NFATc1 signaling drives chronic ER stress responses to promote NAFLD progression

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Materials and Methods

Animal care and Treatments

Mice breeding was performed in the Central Animal Facility of the University Medical Center, Goettingen. Mice were kept under controlled atmosphere in 12 hours light and dark cycles with *ad libitum* supply of food and water. Mice were caged separately based upon gender, treatment, and genotype, and housed in the same facility. At the age of 8 weeks, mice (n=5/group, following RRR principle) were randomly subjected to the different experimental protocols as follows: A) mice of all genotypes were fed with either control diet (CD) or high-fat/high-cholesterol western diet (WD) (ssniff Spezialdiäten GmbH, E15721-347) with 45% (w/w) glucose (Merck) and 55% (w/w) fructose (Carl Roth GmbH) in water) for 4, 12 and 20 weeks. B) *Alb-cre* and *NFATc1*$^{c.a.}$ mice were fed with CD or WD for 20 weeks, and WD treatment was combined with i.p. injections 3x/week of either TUDCA (Merck Millipore) (500 mg/kg of body weight) or vehicle (PBS), respectively. During treatment phase, body weight was recorded once a week. Upon completion of treatment time points liver tissues and blood samples were collected for molecular, histological and serological analysis. Investigators were unaware of allocation of experimental groups and analysis performed. Animal experiments were reported using ARRIVE1 reporting guidelines.

Cell Culture

The alpha mouse liver-12 (AML12, ATCC CRL-2254) cells were cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM)/Nutrient Mixture F-12 (1:1) supplemented with 10% (v/v) FBS (Biowest S181B-500), 40 ng/ml dexamethasone (Sigma-Aldrich, USA) and 1% (v/v) of Insulin-Transferrin-Selenium-Ethanolamine (100X) (Life Technologies 51500-056). At 90% confluency, cells were washed with PBS followed by trypsinization (Gibco, 15400-54 1:10 in PBS). Primary mouse hepatocytes were isolated from 8 weeks old *Alb-cre, NFATc1*$^{c.a.}$ and *NFATc1*$^{\Delta/\Delta}$ mice by two-step collagenase perfusion, as described before [1]. Isolated hepatocytes were maintained in attachment medium (Williams Medium E (1x), Gibco-22551-022, supplemented with 10% (v/v) FBS, 100nM dexamethasone, 2mM L-Glutamine (Sigma-Aldrich, USA) and 1% (v/v) Penicillin/streptomycin (Sigma-Aldrich, USA)) and seeded in 6 well plates (1 million cells/well) followed by incubation at 37 °C with 5% CO_2. After 6 hours incubation, cells were washed with PBS and medium was exchanged with pre-starvation medium (Williams Medium E (1x), supplemented with 100nM dexamethasone, 2mM L-Glutamine and 1% Penicillin/streptomycin) followed by overnight incubation. The following day, plates were washed twice with PBS and cells were provided starvation medium (Williams Medium E (1x) supplemented with 2mM L-Glutamine and 1% Penicillin/streptomycin) and maintained at similar conditions. Following experimental procedures were carried out in cells:

i) Transfection

AML12 cells were transfected for 24 hours with *Nfatc1* siRNA (ambion 288360) or with constitutively active *Nfatc1* construct (HA-tagged MSCV- caNFATc1 bearing Serine to Alanine mutation in conserved serine rich residues). Control cells were treated with lipofectamine 2000 (Invitrogen) as vehicle. Cells were seeded and allowed to grow until 60% confluency. siRNA and c.n.NFATc1 construct were prepared in serum-free media with lipofectamine 2000, respectively.

ii) Fatty acids treatment

AML12 cells were exposed to 200 µM palmitate supplemented in medium for 12 hours. In another approach, AML12 cells were transfected for 24 hours with siRNA for *Nfatc1* or with lipofectamine 2000. After 12 hours of transfection 200 µM palmitate was added in the medium and cells were incubated for next 12 hours. Primary mouse hepatocytes were treated with 100, 200 and 400 µM of either palmitate and oleate alone or in combination, respectively for 12 hours, provided in starvation medium. Control cells were provided BSA.

iii) TUDCA Treatment

AML12 cells were seeded and treated on the following day, fresh medium containing either BSA and H_2O or 200µM Palmitate with 500µM TUDCA was provided for 12 hours [2]. In another approach, AML12 cells were pre-transfected with c.n.NFATc1 construct for 24 hrs. Following 12 hours of transfection, medium containing H_2O/TUDCA (500 µM) was added for next 12 hours. Primary mouse hepatocytes were treated with 200 µM palmitate alone, and in combination with increasing concentrations of TUDCA i.e., 100 µM-500 µM respectively, for 12 hours, provided in starvation medium. Control cells were provided BSA along with H_2O as vehicle control for TUDCA.

Reporter Assay

Dual luciferase reporter gene assay was performed in AML12 cells and primary mouse hepatocytes to verify palmitate induced transcriptional activation of NFATc1. Cells were co-transfected with a NFAT responsive promoter luciferase reporter construct in combination with either an empty vector or a NFATc1 wild-type expression vector, and subsequently treated with 200 µM palmitate (pal) for 24 hours. Renilla luciferase was used as an internal control for transfection efficiency and normalization. Promoter activity is shown as relative firefly luciferase activity normalized to renilla luciferase activity.

Measurement of cytosolic and ER Calcium concentrations

AML12 cells, seeded on 25 mm round (No 1.5, #6310172, VWR) glass coverslips, were loaded with 1 µM Fura-2 AM (#F1221, Thermo Fisher Scientific GmbH) in growth medium for 30 minutes at room temperature. The measurements were performed at room temperature in

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Ringer's buffer (pH 7.4) containing 145 mM NaCl, 4 mM KCl, 10 mM Glucose, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$, or 0 mM $CaCl_2$ with 1 mM EGTA as indicated in the figure legends. Fura-2-based measurements of cytosolic calcium were performed using a Zeiss Axiovert S100TV equipped with a pE-340$_{fura}$ (CoolLED, Andover, United Kingdom) LED light source with LED 340 nm (excitation filter: 340/20) and 380 nm (excitation filter: 380/20) together with a T400 LP dichroic mirror and 515/80 emission filter, a sCMOS pco.edge camera and a Fluar 20x/0.75 objective. AML12 cells were treated with 200 µM palmitate for the indicated time points and basal cytosolic calcium levels were measured in Ringer's buffer containing 0.5 mM calcium. Depletion of ER calcium stores was measured in calcium free Ringer's buffer and depletion of ER stores was achieved by addition of Thapsigargin (Tg, 1 µM) using a perfusion system in untreated cells or cells treated for 12 hours with BSA and 200 µM palmitate. Data were analyzed with VisiView® Software (Visitron Systems GmbH, Puchheim, Germany). The obtained 340 nm/ 380 nm fluorescence ratios were converted to calibrated data using the equation $[Ca^{2+}] = K*(R - R_{min})/(R_{max} - R)$, while the values of K, R_{min}, and R_{max} were determined as described previously [3].

For primary hepatocytes palmitate treated cells were incubated for 30 min with cytoplasmic calcium dye Fluo-8 (K_d 389 nM). For the ER calcium, cells were loaded with Fluo-5N (K_d 90 µM). Time-lapse live-cell calcium imaging was performed in a climate chamber (37°C, 5% CO_2) using an inverted fluorescence microscope (Olympus) with GFP filter (BP 470/20). The calcium levels were represented by changes in fluorescent intensities of the cells subtracted from the background with the help of time series analyzer V 3.0 plugin of Image J and are presented as change in fluorescence units (∆RFU).

Immunoblotting, Immunohistochemistry and Immunofluorescence

Hematoxylin eosin staining, IHC and western blot analysis were performed using standard protocol as described previously [4], using antibodies against HA-tag (#3724; Cell Signaling), NFATc1 (7A6, #sc7294; Santa Cruz Biotechnologies and ab25916; abcam), CD45 (#550539; BD Pharmingen), eif2α (#9722; Cell signaling), p-Eif2α (#9721; Cell Signaling), CHOP(#2895; Cell Signaling), Ddit3 (ab179823; abcam), cleaved caspase-3 (#9661; Cell signaling), cleaved caspase-1 (#67314; Cell signaling), cleaved IL-1β (#52718; Cell signaling), pP53-(s15) (#9284; Cell signaling), NLRP3 (AG-203-0014-C101; Adipogene) and β-actin (A3845; Sigma), Gasdermin D (#39754; Cell signaling), cleaved Gasdermin D (#10137; Cell signaling); TRB3 (#LS-B12111; LSBio), ATF4 (#NB100-852; Novusbio), PERK (#3192; Cell signaling), p-PERK (#3179; Cell signaling), p-PKR (#MBS856680; MyBioSource), PKR (#MBS150276; MyBioSource). For immunoblot analysis of animal tissues each lane represents an individual mouse. For immunostaining and immunofluorescence, scale bar of images are 100 and 200

µm respectively, unless stated differently. For quantification of IHC and IF imageJ software was utilized. For NFATc1 IHC, percentage of NFATc1 positive hepatocytes to a total number of cells was calculated by manual quantification. Percentage of CD45 positive cells were quantified in imageJ using semi-automatic macros. To analyze the fibrosis liver tissue sections were stained with picrosirius red according to the manufacturer's protocol (Polysciences, Inc). For collagen counting percentage of picrosirius red stained area was quantified.

Oil-Red-O staining

Oil red o staining was performed in primary mouse hepatocytes and in liver sections from 20 weeks CD and WD treated mice, respectively, as per manufacturer's instructions (#O1391; Sigma). Briefly, primary hepatocytes were pretreated with 100, 200 and 400 µM of either palmitate and oleate alone or in combination for 12 hours. Later, cells were fixed in 4% paraformaldehyde and stained with oil-red-o. Cells were incubated in 100% isopropanol for 5 mins and OD was measured at 492nm. For mice, 4 µm thick cryosections were stained with oil red o.

RNA isolation and real-time PCR (qRT-PCR)

RNA isolation was performed using phenol-chloroform purification and cDNA was synthesized using iScript cDNA Synthesis Kit (170-8891, BioRad,) as described before [4] [5]. mRNA expression analysis for each sample was performed in triplicates using iTaq Universal SYBR Green Supermix (BioRad, 172-5125) with StepOne Plus Real-Time PCR System (Applied Biosystems). Gene expression values for each sample were normalized to housekeeping gene *Gapdh* and compared to control. Graphical representation and statistical analysis of results was performed in GraphPad Prism, version 9.0. Statistical significance and its method are described in respective results.

Cytokine profiling

Snap-frozen liver tissues from CD and WD treated mice were analysed using a Proteome Profiler Mouse Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA). The samples were prepared as per manufacturer's protocol. The densitometric volume was determined by imageJ software. All the experimental procedures were performed strictly following manufacturers' instructions.

Triglyceride Assay

Liver triglyceride content in 20 weeks CD and Wd treated mice was analysed using a fluorimetric quantification assay (#ab178780; Abcam) according to the manufacturer's instructions. Briefly, lysates from 10 mg liver tissue samples were prepared. These lysates were incubated with lipase at 37 °C for 20 mins. Reaction mix was added, and the samples

were incubated for 30 mins at 37°C. The absorbance was recorded at E_x/E_m = 535/587 nm using a microplate reader.

Chemicals and preparations

Palmitate **(**Sigma Aldrich, 408-35-5**)** and Oleate (Sigma Aldrich, O7501-5G) were dissolved in 150 mM NaCl in conjugation with BSA (Serva, 9048-46-8) with final ratio of 1:6. Briefly, BSA was dissolved in NaCl, to prepare 0.34 mM BSA solution. Half of the BSA was diluted with 150 mM NaCl to make final concentration of 0.17 mM and filtered, for subsequent use as vehicle. 4.4 mM of sodium palmitate and sodium oleate solutions were prepared in 50 ml of 150 mM NaCl, by heating up to 70 °C and 44 ml was transferred to 0.34 mM BSA solution already prepared following stirring at 37 °C for 1 hour. After adjusting pH 7.4, final volume was raised up to 100 ml with 150mM NaCl, to make final concentration 2mM. Palmitate/BSA and oleate/BSA solutions were filtered and stored at -20 °C. Tauroursodeoxycholic Acid (TUDCA, Millipore 580549) was solved in H_2O (20 mM), filtered, and stored at 4 °C for further experiments. For i.p. injections in mice TUDCA was dissolved in PBS at final concentration of 500mg/kg body weight.

Statistical analysis

Statistical analysis was perfomed by Graphpad Prism 9.0 using unpaired t-test, one-way ANOVA and two-way ANOVA, respectively (described in each figure legend). Grubb's test was used to identify the statically significant outlier. Statistical significance was always mentioned as *p <0.05, **p <0.005, ***p< 0.0005, ****p<0.0001.

Supplementary Figure legends

Supplementary Figure 1

Graphs presenting analysis of changes in body weight and liver weight after (A) 4 weeks, (B) 12 weeks and (C) 20 weeks of CD and WD treatment (n=5). (D) Graphs show qualitative analysis of percentage steatosis (n=5), and the liver triglyceride levels (n=4) in 20 weeks CD and WD treated mice. (E) Quantification of serum ALT values in $NFATc1^{\Delta/\Delta}$, *Alb-Cre* and $NFATc1^{c.a.}$ mice treated with CD and WD for 20 weeks (n=5). Statistical analysis (A-E) was performed by Two-way ANOVA and data are shown as mean ± SD, p-values are *p<0.05, **p< 0.005, ***p<0.0005 and ****p<0.0001. (F) Representative images from H&E analysis of liver sections from 4- and 12-weeks of CD and WD treated mice (n=5). Scale bar =100 µm.

Supplementary Figure 2

(A) Western blot analysis of NFATc1 protein expression in primary mouse hepatocytes treated with 100 µM (+), 200 µM (++) and 400 µM (+++) of either palmitate (pal.) and oleate (ol.) alone or in combination (pal./ol.) for 12 hours. (B) Graph represents quantitative analysis of Oil-Red-

O staining in primary mouse hepatocytes pretreated with BSA, 100, 200 and 400 µM of either palmitate (pal.) and oleate (ol.) alone or in combination (pal./ol.) for 12 hours. (C) Changes in ER and cytosolic calcium levels in palmitate (200 µM) treated primary hepatocytes were measured using ER calcium specific Fluo-5N (K_d 90 µM) and cytoplasmic calcium specific dye Fluo-8 (K_d 389 nM). The changes in calcium levels are represented as change in fluorescence units (∆RFU). (D) Cytosolic calcium levels were measured in AML-12 cells using Fura-2 in Ringer's buffer containing 0.5 mM calcium after treatment with 200 µM palmitate for 12 hours. (n values: untreated= 919 cells, 200µM Palmitate=726 cells, N=3). (E) Calcium release from internal ER stores was measured in calcium free Ringer's buffer upon administration of thapsigargin (Tg 1µM) in AML-12 cells using Fura-2-based calcium imaging. Cells were pre-treated for 12 hours with 200µM palmitate. Graph represents quantification of Tg-induced ER calcium store release ((b)-(a)). (n values: untreated= 1537 cells, BSA control=1067 cells, 200 µM palmitate=1491 cells, from 3 independent experiments). (F) Dual luciferase reporter gene assay was performed in AML12 cells to verify palmitate induced transcriptional activation of NFATc1. Cells were co-transfected with a NFAT responsive promoter luciferase reporter construct in combination with either an empty vector or a NFATc1 wild-type ($NFATc1^{wt}$) expression vector, and subsequently treated with 200 µM palmitate (pal.) for 24 hours. Statistical analysis was performed by Two-way ANOVA (B), One-way ANOVA (C, E-F) by unpaired t-test (D). Data are shown as mean ± SD, **p*< 0.05 and ***p*< 0.005, ****p*< 0.0005 and *****p*< 0.0001.

Supplementary Figure 3

(A) Principal component analysis showing differential clustering replicates per groups. (B) Cytokine proteome profiling in liver tissue lysates of 20 weeks treated genetic mice. (n=3) (C) Quantitative analysis of cytokine proteome profiling. Statistical analysis was performed by paired t-test. Data are shown in mean ± SD, p-values are **p* < 0.05, ***p*< 0.005, ****p*< 0.0005, *****p*< 0.0001. Statistical analysis was performed by two-way ANOVA.

Supplementary Figure 4

Schematic representation of ER stress induced PERK-CHOP UPR signaling. (B-D) Densitometry graphs of primary hepatocytes following 12 hours palmitate treatment (+ = 100 µM, ++ = 200 µM) for (B) pEif2α/Eif2 α, (C) pPERK/PERK and (D) pPKR/PKR. (E) Immunoblot analysis of NFATc1, ATF6 F (full length), ATF6 P (partial) and XBP1s (spliced) in AML12 cells following 12 hours palmitate treatment (+ = 200 µM) alone or in combination with siNFATc1. (F) Protein levels of ATF6 F (full length), ATF6 P (partial) and XBP1s (spliced) were measured in 12 hours palmitate treated primary mouse hepatocytes (+ = 100 µM and ++ = 200 µM). (G-H) Immunoblot analysis of NFATc1, NLRP3, CC-1, CC-3, C.IL1β, C.GSDMD and GSDMD in (G) AML12 cells treated with palmitate (+ = 200 µM) alone or in combination with siNFATc1 and in (H) in liver tissue lysates from 20 weeks CD and WD treated mice with differential NFATC1 expression.

Supplementary Figure 5

(A) Immunoblot examination showing protein levels of pPERK, PERK, p-Eif2α, Eif2α, CHOP, NLRP3, C.GSDMD and GSDMD (Gasdermin D) in primary mouse hepatocytes following 12

hours of 100 µM (+), 200 µM (++) and 400 µM (+++) of either palmitate or oleate alone and in combination.

Supplementary Figure 6

(A) Immunoblot analyses showing protein expression of CHOP, NLRP3 and CC-3 in AML12 cells treated with palmitate (pal., 200 µM) alone or along with TUDCA (500 µM) in comparison to control treated cells. (B-C) qRT-PCR analysis of *Chop, IL-1β, Cxcl10 and Cxcl11* in AML12 cells after exposure to palmitate alone or in combination with TUDCA (B) or following transfection with siNFATc1 and c.a. NFATc1 alone or in combination with TUDCA (C). Protein levels of NFATc1, pEif2α and Eif2α were examined in liver tissue lysates of 20 weeks CD, WD and WD+TUDCA treated (C) *Alb-cre* and (D) *NFATc1$^{c.a.}$* mice. Each lane represents individual mouse. Data are shown in mean ± SD, p-values are *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001. Statistical analysis was performed by one-way ANOVA.

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