed no significant differences in the expression of the proteins mentioned above between the TM-treated cells and siUFL1 + TM cells (*P* > 0.05). We also found that the cells transfected with siUFL1 treated with TM showed increased LC3 and decreased p62 expression, when compared with the control cells. Cells transfected with siUFL1 alone showed similar results to those of the cells treated only with TM, a significant increase and decrease in the levels of LC3 and p62, respectively, when compared with the control group (Fig. 4f–h; *P* < 0.05). However, treating cells with TM had the same effect as transfecting them with siUFL1 or combining both the treatments; there were no significant differences in the protein levels of LC3 or p62 (*P* > 0.05). Together, these findings suggest that abrogating *UFL1* production aggravated ER stress and promoted ER stress-induced autophagy.

Fig. 1 Localization of UFL1 in the mammary of bovine. **a** Immunoreactivity of UFL1 in adult mammary of bovine. Negative control was performed with albumin from bovine serum instead of UFL1 antibody. All sections were stained with DAB (brown staining) and all pictures were captured by a Nikon YS100 microscope (Nikon)

with a digital camera (Nikon Tokyo, Japan). **b** Subcellular localization of UFL1 in BMECs. All cells were incubated Alexa Fluor 647 conjugated goat anti-rabbit (red fluorescence). All images were captured on a Zeiss LSM700 META microscope (Carl Zeiss, Oberkochen, Germany). Scale bars: 50 µm. Control: negative control. TM: tunicamycin

UFL1 overexpression alleviates TM-stimulated ER stress

To investigate how UFL1 overexpression might impact ER stress, a UFL1 overexpression plasmid was designed and used to significantly increase the UFL1 protein levels compared with those detected in the control cells (Fig. 5a, b; $P < 0.05$).

As already shown, the *ATF6*, *CHOP*, and *GRP78* levels were significantly higher in cells that received TM treatment than in the control cells. However, UFL1 overexpression significantly attenuated this TM-induced expression of *ATF6*, *CHOP*, and *GRP78* (Fig. 5c–e; $P < 0.05$). Additionally, cells overexpressing UFL1 alone did not activate ER stress when compared with the control group. Together, our data suggested that UFL1 is involved in the activation of ER stress. We showed that significantly higher LC3 and lower p62 levels were found following TM treatment ($P < 0.05$). Furthermore, the TM-induced autophagy was alleviated in cells overexpressing UFL1, which showed reduced expression of LC3 and increased the expression of p62 compared with the TM-

treated normal cells (Fig. 5f–h; $P < 0.05$). Moreover, UFL1 overexpression alone did not affect the expression levels of LC3 and p62 when compared with the control cells ($P > 0.05$). Expression of LC3 in the cells treated with TM was significantly higher than that in the other three groups, and the level of p62 in the cells treated with TM was significantly lower than that in the other three groups ($P < 0.05$). Together, these findings suggest that UFL1 overexpression alleviates ER stress and thereby also prevents ER stress-induced autophagy.

Discussion

In the present study, we clearly illustrated the localization and protective function of UFL1 in BMECs that were induced to undergo ER stress-mediated autophagy. The UFL1 localization pattern in the bovine mammary gland has not been previously reported. In this study, UFL1 expression was concentrated strongly in the cytoplasm, and only weakly in the nuclei of BMECs. Furthermore, silencing of UFL1-induced ER

Fig. 2 Autophagy induced by ER stress. **a–c** qPCR was used for ER stress-related mRNA levels, *ATF6* (a), *CHOP* (b), and *GRP78* (c), in BMECs. All mRNA levels were compared with those of *GAPDH*. **d** Effects of TM on BMEC viability. Cells were incubated with 5 μ g/ml TM for 4 h. **e, f** The ratio of cell apoptosis in response to TM was analyzed by flow cytometric analyses. Quantitative cell apoptosis data

are shown as means $\pm$ SEM, $n=3$. **g–i** Western blot analysis was performed to determine expression levels of LC3 and p62 in BMECs by TM. Control: cells incubated free-serum medium. TM: cells incubated free-serum medium with TM. All works were performed 3 times, respectively. Different letters denote statistical difference at a $P < 0.05$ level of significance

Fig. 3 UFL1 expression increased by TM. **a–c** Effect of TM on UFL1 expression in bovine mammary epithelial cells. **a** Cells treated with TM were processed for protein to analyze UFL1 and GAPDH by western blot. **b** Western blot data were quantified. **c** qPCR analysis of the expression of *UFL1* in bovine mammary epithelial cells treatment with TM. All

experiments were independently done in triplicate. The values are means $\pm$ SEM, $n = 3$. Control: cells incubated free-serum medium. TM: cells incubated free-serum medium with TM. Different letters denotes statistical difference at a $P < 0.05$ level of significance

stress and enhanced ER stress-induced autophagy in BMECs. Conversely, the overexpression of UFL1 prevented the BMECs from undergoing programmed cellular death in response to TM-induced ER stress (Fig. 6). Thus, this study provides novel insights into both the biological significance of UFL1 and the regulation of ER stress-induced autophagy in BMECs.

Protein diversity and functions are often regulated via post-translational modifications such as acetylation, methylation, and ubiquitination; each of which is essential for normal cellular homeostasis. UFL1 functions as a key E3 ligase in the UFM1 system to mediate the conjugation of UFM1 with a target protein (Daniel and Liebau 2014; Komatsu et al. 2014). Ubis were discovered more than a decade ago and have since been characterized to share a similar protein structure, including a ubiquitin or “ β -grasp” fold (Hochstrasser 2000; Pickart and Eddins 2004). While most E3 Ubis contain a “RING” cysteine-rich domain that coordinates a pair of zinc ions, UFL1 does not (Tatsumi et al. 2010; Wei and Xu 2016). Therefore, UFL1 does not share an obvious sequence homology with any other known ubiquitin or Ubl E3 modifiers. However, the results of this study suggest that UFL1 sequences in ruminant species such as *Bos Taurus*, *C. hircus*, and *O. aries* are highly homologous.

While UFL1 was originally identified in a range of murine tissues such as the liver and bone, recent reports have suggested that it may be involved in diverse diseases and

metabolic systems, including cancer, diabetes, and the digestive system (Lemaire et al. 2011; Tatsumi et al. 2011; Yoo et al. 2014; Zhang et al. 2015; Miller et al. 2017). For example, UFL1 and its target protein, ASC1, mediate breast cancer development by regulating estrogen receptor- α transactivation, suggesting that UFL1 may play an important role in human breast homeostasis (Yoo et al. 2014). The mammary gland is similarly defined as a secretory organ, in which mammary epithelial cells form a key component of the secretory alveoli that produce milk (Dessaugue et al. 2011). This study shows, for the first time, that UFL1 is predominantly expressed in the cytoplasm of BMECs. Further studies are required to determine whether and how this impacts milk secretion.

The ER is a critical organelle responsible for protein synthesis and folding and can be induced to undergo stress via stimuli including disease, or an aberrant cellular microenvironment. As in previous in vivo and in vitro animal models, this study used TM to induce an ER stress response in mammary epithelial cells (Zhu et al. 2017). We also confirmed that ER stress induced by TM significantly increased UFL1 expression. Notably, various in vivo and in vitro animal models have previously shown that UFL1 deletion stimulates ER stress. For example, ER stress marker (GRP78, CHOP, ERdi4) protein levels were significantly increased in the bone marrow cells from UFL1 knockout mice (Zhang et al. 2015). Ufbp1 is also a member of the UFM1 conjugation system, with evidence suggesting that Ufbp1 has a similar role as

Fig. 4 UFL1 knockdown aggravates ER stress response by TM. **a, b** Expression of UFL1 in UFL1 knockdown BMECs. Control (vector) and UFL1 siRNA (siUFL1) cells were lysed to perform UFL1 protein expression by western blot analyses. **c–e** ER stress marker gene levels *ATF6*, *CHOP*, and *GRP78* were determined by qPCR compared with those of GAPDH. **f–h** LC3 and p62 protein expressions were detected

by western blot analyses. Control: negative control. TM: cells incubated free-serum medium with TM. siUFL1 + TM: cells co-incubated UFL1-siRNA and TM. siUFL1: cells incubated UFL1-siRNA. All experiments were performed 3 times. Different letters denotes statistical difference at a $P < 0.05$ level of significance

Fig. 5 UFL1 increase alleviates ER stress response by TM. **a, b** Expression of UFL1 in UFL1-overexpressing BMECs. Control (vector) and UFL1 overexpressing (oeUFL1) cells were lysed to perform UFL1 protein expression by western blot analyses. **c–e** ER stress marker gene levels *ATF6*, *CHOP*, and *GRP78* were determined by qPCR compared with those of *GAPDH*. **f–h** LC3 and p62 protein expressions were

detected by western blot analyses. Control: negative control. TM: cells incubated free-serum medium with TM. oeUFL1 + TM: cells co-incubated UFL1 cDNA and TM. oeUFL1: cells incubated UFL1 cDNA. All experiments were performed 3 times. Different letters denotes statistical difference at a $P < 0.05$ level of significance

UFL1 in ER stress. Notably, Ufbp1-depleted hematopoietic stem cells activate ER stress and are essential for maintaining ER homeostasis (Liu et al. 2017). Consistent with the previous studies, silencing UFL1 expression induced ER stress in our system. Moreover, siUFL1 and TM co-treatment of BMECs aggravated the ER stress response. However, the overexpression of UFL1 alleviated the ER stress induced by TM. UFM1 overexpression prevents ER stress-induced increases in CHOP and cleaved caspase-3 levels in mouse macrophage cells (Hu et al. 2014). In this study, the increased UFL1 expression inhibited ER stress in BMECs. Given the established role of UFL1 in the UFM1 system, these effects

are likely a result of the increased ufmylation driven by the increased UFL1 levels. These results suggested that UFL1 might be considered a potential target for activated ER stress.

Autophagy, a kind of programmed cell death, is an evolutionarily conserved and lysosome-mediated cellular degradation process. Autophagy is activated by various cellular stresses, including ER stress (Levine and Kroemer 2008; Deegan et al. 2014; Song et al. 2017). Upon activation of autophagy, the LC3 levels are increased due to an increase in autophagosome formation (Kabeya et al. 2000), making it a good marker for the autophagy process. Decreased p62 expression is also considered an important

Fig. 6 A model of the role of UFL1 in ER stress-induced autophagy in BMECs

marker of autophagy (Song et al. 2017). In this study, we disrupted UFL1 production and demonstrated an increase in autophagy via increased LC3 and decreased p62 expression. These expression levels were further significantly aggravated via concomitant TM treatment. However, the overexpression of UFL1 plasmid in BMECs induced the opposite effect upon TM treatment. Thus, UFL1 may be exploited as a potential target protein that alleviates autophagy activated by ER stress.

Deleting UFL1 in pancreatic beta cells affects the expression of the UFM1-UBBP1 conjugation complex and thereby aggravates ER stress to cause a significant increase in the rate of apoptosis (Lemaire et al. 2011). Bone marrow and hematopoietic stem cells from UFL1-deficient mice accumulated proteins such as LC3 and p62, which affected cellular homeostasis. There is not enough evidence suggesting that UFL1-induced ER stress directly regulated the protein levels of FIP200 and ATG7, which are markers for the early stages of autophagy. Therefore, UFL1 may be involved in the later stages of autophagy (Zhang et al. 2015). However, in our study, the UFL1 expression decreased the expression of p62, which might be attributed to the different cells and treatments. Nonetheless, our results suggested that UFL1 has similar roles as the other members of the UFM1 conjugation system in autophagy induced by ER stress. However, the exact

molecular mechanisms underlying this role of UFL1 in ER-induced autophagy need further investigation.

In conclusion, we determined that UFL1 is strongly expressed in BMECs, suggesting that UFL1 may be involved in BMEC homeostasis. This study is the first to indicate that increase in UFL1 expression can protect cellular homeostasis via downregulation of ER stress and autophagy-related gene expression in BMECs. Our results indicate that UFL1 plays a critical protective role against ER stress in BMECs and is important to maintain cellular homeostasis.

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Compliance with ethical standards All experiments were approved in accordance with the manual of Institutional Animal Care and the Use of Laboratory Animals (SYXK2011-0036) published by the Committee of Nanjing Agricultural University and were prepared in accordance with the guidelines of the Care and Use of Laboratory Animals.

Conflict of interest The authors declare that they have no conflict of interest.

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