Diabetologia (2015) 58:809–818
DOI 10.1007/s00125-014-3475-6

ARTICLE

Fibroblast growth factor 21 participates in adaptation to endoplasmic reticulum stress and attenuates obesity-induced hepatic metabolic stress

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Received: 30 July 2014 / Accepted: 25 November 2014 / Published online: 24 December 2014

Abstract
Aims/hypothesis Fibroblast growth factor 21 (FGF21) is an endocrine hormone that exhibits anti-diabetic and anti-obesity activity. FGF21 expression is increased in patients with and mouse models of obesity or nonalcoholic fatty liver disease (NAFLD). However, the functional role and molecular mechanism of FGF21 induction in obesity or NAFLD are not clear. As endoplasmic reticulum (ER) stress is triggered in obesity and NAFLD, we investigated whether ER stress affects FGF21 expression or whether FGF21 induction acts as a mechanism of the unfolded protein response (UPR) adaptation to ER stress induced by chemical stressors or obesity.

Methods Hepatocytes or mouse embryonic fibroblasts deficient in UPR signalling pathways and liver-specific *eIF2α* mutant mice were employed to investigate the in vitro and in vivo effects of ER stress on FGF21 expression, respectively. The in vivo importance of FGF21 induction by ER stress and obesity was determined using inducible *Fgf21*-transgenic mice and *Fgf21*-null mice with or without leptin deficiency.
Results We found that ER stressors induced FGF21 expression, which was dependent on a PKR-like ER kinase–eukaryotic translation factor 2α–activating transcription factor 4 pathway both in vitro and in vivo. *Fgf21*-null mice exhibited increased expression of ER stress marker genes and augmented hepatic lipid accumulation after tunicamycin treatment. However, these changes were attenuated in inducible *Fgf21*-transgenic mice. We also observed that *Fgf21*-null mice with leptin deficiency displayed increased hepatic ER stress response and liver injury, accompanied by deteriorated metabolic variables.
Conclusions/interpretation Our results suggest that FGF21 plays an important role in the adaptive response to ER stress- or obesity-induced hepatic metabolic stress.

Keywords ATF4 · eIF2α · ER stress · FGF21 · Insulin resistance · Lipid accumulation · Liver injury · Obesity

Abbreviations

ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
ATF4	Activating transcription factor 4
ATF6α	Activating transcription factor 6α
BAT	Brown adipose tissue
BIP	Binding immunoglobulin protein
CHOP	CCAAT/enhancer binding protein homologous protein

Electronic supplementary material The online version of this article (doi:10.1007/s00125-014-3475-6) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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eIF2α	Eukaryotic translation factor 2α
ER	Endoplasmic reticulum
FGF21	Fibroblast growth factor 21
IRE1α	Inositol-requiring 1α
ITT	Insulin tolerance test
MEFs	Mouse embryonic fibroblasts
NAFLD	Nonalcoholic fatty liver disease
PERK	PKR-like endoplasmic reticulum kinase
PTT	Pyruvate tolerance test
SIRT1	Sirtuin1
TG	Triacylglycerol
UPR	Unfolded protein response
WAT	White adipose tissue
XBP1	X-box binding protein 1
XBP1s	Spliced X-box binding protein 1

Introduction

Fibroblast growth factor 21 (FGF21) is an endocrine hormone produced predominantly in the liver but also in white adipose tissue (WAT), brown adipose tissue (BAT), pancreas and skeletal muscle [1, 2]. In starvation, FGF21 promotes lipolysis, beta oxidation or ketogenesis [3], indicating that FGF21 is a critical regulator of lipid homeostasis in adaptation to starvation. FGF21 can improve deteriorated metabolic variables in obese diabetic humans and in animal models of obesity and diabetes [4–7]. In addition, FGF21 plays a protective role in toxin-induced tissue injury [8, 9].

Protein folding occurs in the endoplasmic reticulum (ER). Perturbations of ER homeostasis cause accumulation of misfolded proteins in the ER lumen, triggering the unfolded protein response (UPR), an adaptive programme to resolve misfolded protein accumulation in the ER [10]. The UPR is regulated through three ER transmembrane sensors: inositol-requiring 1α (IRE1α), activating transcription factor 6α (ATF6α) and PKR-like ER kinase (PERK) [10]. X-box binding protein 1 (XBP1) and eukaryotic translation factor 2α (eIF2α)-activating transcription factor 4 (ATF4) participate in various adaptive responses to ER stress downstream of IRE1α and PERK, respectively. Unresolved ER stress leads to the development and progression of various diseases such as obesity and diabetes [11, 12].

Recent studies reported that FGF21 is increased in the liver in mouse models of and humans with obesity or nonalcoholic fatty liver disease (NAFLD) [13–15]. However, little is known about the functional role and molecular mechanism of FGF21 induction in these conditions. As ER stress is elevated in obesity [16], we investigated the relationship between ER stress and FGF21 induction. We found that FGF21 is induced by ER stress in a PERK-eIF2α-ATF4-dependent manner. In addition, we observed that FGF21 deletion accelerates ER stress-induced hepatic injury or lipid accumulation, and exacerbates obesity-induced ER stress and metabolic deterioration. These results suggest that FGF21 may play a role in the adaptive response to ER stress induced by a pharmacological ER stressor or obesity.

Methods

Animal experiments $Fgf21^{+/+}$ and $Fgf21^{-/-}$ mice have been described elsewhere [17]. $Fgf21^{+/-}$ mice were crossed with *ob/w* mice (Jackson Laboratory, Bar Harbor, ME, USA) to generate $Fgf21^{+/+}ob/ob$ and $Fgf21^{-/-}ob/ob$ mice. Mice with a liver-specific defect in eIF2α phosphorylation ($Eif2\alpha^{A/A}$/*fTg*/*Alfp-Cre*) were generated by breeding $Eif2\alpha^{A/A}$/*fTg* mice [18] with $Eif2\alpha^{S/A}$/*Alfp-Cre* mice. Liver-specific inducible *Fgf21*-transgenic (*Apoe-rtTA*M2*/*TetO-Fgf21*) mice were generated using a tetracycline-inducible system. *Fgf21*-null and inducible *Fgf21*-transgenic mice were maintained in a specific pathogen-free facility of Samsung Biomedical Research Institute. *Eif2α* mutant mice were maintained under a specific pathogen-free condition in the laboratory animal care facility of the University of Ulsan. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute or University of Ulsan. See Electronic Supplementary Material (ESM) Methods for details.

GTT, insulin tolerance test and pyruvate tolerance test GTTs and pyruvate tolerance tests (PTTs) were performed in overnight-fasted mice with intraperitoneal injection of glucose (1 g/kg) and pyruvate (1 g/kg), respectively. An insulin tolerance test (ITT) was conducted in 6 h-fasted mice with an intraperitoneal injection of insulin (1 U/kg). Blood glucose levels were measured with an Accu-Check glucometer (Roche, Mannheim, Germany).

Ex vivo glucose-stimulated insulin secretion assay Pancreatic islets were isolated from female $Fgf21^{+/+}ob/ob$ and $Fgf21^{-/-}ob/ob$ mice by collagenase digestion and Biocoll (Biochrom AG, Berlin, Germany) gradient centrifugation. See ESM Methods for details.

Plasmid constructs and reagents See ESM Methods for details.

Generation of adenovirus Adenoviruses expressing *ATF4* or Flag-*XBP1s* were generated by homologous recombination between a linearised transfer vector (pAd-Track-*ATF4* or

pAd-Track-Flag-*XBP1s*) and an adenoviral backbone vector (pAd-Easy).

Cell culture FaO, AML12, HepG2 cells, primary mouse hepatocytes and mouse embryonic fibroblasts (MEFs) deficient in UPR genes were maintained at 37°C in a humid atmosphere of 5% CO_2. See ESM Methods for details.

Luciferase assay See ESM Methods for details.

siRNA transfection See ESM Methods for details.

RNA analysis Real-time RT-PCR was conducted using SYBR Green Master Mix (Takara, Otsu, Shiga, Japan) and gene-specific primers (ESM Table 1) in ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA). Relative expression values of specific genes were normalised to L32 mRNA. See ESM Methods for details.

Immunoblot analysis See ESM Methods for details.

Histology and staining analysis Liver and pancreas tissues were fixed with 10% neutral buffered formalin and 4% paraformaldehyde to make paraffin- and optimal cutting temperature-embedded blocks, respectively. See ESM Methods for details.

Blood chemistry and metabolite analysis See ESM Methods for details.

Statistical analysis All values are expressed as mean±SEM. Statistical significance was tested with the unpaired two-tailed Student's *t* test using GraphPad Prism Version 5.02 Software (La Jolla, CA, USA). A p value of less than 0.05 was considered significant.

Results

ER stress induces FGF21 expression through PERK–eIF2α–ATF4 pathway in vitro To analyse the effect of ER stress on FGF21 expression in hepatocytes, mouse AML12 cells were treated with chemical ER stressors, tunicamycin and thapsigargin. We found that, remarkably, both tunicamycin and thapsigargin induced *Fgf21* gene expression as well as upregulation of several UPR genes, including spliced *Xbp1* (*Xbp1s*), *Bip*, *Chop* (also known as *Ddit3*) and *Grp94* (Fig. 1a, b). Additionally, *FGF21* expression was increased in rat FaO, human HepG2 and primary mouse hepatocytes treated with ER stressors (Fig. 1b, c). In parallel, FGF21 level was augmented in culture supernatant fractions of FaO cells treated with ER stressors (Fig. 1d). These results demonstrate that ER stress induces FGF21 expression in various hepatocyte cell lines and primary hepatocytes.

We next investigated the mechanisms by which ER stress increases FGF21 expression using MEFs deficient in ER stress sensors. *Fgf21* induction by ER stressors was much weaker in $Perk^{-/-}$ MEFs compared with control MEFs (Fig. 1e), while ER stressor-induced *Fgf21* expression in $Ire1\alpha^{-/-}$, $Atf6\alpha^{-/-}$ or $Xbp1^{-/-}$ MEFs was comparable with that in control MEFs (ESM Fig. 1a–c). Furthermore, adenovirus-mediated overexpression of XBP1s had no effect on *FGF21* expression (ESM Fig. 1d). We next tested whether eIF2α and ATF4 downstream of PERK induce FGF21 expression. As expected, two ER stressors increased *Fgf21* mRNA levels in $Eif2\alpha^{S/S}$ (wild-type) and $Atf4^{+/+}$ MEFs, while *Fgf21* induction was markedly attenuated in $Eif2\alpha^{A/A}$ MEFs (harbouring S51A mutation) and $Atf4^{-/-}$ MEFs (Fig. 1f, g). ATF4 knockdown consistently suppressed tunicamycin-induced *Fgf21* expression (Fig. 1h, i), while ATF4 overexpression increased *FGF21* mRNA expression and *Fgf21* promoter activity (Fig. 1j, k). However, XBP1s or ATF6α overexpression had no effect on luciferase activity (Fig. 1k). Moreover, mutations of putative ATF4-response elements abolished the increase in *Fgf21* promoter activity by ER stressors (Fig. 1l), indicating that two ATF4-response elements are required for ER stress-induced FGF21 expression. Together, these findings suggest that the PERK–eIF2α–ATF4 pathway is critical for ER stress-induced FGF21 expression in vitro.

Hepatic eIF2α–ATF4 pathway is required for ER stress-induced FGF21 expression in vivo We investigated whether or not ER stress stimulates FGF21 expression in vivo. When we injected tunicamycin into male C57BL/6 mice, hepatic *Fgf21* and UPR gene expression was induced accompanied by elevated serum FGF21 levels (Fig. 2a–c, ESM Fig. 2). However, we did not observe a difference in *Fgf21* expression in other tissues such as WAT or skeletal muscle (Fig. 2b), whereas *Fgf21* expression in BAT appeared to be increased by tunicamycin administration (Fig. 2b). This finding indicates that hepatic FGF21 induction is largely responsible for elevated circulating FGF21 level after tunicamycin administration.

To study the importance of the eIF2α–ATF4 axis in FGF21 induction by ER stress in vivo, we generated liver-specific $Eif2\alpha^{A/A}$ mice ($Eif2\alpha^{A/A}$/*fTg*/*Alfp-Cre*) with a homozygous Ser51Ala mutation in *Eif2α* alleles of the liver (Fig. 2d). Phosphorylation of eIF2α in the liver was not observed in $Eif2\alpha^{A/A}$/*fTg*/*Alfp-Cre* mice (Fig. 2e), confirming a defect in hepatic eIF2α phosphorylation in these mice. Importantly, we observed that hepatic expression of FGF21 or ATF4 and serum FGF21 level were markedly decreased in liver-specific $Eif2\alpha^{A/A}$ mice compared with control heterozygous $Eif2\alpha^{S/A}$/*fTg* mice under ER stress condition (Fig. 2e–g). These data indicate that the eIF2α–ATF4 pathway is critical for FGF21 induction in response to ER stress in vivo.

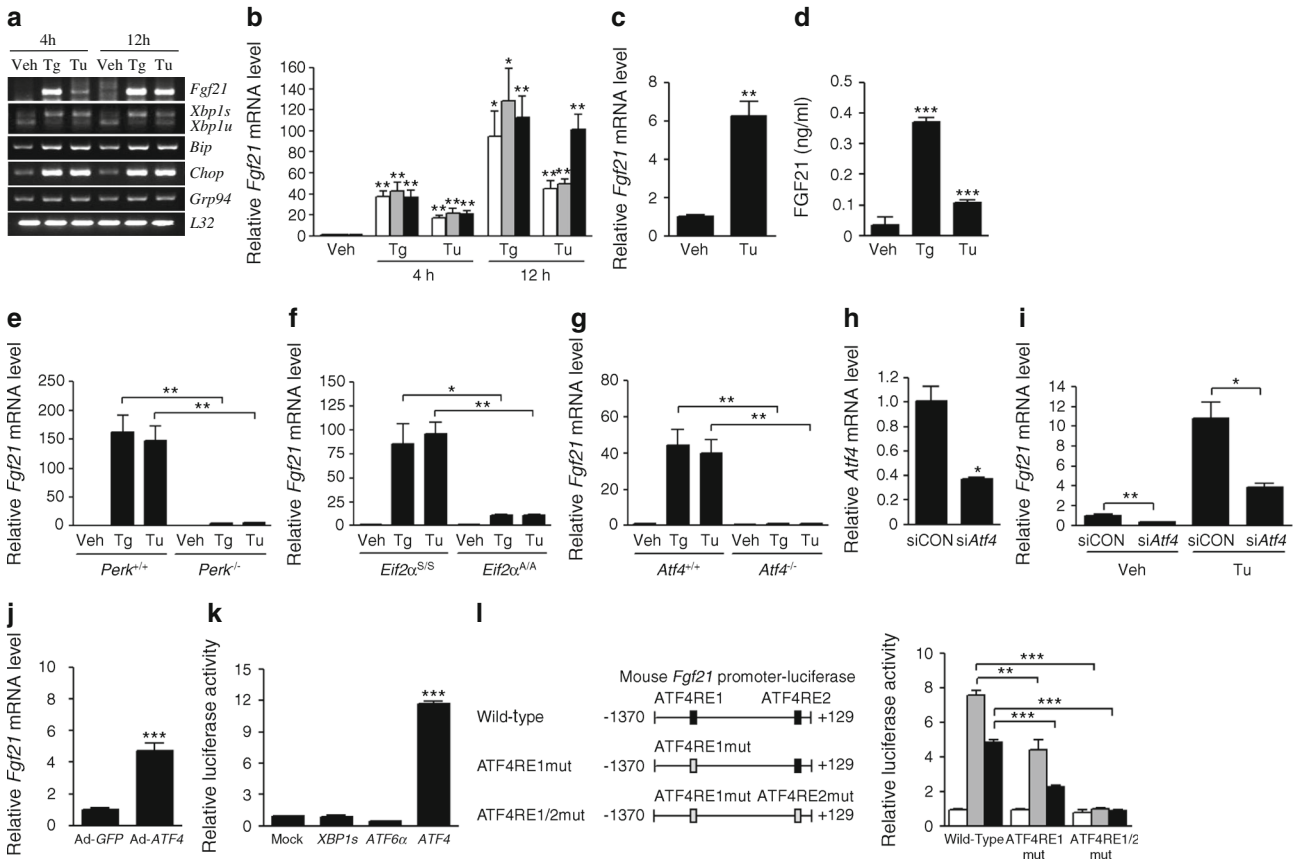

Fig. 1 The PERK–eIF2α–ATF4 pathway is indispensable for ER stress-induced FGF21 expression in vitro. (**a**) RT-PCR showing mRNA expression of *Fgf21* and ER stress markers in AML12 cells treated with tunicamycin (Tu, 5 μg/ml), thapsigargin (Tg, 1 μmol/l) or dimethyl sulfoxide (DMSO, Veh) for indicated times (n=3). (**b**) Relative *Fgf21* mRNA level in FaO (white bars), AML12 (grey bars) and HepG2 (black bars) cells treated with ER stressors (n=3). *Fgf21* mRNA level normalised to *L32* mRNA in Veh-treated cells is presented as 1; *p<0.05, **p<0.01 vs Veh. (**c**) Relative *Fgf21* mRNA level in primary mouse hepatocytes treated with Tu for 4 h (n=3). *Fgf21* mRNA level normalised to *L32* mRNA in Veh-treated hepatocytes is presented as 1. (**d**) FGF21 level in culture supernatant fractions of FaO cells treated with ER stressors for 16 h (n=3); ***p<0.001 vs Veh. Relative *Fgf21* mRNA level in (**e**) *Perk*$^{-/-}$, (**f**) *Eif2α*$^{-/-}$ and (**g**) *Atf4*$^{-/-}$ MEFs treated with ER stressors for 12 h (n=3). *Fgf21* mRNA level normalised to *L32* mRNA in Veh-treated wild-type MEFs is presented as 1. (**h**, **i**) Relative *Atf4* or *Fgf21* mRNA level in siCON- or si*Atf4*-transfected AML12 cells treated with Tu for 8 h (n=3). *Atf4* and *Fgf21* mRNA level normalised to *L32* mRNA in siCON-transfected cells and those treated with Veh are presented as 1, respectively. (**j**) Relative *FGF21* mRNA level in HepG2 cells infected with adenovirus expressing *GFP* or *ATF4* for 36 h (n=3). *FGF21* mRNA level normalised to *L32* mRNA in Ad-*GFP*-infected cells is presented as 1. (**k**) Luciferase activity of *Fgf21* promoter in FaO cells overexpressing *XBP1s*, *ATF6α* or *ATF4* for 24 h (n=3); ***p<0.001 vs Mock. (**l**) Luciferase activity of ATF4-response element (ATF4RE)-mutated *FGF21* promoter in FaO cells treated with vehicle (white bars), Tg (grey bars) or Tu (black bars) for 12 h (n=3). Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001

FGF21 suppresses ER stress response and alleviates ER stress-induced hepatic injury and lipid accumulation To study the physiological role of FGF21 induction by ER stress, we challenged *Fgf21*$^{-/-}$ mice with tunicamycin and investigated changes in UPR signalling. As expected, there was no increase in serum FGF21 level in *Fgf21*$^{-/-}$ mice after tunicamycin administration (ESM Fig. 3a). Importantly, expression of UPR genes after tunicamycin treatment was significantly increased in the liver of *Fgf21*$^{-/-}$ mice compared with control *Fgf21*$^{+/+}$ mice (Fig. 3a). In parallel, levels of phosphorylated eIF2α, ATF4 and binding immunoglobulin protein (BIP) in the liver of *Fgf21*$^{-/-}$ mice were higher compared with levels in control mice after tunicamycin treatment (Fig. 3b). Furthermore, induction of the pro-apoptotic transcription factor CCAAT/enhancer binding protein homologous protein (CHOP) by tunicamycin was elevated in the liver of *Fgf21*$^{-/-}$ mice compared with that of *Fgf21*$^{+/+}$ mice (Fig. 3b), implying that FGF21 deletion may exacerbate liver injury caused by ER stress. In parallel, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were increased in *Fgf21*$^{-/-}$ mice under ER stress condition (Fig. 3c). These results suggest that FGF21 plays a protective role in ER stress-induced liver injury.

As ER stress can induce hepatic lipid accumulation [12], we next studied the physiological role of FGF21 in ER stress-mediated hepatosteatosis. Importantly, *Fgf21*$^{-/-}$ mice exhibited elevated hepatic triacylglycerol (TG) accumulation compared with *Fgf21*$^{+/+}$ mice after tunicamycin administration

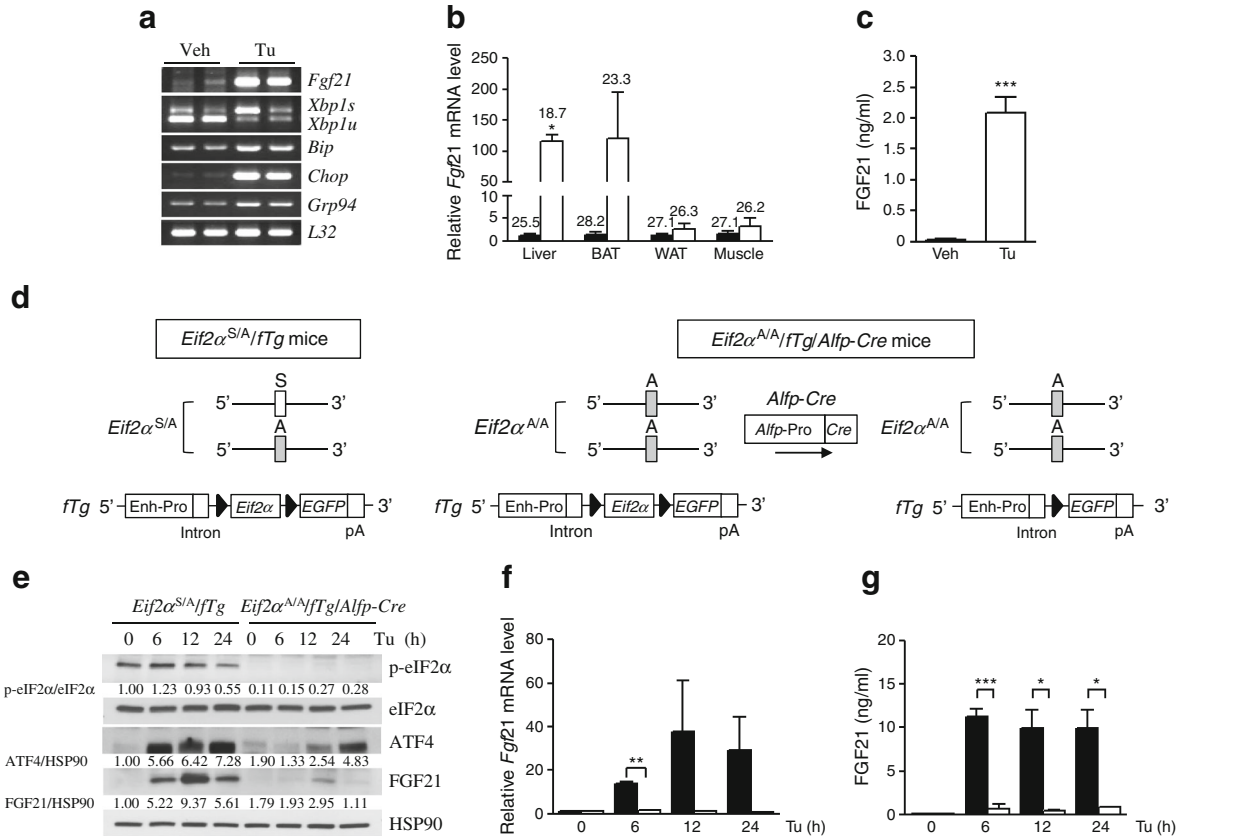

Fig. 2 Disruption of eIF2α–ATF4 pathway abrogates FGF21 induction in response to ER stress in vivo. (**a**) RT-PCR showing the mRNA expression of *Fgf21* and ER stress markers in the liver of C57BL/6 mice after tunicamycin administration (Tu, 2 mg/kg) or 2% DMSO (Veh) for 8 h. (**b**) Relative *Fgf21* mRNA level (n=4) and (**c**) serum FGF21 level (n=6) in C57BL/6 mice after Veh (black bars) or Tu (white bars) administration for 24 h. The number above the bars represents the mean C_t value. *Fgf21* mRNA level normalised to *L32* mRNA in Veh-treated tissue is presented as 1; *p<0.05 vs Veh. (**d**) Schematic diagram for the generation of liver-specific $Eif2\alpha^{A/A}$ mice. $Eif2\alpha^{S/A}$/*fTg* mice have a Ser51Ala mutation in one *Eif2α* allele and LoxP sequences (black arrowheads)-flanked wild-type *Eif2α* transgene driven by the cytomegalovirus enhancer and chicken β-actin promoter (Enh-Pro). In $Eif2\alpha^{A/A}$/*fTg*/*Alfp-Cre* mice, excision of LoxP sites by *Cre* recombinase expressed under the control of the albumin promoter and α-fetoprotein enhancer (*Alfp*) causes deletion of wild-type *Eif2α* transgene and induces *EGFP* expression. (**e**) Immunoblotting of the liver, and (**f**) relative hepatic *Fgf21* mRNA level and (**g**) serum FGF21 level in $Eif2\alpha^{S/A}$/*fTg* (black bars) and $Eif2\alpha^{A/A}$/*fTg*/*Alfp-Cre* mice (white bars) after Tu administration (1 mg/kg). *Fgf21* mRNA level normalised to *L32* mRNA in Veh-treated sample is presented as 1. Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001

(Fig. 3d, e). When we analysed changes in the expression of genes involved in lipid metabolism, the expression of fatty acid and TG synthesis genes such as *Scd1*, *Adrp* (also known as *Plin2*) and *Dgat2* appeared to be increased in the liver of $Fgf21^{-/-}$ mice compared with that of $Fgf21^{+/+}$ mice under ER stress condition (Fig. 3a). By contrast, we did not observe differences in the expression of genes such as *Pparγ*, *Srebp1c* (also known as *Srebf1*) and *Acc1* (Fig. 3a). The expression of *Cd36*, which is involved in lipid uptake, was also significantly increased in the liver of $Fgf21^{-/-}$ mice compared with $Fgf21^{+/+}$ mice (Fig. 3a). Additionally, we observed a tendency towards increased expression of the genes involved in beta oxidation, including *Pparα* and *Mcad* (also known as *Acadm*) in the liver of $Fgf21^{-/-}$ mice compared with control mice after tunicamycin administration (ESM Fig. 3b), which is likely to be an adaptive response to excessive TG accumulation caused by FGF21 deletion. These results indicate that enhanced hepatic lipid synthesis and uptake may contribute to increased hepatic TG accumulation in $Fgf21^{-/-}$ mice under ER stress condition.

We next investigated the effect of exogenous FGF21 on tunicamycin-induced ER stress and hepatic lipid accumulation using liver-specific inducible *Fgf21*-transgenic mice. As expected, inducible *Fgf21*-transgenic mice had increased serum FGF21 levels and elevated hepatic *Fgf21* gene expression when fed chow diet containing doxycycline (Fig. 3f, ESM Fig. 4). Importantly, these mice showed attenuated expression of UPR genes and decreased hepatic lipid accumulation after tunicamycin administration (Fig. 3g, h). Together, these findings suggest that FGF21 expression is an adaptive response to ER stress to alleviate excessive liver injury and lipid accumulation.

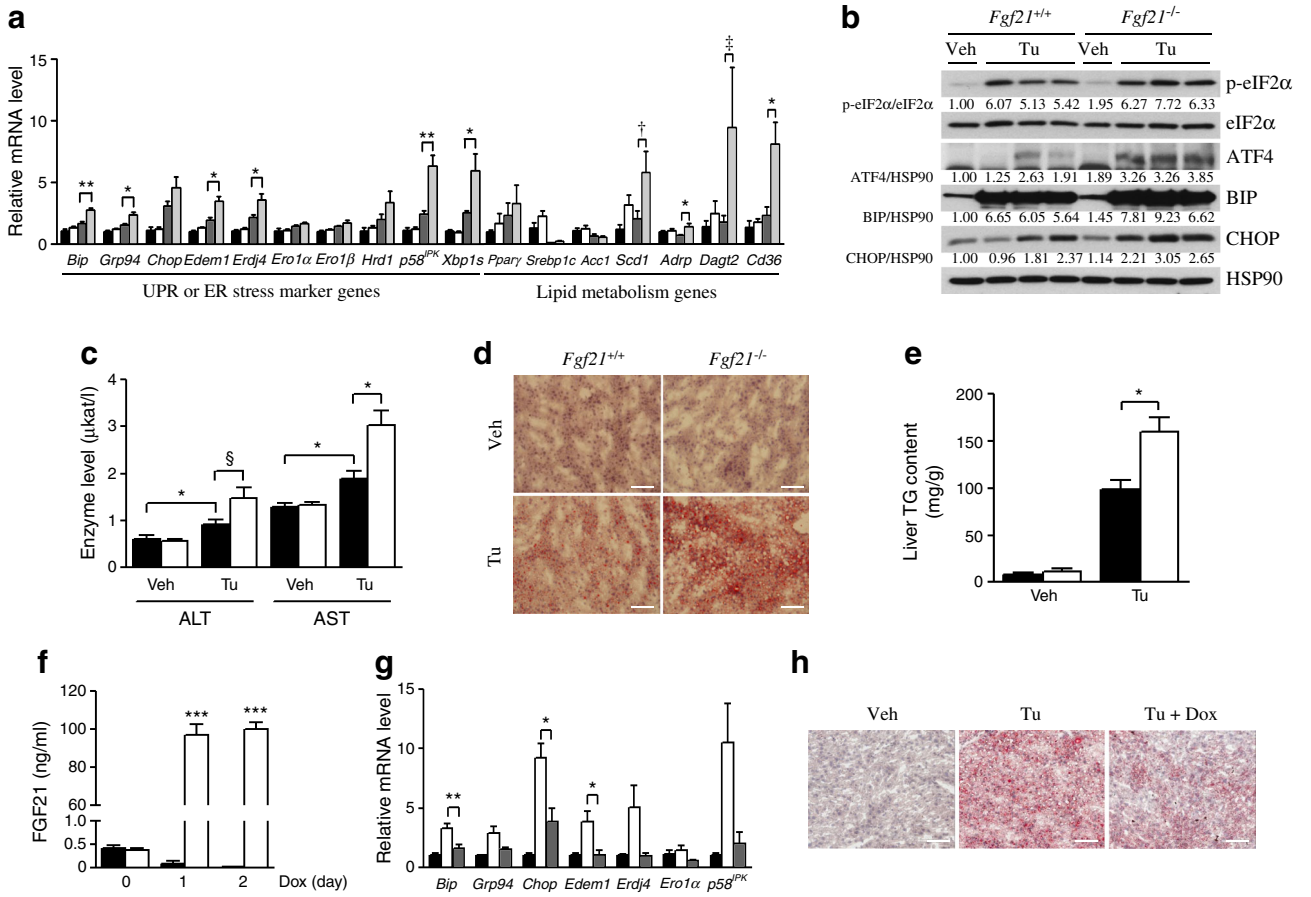

Fig. 3 FGF21 alleviates upregulation of ER stress marker genes and attenuates liver injury or hepatic steatosis in response to ER stressors. (**a**) Relative mRNA level of genes of ER stress markers and lipid metabolism (n=4–7), and (**b**) immunoblotting of ER stress markers in mice after administration of Vehicle (Veh) or tunicamycin (Tu, 1 mg/kg) for 24 h. mRNA levels normalised to *L32* mRNA in the liver of Veh-treated $Fgf21^{+/+}$ mice are presented as 1. Black bars, $Fgf21^{+/+}$ + Veh; white bars, $Fgf21^{-/-}$+Veh; dark grey bars, $Fgf21^{+/+}$ + Tu; light grey bars, $Fgf21^{-/-}$+ Tu. (**c**) Serum ALT and AST levels (n=5–11), (**d**) Oil Red O staining in the liver and (**e**) hepatic TG content (n=5–11) in $Fgf21^{+/+}$ (black bars) or $Fgf21^{-/-}$ mice (white bars) after Tu administration (1 mg/kg) for 24 h. Scale bars, 100 μm. (**f**) Serum FGF21 level in inducible *Fgf21*-transgenic (white bars) or control mice (black bars) with or without dietary doxycycline (Dox) for 1–2 days (n=3–11); ***p<0.001 vs time-matched control mice. (**g**) Relative mRNA level of ER stress marker genes (n=3) and (**h**) Oil Red O staining in the liver of inducible *Fgf21*-transgenic mice after Tu administration (1 mg/kg) for 24 h. mRNA levels normalised to *L32* mRNA in the liver of Veh-treated mice are presented as 1. Black bars, Veh; white bars, Tu; grey bars, Tu+Dox. Scale bars, 100 μm. Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001, $^{\dagger}p$=0.08, $^{\ddagger}p$=0.17, $^{\S}p$=0.09

Obesity-mediated FGF21 induction alleviates lipid-induced ER stress, liver injury and metabolic deterioration Since ER stress occurs in the liver of obese mice [16], we investigated the pathophysiological role of FGF21 in obesity-induced hepatic ER stress using $Fgf21^{-/-}ob/ob$ mice. Consistent with previous reports [15, 16], *ob/ob* mice exhibited increased hepatic *Fgf21* expression and serum FGF21 levels compared with lean mice, together with upregulation of UPR genes and aggravated liver injury (Fig. 4a–c). Importantly, we found that both male and female $Fgf21^{-/-}ob/ob$ mice displayed increased serum AST and ALT levels compared with their respective control $Fgf21^{+/+}ob/ob$ mice, although differences in male mice were marginal (Fig. 4d, e). In parallel, the expression of UPR genes was upregulated in the liver of female $Fgf21^{-/-}ob/ob$ mice compared with female controls (Fig. 4f). These results suggest that obesity-mediated FGF21 induction serves as a protective mechanism to ameliorate obesity-induced ER stress and liver injury.

Given the role of ER stress in obesity-related metabolic diseases [12], we next studied the role of FGF21 on obesity-mediated metabolic derangement. Notably, both male and female $Fgf21^{-/-}ob/ob$ mice displayed increased random-fed or fasting glucose levels and worsened glucose tolerance/insulin resistance compared with their respective control $Fgf21^{+/+}ob/ob$ mice without changes in body weight (Fig. 4g–j, ESM Fig. 5a–c), although the differences in fasting glucose level in female mice were marginal. However, we did not observe any difference in metabolic profile between nonobese $Fgf21^{+/+}$ and $Fgf21^{-/-}$ mice of either sex (ESM Fig. 6), suggesting that FGF21 deletion exacerbates metabolic variables only in the metabolically stressed condition.

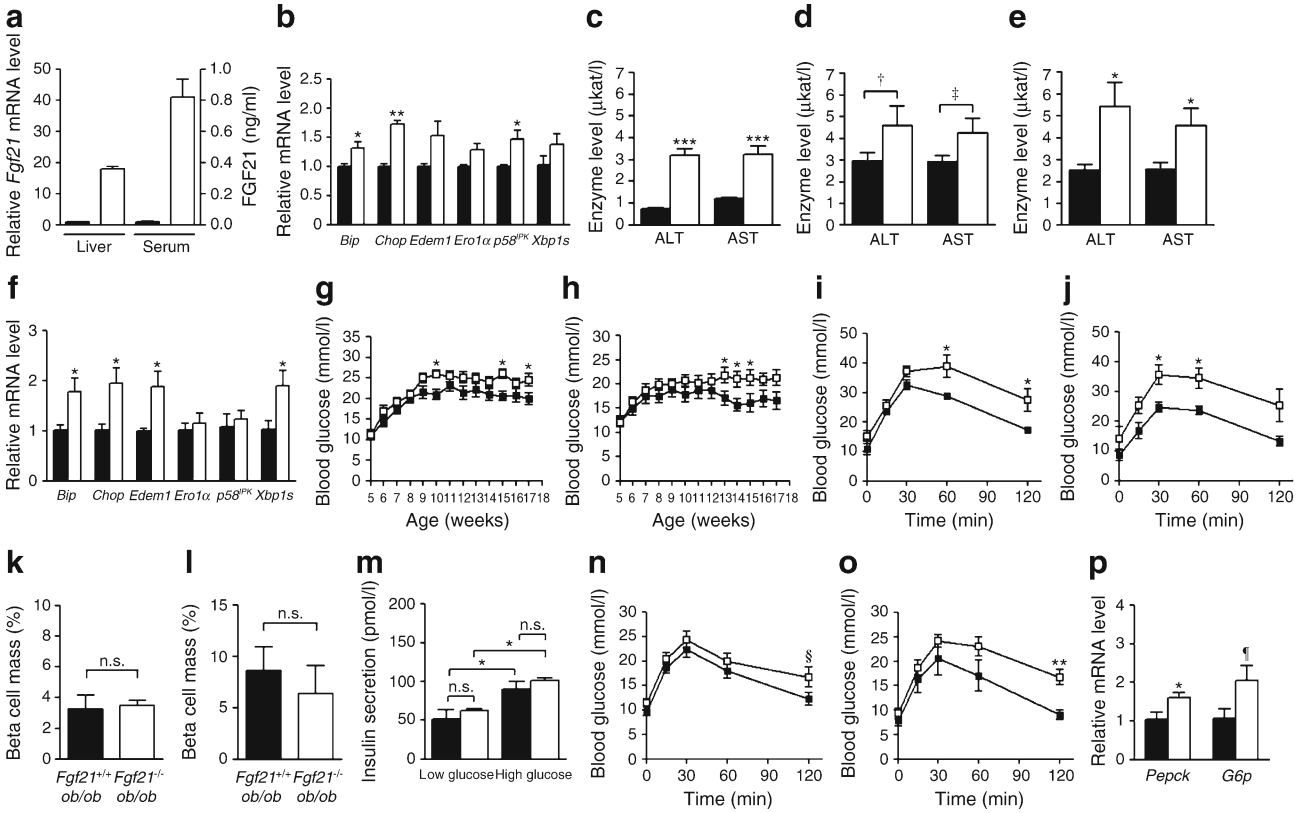

Fig. 4 FGF21 deletion aggravates obesity-induced ER stress and metabolic deterioration. (**a**) Hepatic *Fgf21* mRNA level (n=3), serum FGF21 level (n=5), (**b**) relative mRNA level of hepatic ER stress marker genes (n=3) and (**c**) serum ALT and AST levels (n=5) in lean (black bars) and *ob/ob* mice (white bars). mRNA levels normalised to *L32* mRNA in the liver of lean mice are presented as 1. (**d**, **e**) Serum ALT and AST levels in $Fgf21^{+/+}ob/ob$ (black bars) and $Fgf21^{-/-}ob/ob$ mice (white bars) (**d**, male, n=4; **e**, female, n=5). (**f**) Relative hepatic mRNA level of ER stress marker genes in female mice (n=4–5). mRNA levels normalised to *L32* mRNA in the liver of $Fgf21^{+/+}ob/ob$ mice are presented as 1. Black bars, $Fgf21^{+/+}ob/ob$; white bars, $Fgf21^{-/-}ob/ob$. (**g**–**l**) Random-fed blood glucose level (**g**, male, n=16–20; **h**, female, n=18–21), GTT (**i**, male, n=4; **j**, female, n=5) and beta cell mass (**k**, male, n=3; **l**, female, n=3) in both sexes; beta cell mass is measured as beta cell area/pancreas area. *p<0.05 vs age- or time-matched $Fgf21^{+/+}ob/ob$ mice. Black bars or squares, $Fgf21^{+/+}ob/ob$; white bars or squares, $Fgf21^{-/-}ob/ob$. (**m**) Ex vivo glucose-stimulated insulin secretion from islets of female mice (n=5). Black bars, $Fgf21^{+/+}ob/ob$; white bars, $Fgf21^{-/-}ob/ob$. (**n**, **o**) PTT (**n**, male, n=10–12; **o**, female, n=5–11). Black squares, $Fgf21^{+/+}ob/ob$; white squares, $Fgf21^{-/-}ob/ob$. **p<0.01 vs time-matched $Fgf21^{+/+}ob/ob$ mice. (**p**) Relative hepatic mRNA level of gluconeogenesis-related genes in female mice (n=4–5). mRNA levels normalised to *L32* mRNA in the liver of $Fgf21^{+/+}ob/ob$ mice are presented as 1. Black bars, $Fgf21^{+/+}ob/ob$; white bars, $Fgf21^{-/-}ob/ob$. Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001, $^{\dagger}p$=0.12, $^{\ddagger}p$=0.14, $^{\S}p$=0.09, $^{\P}p$=0.08. n.s., not significant

Despite deteriorated glucose tolerance and insulin resistance, serum insulin level, beta cell mass, beta cell apoptosis/proliferation and glucose-stimulated insulin secretion from islets were not different between $Fgf21^{-/-}ob/ob$ and $Fgf21^{+/+}ob/ob$ mice (Fig. 4k–m, ESM Fig. 5d–f), suggesting that beta cell dysfunction is not involved in metabolic deterioration of $Fgf21^{-/-}ob/ob$ mice. As ER stress contributes to the increased gluconeogenesis in obesity and diabetes [19], we conducted a PTT in $Fgf21^{-/-}ob/ob$ mice. As expected, both male and female $Fgf21^{-/-}ob/ob$ mice exhibited impaired pyruvate tolerance compared with $Fgf21^{+/+}ob/ob$ mice, accompanied by elevated expression of gluconeogenesis-related genes. However, differences in the PTTs of male mice were marginal (Fig. 4n–p), implying that deteriorated glucose tolerance is probably due to increased hepatic glucose production. Nonetheless, hepatic lipid content and serum levels of metabolites, such as TG, cholesterol and NEFA, were not different between the two groups in both sexes (ESM Fig. 7a–d). Together, these data suggest that obesity-mediated FGF21 induction plays a protective role in obesity-induced ER stress and metabolic deterioration.

Discussion

Recent studies showed that FGF21 expression is increased in the liver of patients with and mouse models of obesity and NAFLD [13–15]. However, the molecular mechanism of FGF21 induction and its pathophysiological function in these conditions is unclear. Here, we demonstrated that a PERK–eIF2α–ATF4 pathway is critical for ER stress-induced FGF21 expression in vitro and in vivo. Additionally, we showed a protective role of FGF21 in chemical- or obesity-induced hepatic ER stress.

It has been reported that FGF21 is induced by various stresses, including glucose starvation, cold and autophagy deficiency, which may play an important role in adaptation to these stresses [3, 20, 21]. Additionally, a recent paper has reported that FGF21 levels are increased in hepatocytes and in mouse liver after treatment with an ER stressor [22]. However, the physiological or pathological significance of ER stress-mediated FGF21 induction is unknown. Furthermore, molecular mechanisms of in vitro FGF21 induction by ER stress are unclear, although the effects of ATF4 overexpression on *FGF21* mRNA expression and *FGF21* promoter activity have been evaluated [22, 23]. Here, we demonstrated the importance of a PERK–eIF2α–ATF4 pathway in ER stress-induced *Fgf21* expression in vivo and in vitro, and the physiological role of endogenous FGF21 induction caused by ER stress. In addition to ATF4, CHOP, the downstream target of ATF4, has been shown to be partially involved in ER stress-induced *Fgf21* expression in primary hepatocytes [23]. However, we did not observe differences in *Fgf21* expression between $Chop^{+/+}$ and $Chop^{-/-}$ MEFs under ER stress conditions (ESM Fig. 8). During the course of the revision of our paper, the IRE1α–XBP1 pathway has been reported to mediate ER stress-induced FGF21 expression [24], which is in contrast to our findings showing no significant role of IRE1α and XBP1 in FGF21 induction by ER stress. There is no clear explanation regarding these discrepancies, but they might be attributed to the differences in cell lines (hepatocytes vs MEFs) or experimental procedures.

$Fgf21^{-/-}$ mice exhibited an aggravated ER stress response and hepatic cell death compared with $Fgf21^{+/+}$ mice after tunicamycin administration, while inducible *Fgf21*-transgenic mice had an attenuated ER stress response, indicating that FGF21 plays a protective role against ER stress. However, we did not further address the mechanisms by which FGF21 alleviates ER stress or ER stress-induced liver injury in the present study. Several possible mechanisms may be proposed. First, downstream target proteins of FGF21 may participate in FGF21-mediated attenuation of ER stress. It has been reported that FGF21 activates the AMP-activated protein kinase (AMPK)–Sirtuin1 (SIRT1) pathway [25], and AMPK or SIRT1 has a protective role against ER stress [26, 27]. We, therefore, hypothesise that AMPK or SIRT1 may mediate the effects of FGF21 on ER stress or hepatic injury due to ER stress. Another possibility is that reduced ER stress is a result of FGF21-induced suppression of lipid accumulation, since excessive lipid overloading can cause ER stress in the liver [28]. Given the protective role of FGF21 against oxidative stress in cardiac damage [29], reduced oxidative stress may be a mechanism by which FGF21 alleviates ER stress-induced hepatic injury. Intriguingly, FGF21 has been reported to exert metabolic effects via its actions in other target organs such as brain and adipose tissue [30–32]. In particular, increased adiponectin levels derived from adipose tissue contribute to the metabolic improvement elicited by FGF21 [31, 32]. However, we did not observe changes of circulating adiponectin levels or adipose tissue *Adipoq* mRNA level in tunicamycin-treated mice (ESM Fig. 9a, b). In addition, serum adiponectin level in $Fgf21^{-/-}$ mice was not different compared with $Fgf21^{+/+}$ mice after tunicamycin administration (ESM Fig. 9c). Thus, we could exclude the contribution of adiponectin in the adaptive effect of FGF21 in response to ER stress. However, we still cannot eliminate the possibility that other changes in non-hepatic tissues contribute to the amelioration of hepatic ER stress by FGF21. Further studies will be necessary to elucidate the mechanisms underlying the protective role of FGF21 against ER stress.

As ER stress contributes to obesity-associated metabolic disease [16] and FGF21 expression is increased in patients with and mouse models of obesity [13–15], we studied the pathophysiological function of FGF21 in ER stress and metabolic alterations caused by obesity using *ob* mice. While the metabolic effects of FGF21 deletion on diet-induced obesity models were variable [33, 34], FGF21 deletion exacerbated hepatic ER stress and worsened glucose tolerance/insulin resistance in *ob* mice. However, alterations of these metabolic variables were not associated with beta cell failure. Our findings are inconsistent with previous reports showing beneficial effects of FGF21 on beta cell function and impairment of FGF21 signalling in islets of obese mice [35, 36]. This discrepancy is probably due to differences between exogenous FGF21 administration vs endogenous FGF21. Although the role of FGF21 in the regulation of gluconeogenesis is controversial [37], we observed elevated gluconeogenesis in $Fgf21^{-/-}ob/ob$ mice, which is consistent with previous reports showing suppressive effects of FGF21 on gluconeogenesis in obese conditions [5]. Together, our results suggest that endogenous FGF21 is important in alleviating ER stress and metabolic deterioration due to obesity.

In conclusion, our results indicate that FGF21 is induced in response to ER stress through the eIF2α–ATF4 axis, which serves as a compensatory mechanism to attenuate ER stress-induced liver injury and hepatic lipid accumulation. We also showed that FGF21 induction plays a protective role in obesity-related ER stress and metabolic deterioration. Thus, our findings provide new insights into the role of FGF21 in ER stress response and suggest an innovative therapeutic strategy for the treatment of ER stress-associated diseases such as NAFLD, obesity and diabetes.

Acknowledgements We thank D. Ron (University of Cambridge, Cambridge, UK) for *Atf4* and *Perk* MEFs, R. J. Kaufman (Sanford Burnham Medical Research Institute, La Jolla, CA, USA) for *Ire1α*, *Atf6α*, *Chop*, *Eif2α* and *Xbp1* MEFs and L. H. Glimcher (Weill Cornell Medical College, New York, NY, USA) for *Xbp1* MEFs.

Funding This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (2013R1A6A3A04065825 to KHK and 2014R1A6A1030318 to SHB) and the Global Research Laboratory Grant of the National Research Foundation of Korea (K21004000003-10A0500-00310 to M-SL). M-SL received the National Research Foundation of Korea grant funded by the Korea government (MSIP) (2014M3A9D8034459).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement KHK and M-SL are the guarantors of the work. SHK, KHK and M-SL conceived and designed the experiments. SHK, KHK, H-KK, M-JK, SHB, MK and NI contributed to the acquisition of data. SHK, KHK and M-SL analysed and interpreted the data. SHK, KHK, SHB and M-SL wrote the manuscript. All authors have revised the manuscript critically for important intellectual content and approved the final version to be published.

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