Brief Communication

MOLECULAR METABOLISM

Point mutation of *Ffar1* abrogates fatty acid-dependent insulin secretion, but protects against HFD-induced glucose intolerance

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ABSTRACT

Objective: The fatty acid receptor 1 (FFAR1/GPR40) mediates fatty acid-dependent augmentation of glucose-induced insulin secretion (GIIS) in pancreatic β-cells. Genetically engineered *Ffar1*-knockout/congenic mice univocally displayed impaired fatty acid-mediated insulin secretion, but *in vivo* experiments delivered controversial results regarding the function of FFAR1 in glucose homeostasis and liver steatosis. This study presents a new coisogenic mouse model carrying a point mutation in *Ffar1* with functional consequence. These mice reflect the situations in humans in which point mutations can lead to protein malfunction and disease development.

Methods: The Munich *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis-derived F1 archive containing over 16,800 sperms and corresponding DNA samples was screened for mutations in the coding region of *Ffar1*. Two missense mutations (R258W and T146S) in the extracellular domain of the protein were chosen and homozygote mice were generated. The functional consequence of these mutations was examined *in vitro* in isolated islets and *in vivo* in chow diet and high fat diet fed mice.

Results: Palmitate, 50 μM, and the FFAR1 agonist TUG-469, 3 μM, stimulated insulin secretion in islets of *Ffar1*$^{T146S/T146S}$ mutant mice and of wild-type littermates, while in islets of *Ffar1*$^{R258W/R258W}$ mutant mice, these stimulatory effects were abolished. Insulin content and mRNA levels of *Ffar1*, *Glp1r*, *Ins2*, *Slc2a2*, *Ppara*, and *Ppard* were not significantly different between wild-type and *Ffar1*$^{R258W/R258W}$ mouse islets. Palmitate exposure, 600 μM, significantly increased *Ppara* mRNA levels in wild-type but not in *Ffar1*$^{R258W/R258W}$ mouse islets. On the contrary, *Slc2a2* mRNA levels were significantly reduced in both wild-type and *Ffar1*$^{R258W/R258W}$ mouse islets after palmitate treatment. HFD feeding induced glucose intolerance in wild-type mice. *Ffar1*$^{R258W/R258W}$ mutant mice remained glucose tolerant although their body weight gain, liver steatosis, insulin resistance, and plasma insulin levels were not different from those of wild-type littermates. Worth mentioning, fasting plasma insulin levels were lower in *Ffar1*$^{R258W/R258W}$ mice.

Conclusion: A point mutation in *Ffar1* abrogates the stimulatory effect of palmitate on GIIS, an effect that does not necessarily translate to HFD-induced glucose intolerance.

Keywords FFAR1/GPR40; Free fatty acids; Insulin secretion; ENU-mutated *Ffar1*; FFAR1 deficient mice; High fat diet

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Abbreviations: CD, regular chow diet; ENU, *N*-ethyl-*N*-nitrosourea; FFAR1, free fatty acid receptor 1; GIIS, glucose-induced insulin secretion; GLP-1, glucagon like peptide-1; Glut-2, glucose transporter 2; GTT, glucose tolerance test; HEK-EM 293 cells, human embryonic kidney macrophage scavenger receptor-expressing (TRH-R) cells; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HFD, high fat diet; ipITT, intraperitoneal insulin tolerance test; PAX6, paired box protein, also known as aniridia type II protein (AN2) or oculorhombin; *Ppara*/*Ppard*, peroxisome proliferator activated receptor α/δ genes; *Slc2a2*, solute carrier family 2 member 2 gene; TAK875 and TUG-469, FFAR1 agonists

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1. INTRODUCTION

Free fatty acid receptor-1 (FFAR1, formerly GPR40) promotes long chain fatty acid-mediated augmentation of glucose-induced insulin secretion (GIIS) [1–3]. In humans and rodents, high expression of FFAR1 is restricted to pancreatic and gastric endocrine cells, while expression in other tissues, including brain, is much lower [1,2,4,5]. These features make FFAR1 an attractive drug target for the treatment of insufficient insulin secretion, which is the ultimate cause for the onset of hyperglycemia and type-2 diabetes mellitus [6,7]. Until today, multiple agonists have been generated and tested for their efficacy to treat hyperglycemia in humans [6]. Although FFAR1 agonists counteract glucose intolerance in mice and humans, the beneficial effect of these new therapeutic drugs is still a matter of debate [8,9]. Thus, the promising drug TAK875 was discontinued after clinical phase III due to its liver toxicity. Confirming this side effect, FFAR1-deficient mice are protected against diet-induced liver steatosis [10]. This observation prompted the investigation of FFAR1-antagonists as therapeutic tools against fatty liver disease.

In addition, different FFAR1 agonists exert their effects through different cellular pathways. Thus, fatty acids stimulate insulin secretion mainly via Gq proteins, while TAK875 stimulation is mediated by β-arrestin-2 [11]. An additional, but indirect, stimulatory effect of FFAR1-agonists on insulin secretion is caused by the activation of FFAR1 expressed in intestinal endocrine cells which leads to GLP-1 secretion [12].

Several transgenic and knockout/congenic mouse models have been generated in order to assess the role of FFAR1 for proper insulin secretion and maintenance of glucose homeostasis. The results obtained with three different receptor knockout mouse models were not consistent. The protection against high fat feeding-induced fatty liver and glucose intolerance, as observed by Steneberg and colleagues, could not be reproduced using other *Ffar1* KO mouse models [10,13,14]. Such differences may be explained by undesirable side effects generated by insertion of exogenous DNA, deletion of non-coding regions with specific functions, e.g. microRNA, and the additional role of the *Ffar1* promoter for the expression of FFAR2 (GPR43) and FFAR3 (GPR41) [15,16]. Congenic mice differ not only in the ablated gene but also in a flanking segment on either side of the ablated locus [17]. Furthermore, a complete deletion of a protein may generate a compensatory up-regulation of other proteins. To circumvent such problems, we searched for a coisogenic mouse model with a minimal genetic alteration producing a maximal effect. Using site-directed mutagenesis, several point mutations in *Ffar1* with functional consequences have been identified, including R258 [18,19]. We screened the Munich ENU-mutagenesis-derived F1 sperm and corresponding DNA archive for point mutations in *Ffar1*. The archive comprises more than 16,800 samples from individual F1-mutagenized mice on the C3HeB/FeJ genetic background [20,21]. Two mouse models carrying point mutations in the coding region of *Ffar1* are presented in this study of which the R258W mutation prevents the stimulation of insulin secretion by palmitate and the FFAR1 agonist TUG-469.

2. MATERIALS, ANIMALS AND METHODS

2.1. Materials

TUG-469, a specific FFAR1 agonist, was a kind gift of Trond Ulven, Southern University of Denmark, Odense M, Denmark. All other materials, unless otherwise stated, were from Sigma–Aldrich (Deisenhofen, Germany) and of analytical grade.

2.2. Generation of mice

ENU mutagenesis was performed as described previously [22]. Briefly, male C3HeB/FeJ mice were treated weekly by one 90 mg/kg ENU-injection for three consecutive weeks. First generation F1 mice were phenotyped, and male mice were cryo-archived by their sperm and spleen-derived DNA samples. The DNA archive was screened for alleles of interest using a LightScanner® device originally from Idaho Technology Inc. (distributed by Bioke, Leiden, Netherlands). *In vitro* fertilization, fusing sperm of mutated F1 mice and mating with wild-type C3HeB/FeJ mice were performed as described elsewhere [23]. During maintenance the mutant mice were repeatedly backcrossed to wild-type C3HeB/FeJ mice in order to eliminate unwanted ENU mutations. Mice were kept under a 12 h light/dark cycle and had *ad libitum* access to chow diet (CD) and water. High fat diet (HFD) containing 45% fat/kcal from lard and soybean (Research Diets D12451; New Brunswick; NJ; USA) was fed for 8 weeks starting at age of 4 weeks. Mouse holding and handling were done according to the federal animal welfare guidelines and the state ethics committee and approved by the governments of Upper Bavaria and Baden-Württemberg.

2.3. Glucose and insulin tolerance tests

Blood glucose concentrations were measured after intra-peritoneal injection of 2 g glucose/kg body weight (ipGTT) or of intra-peritoneal injection of 1 unit/kg body weight insulin (ipITT) in mice fed CD or HFD for 8 weeks. Before GTT, mice were fasted overnight. For determination of plasma insulin levels blood samples were collected in heparinized capillary from tail vein. Blood glucose was measured with a glucometer. During oral glucose tolerance test (oGTT), 2 g glucose/kg body weight was administered via gavage in overnight fasted animals. The tests were performed with the same animals keeping an interval of 1 week between the tests. Plasma insulin and glucagon were measured using ELISA kits (Mercodia, Sweden). Plasma leptin and resistin were quantified using a ProcartaPlex™ immunoassay (Luminex™ xMAP technology, Invitrogen).

2.4. Isolation of islets and insulin secretion

Mouse islets were isolated via collagenase digestion (1 mg/ml collagenase, Serva, Heidelberg, Germany) and thereafter purified by handpicking. Islets were cultured overnight in RPMI 1640 medium containing 11 mM glucose supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, and 1 mM Na-pyruvate without antibiotics. Thereafter, islets were washed twice and pre-incubated for 1 h at 37 °C with Krebs Ringer buffer (KRB) containing (in mM): 135 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 5 $NaHCO_3$, 2.8 glucose, 10 HEPES, and 5 g/l bovine serum albumin (fatty acid free, low endotoxin, Sigma, Deisenhofen, Germany), pH 7.4. Subsequently, islets were incubated in fresh KRB containing 0.5 g/l bovine serum albumin supplemented with test substances as indicated for 1 h at 37 °C. Palmitate was added from a stock solution of 50 mM in DMSO. Secreted insulin and islet insulin content after insulin extraction in acid ethanol (1.5% [vol/vol] HCl/75% [vol/vol] ethanol) were measured via radioimmunoassay (Millipore, Biotrend Chemikalien GmbH, Germany).

2.5. Semiquantitative analysis of cellular mRNA levels

Islets were isolated and directly lysed in RNA lysis buffer (Macherey–Nagel, Düren, Germany, Figure 1C), or islets were cultured before lysis in medium supplemented with 10% FCS ± 600 μM palmitate. Palmitate, from a stock solution of 100 mM in DMSO, was coupled to FCS at a final concentration of 6 mM before addition to the culture medium. 50–200 islets were collected for total RNA-isolation using the commercial RNeasy kit (Qiagen, Hilden, Germany). Residual DNA

A

No	Id	Location	Mutation		Consequence	Type of AS exchange	Predicted 2nd structure
1	14004179	c.69C>A	Asn23Lys	N23K	missense	polar > basic	helical, TM domain 1
2	21027588	c.71T>A	Leu24Stop	L24*	nonsense		helical, TM domain 1
3	10093889	c.111A>C	Arg37Arg	R37R	silent		
4	21040433	c.194A>T	Glu65Val	E65V	missense	acidic > nonpolar	helical, TM domain 2
5	21082700	c.252G>T	Leu84Phe	L84F	missense	nonpolar > nonpolar	helical, TM domain 3
6	21050574	c.280G>A	Gly94Arg	G94R	missense	nonpolar > basic	helical, TM domain 3
7	20064907	c.317T>C	Leu106Pro	L106P	missense	nonpolar > nonpolar	border TM domain 3 to loop C-II
8	20049658	c.317T>C	Leu106Pro	L106P	missense	nonpolar > nonpolar	border TM domain 3 to loop C-II
9	21033736	c.436A>T	Thr146Ser	T146S	missense	polar > polar	border TM domain 4 to loop E-II
10	10087466	c.772A>T	Arg258Trp	R258W	missense	basic > nonpolar	border loop E-III to TM domain 7

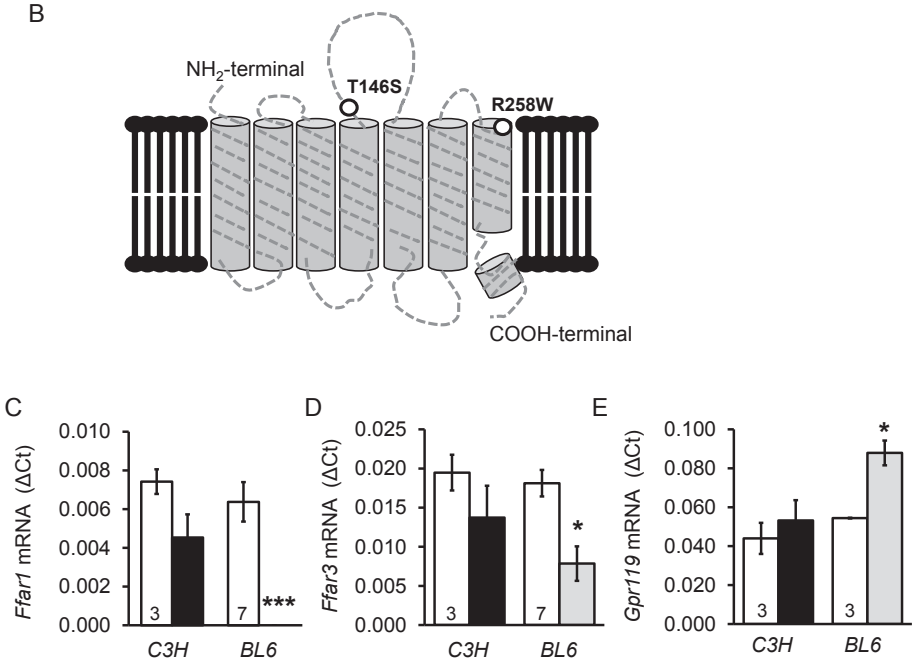

Figure 1: Generation of mice with mutations in *Ffar1*. (A) Mutations in *Ffar1* induced by ENU in mice. Sperm of mice carrying the mutation 9 and 10 were chosen for *in vitro* fertilization and generation of mutant mice. (B) Extracellular locations of the mutations 9 and 10 in FFAR1. Note that R258 is located in the agonist-binding domain of the receptor. (C, D, E) Relative mRNA levels in freshly isolated islets from *Ffar1*$^{R258W/R258W}$ (black bars) and *Ffar1* KO mice (gray bars) compared to their respective wild-type mice expressed as means ± SEM. The number of mice is given in the respective columns. *Rps13* was used as housekeeping gene.

was removed using on column DNAse treatment (Qiagen, Hilden, Germany). Eluted RNA was transcribed into cDNA using Oligo(dT)12–18 as primer (Roche Diagnostics GmbH, Mannheim, Germany).

PCR was performed using the LightCycler 480 Probes Master system (Roche Diagnostics GmbH, Mannheim, Germany). Quantification was performed by the $2^{-(\Delta CT)}$ method relative to the housekeeping gene *Rps13*. Specific primers used were: for m*Ffar1* up: 5′-CATCACTCTGCCCCTGAAG-3′ down: 5′-AAGGCAAAGACTGGGCAGA-3′, probe #50; for m*Ffar2* up: 5′-AAAGGAGCTGACAGGGGTTC-3′ down: 5′-GCAAGTTCAGGGGTTTCTTCT-3′, probe #82; for m*Ffar3* up: 5′-GTGCACTCACAAGGACTCTCC-3′ down: 5′-AAATTCGGGGTTTATGAGAGG-3′, probe #12; for m*Ffar4* up: 5′-TTGGTGTTGAGCGTCGTG-3′ down: 5′-CCAGCAGTGAGACGACAAAG-3′, probe #45; for m*Gpr119* up: 5′-TTCACTTCAATCCTCCTCCTTC-3′ down: 5′-TGCATGTTCTTGAGAGAAGTCC-3′, probe #72; for m*GLP1-R* up: 5′-GGACAACTGGGTCAAGCATT-3′, down: 5′-CTTTTCTCCCCTCATGGACA-3′, probe #12; for m*Ins2* up: 5′-GAAGTGGAGGACCCACAAGT-3′, down: 5′-AGTGCCAAGGTCTGAAGGTC-3′ probe #32; for m*Ppara* up: 5′-CACGCATGTGAAGGCTGTAA-3′, down: 5′-CAGCTCCGATCACACTTGTC-3′, probe #41; for m*Ppard* up: 5′-ATGGGGGACAGAACACAC-3′, down: 5′-GGAGGAATTCTGGGAGAGGT-3′, probe #11; for m*Rps13* up: 5′-TGCTCCCACCTAATTGGAAA-3′, down: 5′-CTTGTGCACACAACAGCATTT-3′, probe #110; for m*Slc2a2* up: 5′-TCTGCTACTGCTCTTCTGTCCA-3′, down: 5′-GGTGACATCCTCAGTTCCTCTTA-3′, probe #45.

2.6. Oil red staining and measurement of liver triglyceride content

For oil red staining, liver cryosections (20 μm thick) were fixed with 4% formalin in phosphate-buffered saline and dehydrated in 100% propylene glycol. Staining was carried out with 0.5% oil red in

propylene glycol for 15 min and hematoxylin was used as counterstaining. For the assessment of triglyceride concentrations, cryoconserved liver samples were homogenized in phosphate-buffered saline containing 1% Triton-X100 (20 µl buffer/mg tissue) using a TissueLyser (Qiagen, Hilden, Germany). Triglyceride content in the lysates was measured by a fully automatic enzymatic method on an ADVIA Chemistry XPT system (Siemens Healthcare GmbH, Erlangen, Germany).

2.7. Statistical analysis

All data were examined using ANOVA with Tukey's multiple comparisons test as post-hoc test. The level of significance was set to $p < 0.05$.

3. RESULTS

3.1. Generation of mice with point mutations in *Ffar1*

Mutations of *Ffar1* were selected from the F1 repository of ENU mutated mice. Ten point mutations were detected in the coding region of *Ffar1* (Figure 1A). We chose two missense mutations (T146S and R258W) in the extracellular domain of the receptor for generation of mutant mice strains (Figure 1B). Of note, the amino acid R258 is located in the agonist binding domain [19].

Heterozygous mutant mice of both lines developed normally under chow diet and did not display any metabolic phenotype regarding body weight gain, glucose and insulin tolerance as well as fasting and fed plasma insulin concentrations (data not shown). Therefore, homozygous mice (*Ffar1*$^{R258W/R258W}$ and *Ffar1*$^{T146S/T146S}$) were generated for further analysis.

The expression of *Ffar1* and the adjacent genes *Ffar2* and *Ffar3* were estimated by RT-PCR in freshly isolated islets from wild-type (C3HeB/FeJ) and homozygote mutant mice and compared to the mRNA levels of *Ffar1*$^{(-/-)}$ and respective wild-type (C57/BL6) mouse islets [10]. As shown in Figure 1C–E, mRNA levels of *Ffar1*, *Ffar3*, and *Gpr119* were not significantly different between C3HeB/FeJ and *Ffar*$^{R258W/R258W}$ mutant mouse islets. In contrast, in *Ffar1* deficient mice, *Ffar3* mRNA levels were significantly lower and *Gpr119* mRNA higher than in the respective wild-type mouse islets. The mRNA levels of *Ffar2* and *Ffar4* (GPR120) were 1000- and 50-times lower than *Ffar1* mRNA levels, respectively, and no differences were detected (data not shown).

3.2. R258W mutation of *Ffar1* prevents FFAR1-dependent stimulation of insulin secretion in isolated mouse islets

As suggested by the use of *Ffar1* knockout mice, FFAR1 contributes to palmitate-dependent augmentation of insulin secretion. Therefore, the functional relevance of the mutations was analyzed in isolated islets (Figure 2). FFAR1 was activated by a physiological agonist, palmitate, and a synthetic agonist, TUG-469 [24]. The concentrations of palmitate, 50 µM, and TUG-469, 3 µM, were adapted to the low concentration of albumin (0.05%) since albumin interferes with agonist (TUG-469)-receptor interaction [1].

In islets of wild-type littermates of both mutant mouse strains (*Ffar1*$^{R258W/R258W}$, Figure 2A and *Ffar1*$^{T146S/T146S}$, Figure 2B), palmitate, 50 µM, or TUG-469, 3 µM, significantly augmented insulin secretion in the presence of 12 mM glucose. In mouse islets carrying the R258W mutation the effects of palmitate and TUG-469 on insulin secretion were abrogated (Figure 2A), while the mutation T146S had no impact on fatty acid- or agonist-induced insulin secretion (Figure 2B). Neither mutation (T146S; R258W) affected glucose-stimulated insulin secretion or insulin content (46.8 ± 7.9 and 53.4 ± 8.2 ng insulin/islet of wild-type and *Ffar1*$^{R258W/R258W}$ mice, respectively). These data reveal that mice with the R258W mutation represent a model with dysfunctional FFAR1, while the T146S mutation has no functional consequence. Therefore, further analyses were performed with *Ffar1*$^{R258W/R258W}$ mice only.

3.3. R258W mutation of *Ffar1* prevents palmitate-induced up-regulation of peroxisome proliferator-activated receptor α (*Ppara*) mRNA levels in isolated mouse islets

To further investigate the specificity of the R258W mutation in FFAR1, palmitate-induced changes in gene expression were analyzed. Previous works reported the involvement of FFAR1 in activation of peroxisome proliferator-activated receptor α (*Ppara*) [10]. When isolated islets were exposed to palmitate (600 µM, adapted to the albumin concentration in culture medium) for 24 h, *Ppara* mRNA levels were augmented in wild-type islets (Figure 2C). This effect was absent in islets of *Ffar1*$^{R258W/R258W}$ mice, confirming that the mutation impairs receptor function. The effect is specific for *Ppara*, in that *Ppard* mRNA levels remained unchanged (Figure 2D). Interestingly, palmitate significantly reduced mRNA levels of *Slc2a2* (Glut-2 gene) in wild-type and mutant islets, indicating that this effect is FFAR1-independent (Figure 2E). Neither the R258W mutation nor palmitate altered the mRNA levels of *Ffar1*, *Glp1r*, and *Ins2* (Figure 2F,D, data not shown).

In freshly isolated, non-cultured, wild-type islets from CD and HFD fed mice, *Ffar1* mRNA levels were 5-fold lower after high fat feeding (ΔCt*1000: 0.9 ± 0.2, n = 6 vs 4.5 ± 1.2, n = 5; HFD vs CD, respectively) while *Slc2a2* mRNA levels remained unchanged (ΔCt*1000: 48.2 ± 7.9, n = 6 vs 57.7 ± 2.1, n = 6; HFD vs CD, respectively). *Ppara* mRNA levels appearing at >35 cycles were 10-times lower than *Ffar1* mRNA levels, which made the quantification unreliable.

These observations substantiate that the R258W mutation specifically inhibits FFAR1 function.

3.4. *Ffar1*$^{R258W/R258W}$ mice are protected against HFD-induced glucose intolerance in spite of insulin resistance and fatty liver

In regular CD fed wild-type and *Ffar1*$^{R258W/R258W}$ mutant mice, fasting blood glucose and plasma insulin levels were identical (Figure 3A,B). During ipGTT, the rise in blood glucose and plasma insulin was also not significantly different between wild-type and mutant mice. As expected, after HFD wild-type male mice but, surprisingly, not *Ffar1*$^{R258W/R258W}$ mutant mice, became glucose intolerant (Figure 3C). Due to significantly lower fasting insulin levels of *Ffar1*$^{R258W/R258W}$ mice, the increase in plasma insulin during ipGTT was 4-fold in mutant mice compared to 2-fold in wild-type mice (Figure 3D). The *Ffar1* mutation protected against HFD-induced glucose intolerance, although wild-type and mutant mice developed a similar insulin resistance upon high fat feeding (Figure 3E). During high fat feeding, the mice became overweight regardless of their genotype (Figure 3F). Body weights of 12 weeks old wild-type mice were 26.5 ± 0.4 g (n = 4) and 34.1 ± 1.6 g (n = 5, $p < 0.05$) after CD and HFD feeding, respectively. The corresponding weights of *Ffar1*$^{R258W/R258W}$ mutant mice were 27.0 ± 0.5 g (n = 3) and 35.0 ± 2.0 g (n = 4, $p < 0.05$).

In agreement, plasma leptin and resistin were significantly higher in mice fed HFD compared to CD regardless of the genotype (Figure 4A,B). Furthermore, liver steatosis was detectable after HFD in both wild-type and mutant mice and the mean hepatic triglyceride content was not significantly different (Figure 4C–E).

Finally, when HFD-fed mice were subjected to an oral glucose tolerance test, the glucose excursions were not significantly different between the genotypes (Figure 4F). Also, fasting plasma glucagon levels were 2.8 ± 0.3 pM and 3.8 ± 0.7 pM in wild-type and mutant mice

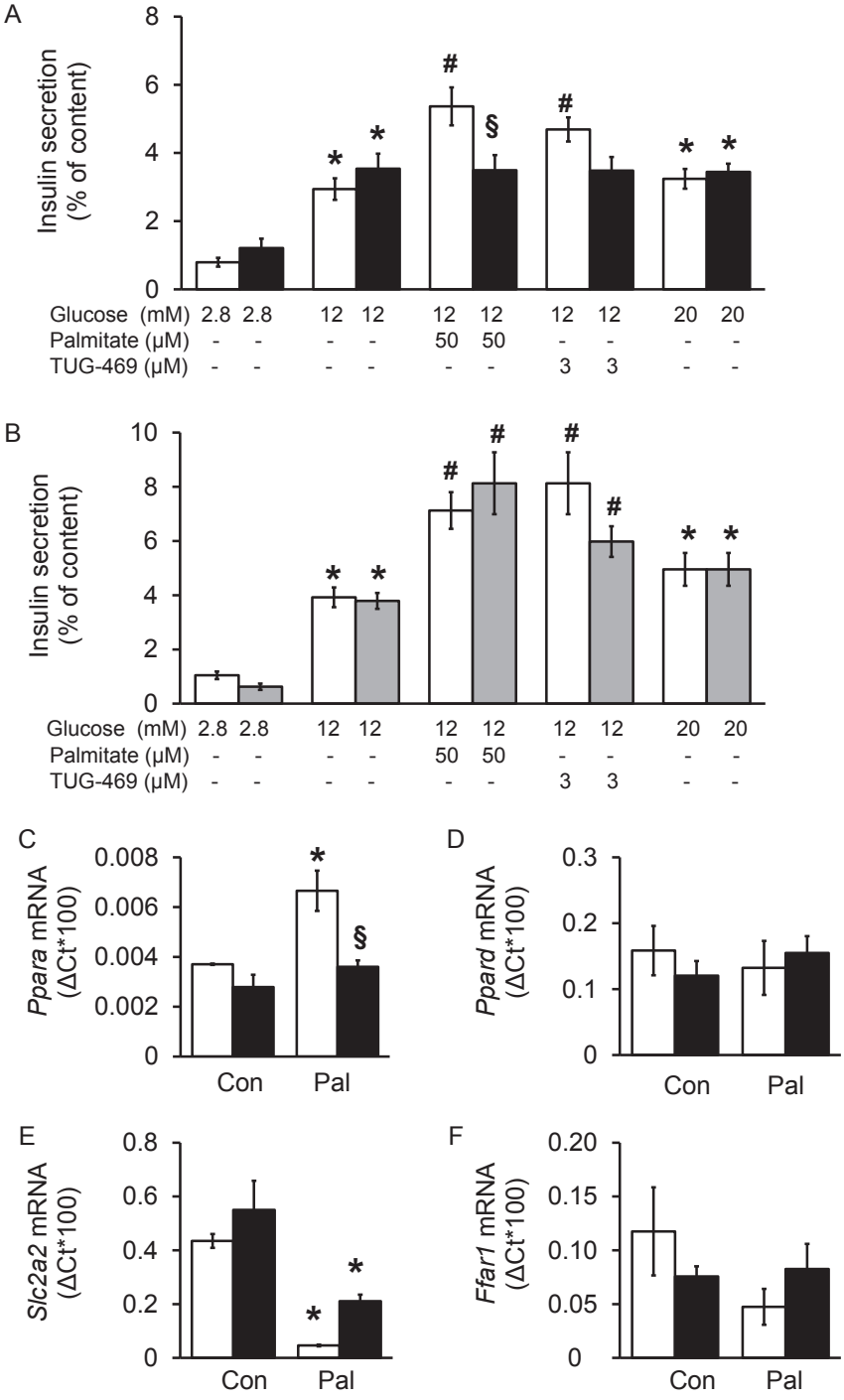

Figure 2: Mutation R258W but not T146S of FFAR1 abrogates palmitate- and TUG-469-induced stimulation of insulin secretion and the effect of palmitate on *Ppara* mRNA levels. (A, B) Insulin secretion of isolated islets from (A) wild-type littermates (white bars) and *Ffar1*$^{R258W/R258W}$ mice (black bars) as well as (B) wild-type littermates (white bars) and *Ffar1*$^{T146S/T146S}$ mice (gray bars) measured after 1 h static incubation with substances as indicated. Results are presented as means ± SEM of n = 3–5 independent experiments. (C–F) Isolated islets from wild-type (white bars) and *Ffar1*$^{R258W/R258W}$ mice (black bars) were cultured under control condition or exposed to palmitate, 600 μM for 24 h. Relative mRNA levels are expressed as means ± SEM of n = 3–4 independent experiments. *Rps13* was used as housekeeping gene. * denotes significance vs respective 2.8 mM glucose or Control (Con). # denotes significance vs. 12 mM glucose, § denotes significance between genotypes.

fed CD, respectively. After HFD, plasma glucagon concentrations dropped under the detection level.

In conclusion, the *Ffar1*$^{R258W/R258W}$ mutation generated a functional phenotype, i.e. the repression of fatty acid-induced insulin secretion along with minimal genetic modification. The unexpected protection against HFD-induced impaired glucose tolerance suggests an unmasking of a glucose lowering mechanism in the mutant mouse.

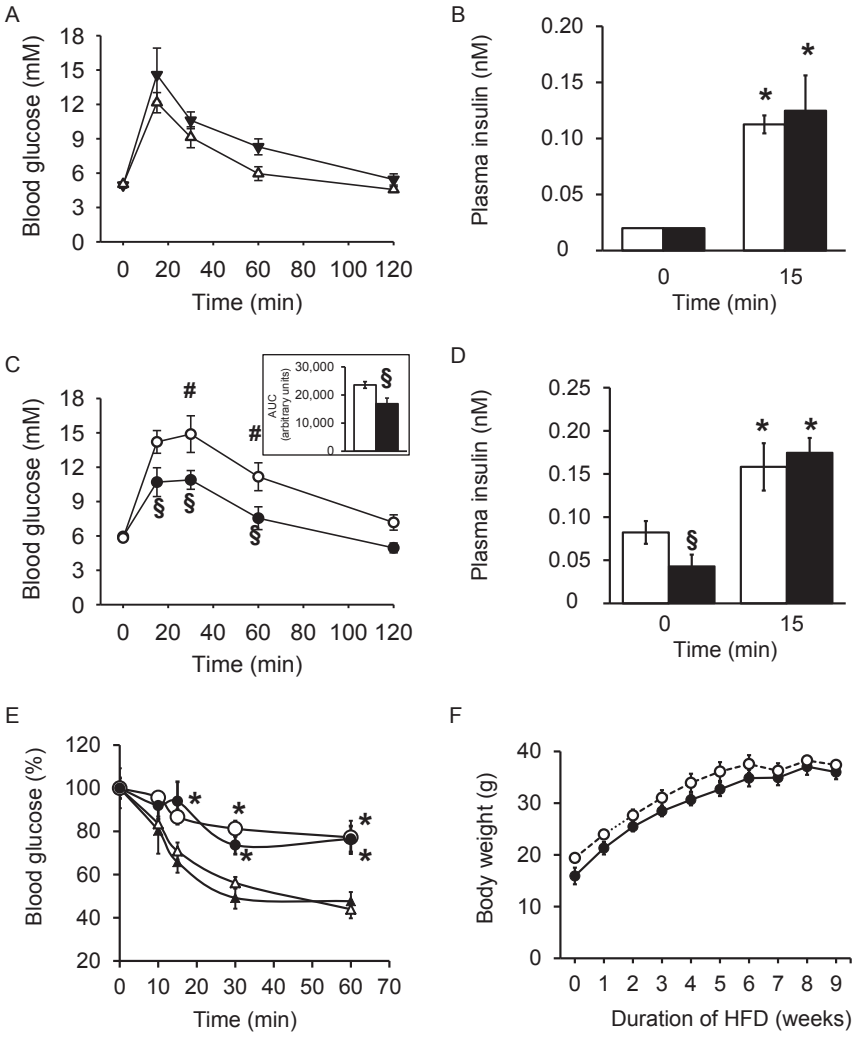

Figure 3: R258W mutation in FFAR1 protects against HFD-induced glucose intolerance. (A and C) Blood glucose and (B and D) plasma insulin concentrations during ipGTT of wild-type (white symbols and bars) and *Ffar1*$^{R258W/R258W}$ littermates (black symbols and bars) after (A and B) CD and (C and D) HFD feeding expressed as means ± SEM, n = 4–6 (CD) and 6–8 (HFD) male mice. * denotes significance vs respective 0 min time point. # denotes significance between HFD and CD of wild-type mice at the same time point; § significance between genotypes at the same condition. (E) Blood glucose in wild-type (white symbols) and *Ffar1*$^{R258W/R258W}$ (black symbols) mice during ipITT after CD (triangles, n = 4–6) and HFD (circles, n = 6–8). (F) Body weight gain during high fat feeding.

4. DISCUSSION

This study presents mice with a missense point mutation in R258 of FFAR1 that has functional consequences. Firstly, in islets of *Ffar1*$^{R258W/R258W}$ mice, both the physiological agonist palmitate and the synthetic agonist TUG-469 were unable to augment GIIS. Secondly, palmitate-mediated increase of *Ppara* mRNA levels was abrogated. The findings that FFAR1 mediates FFA effects on insulin secretion and *Ppara* mRNA are consistent with previous observations [1,3,10]. The loss of function of FFAR1 in *Ffar1*$^{R258W/R258W}$ mouse islets was not accompanied by a change of *Ffar1* mRNA levels, indicative of a normal expression of the non-functional receptor. Whether protein trafficking to the plasma membrane remains unaltered needs further experimental evidence. HEK-EM 293 cells that overexpress R258A-mutated FFAR1 exhibit an unaltered receptor abundance at the plasma membrane and an abrogation of FFAR1-agonist GW9508-induced Ca^{2+}-flux when compared to cells expressing wild-type receptors [19]. In contrast, in isolated islets of *Ffar1*$^{(-/-)}$ mice, *Ffar1* mRNA was undetectable. That the deletion affected the expression of adjacent genes is suggested by concomitant reduction of *Ffar3* mRNA levels. The functional consequence of the reduction of *Ffar3* and the increase of *Gpr119* mRNA levels is unknown. In respect to undesirable side effects, the mutant mouse represents a more reliable model.

The functional impact of *Ffar1*$^{R258W/R258W}$ became visible only in homozygous mice, while heterozygous mice did not develop any phenotype revealing a recessive character of the mutation (data not shown). Furthermore, GIIS was unaffected. Comparably, in humans, mutations (single nucleotide polymorphisms) in *FFAR1* link to minor, but significant metabolic changes [24].

The *Ffar1*$^{R258W/R258W}$ mouse model provided new insight into FFAR1-dependent and -independent effects of palmitate. Thus, in contrast to the FFAR1-mediated effect on *Ppara* mRNA, the palmitate-induced reduction of *Slc2a2* mRNA levels was independent of functional FFAR1. An effect of palmitate on Glut-2 expression has been previously reported but the underlying signaling pathways remained unexplored [25]. However, HFD did not alter *Slc2a2* mRNA levels,

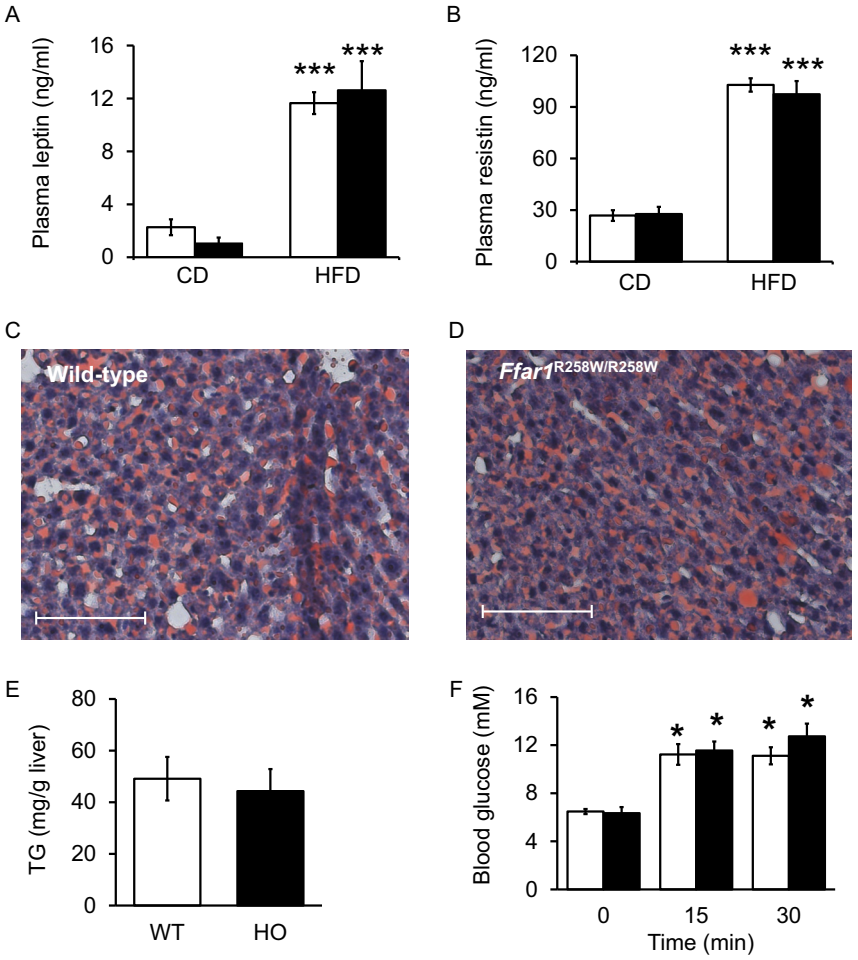

Figure 4: R258W mutation of FFAR1 did not influence HFD-stimulation of adipocyte hormone release nor liver fat accumulation. Fasting plasma (A) leptin and (B) resistin concentrations after CD (n = 4–6) and HFD (n = 6–8) feeding of wild-type (white bars) and *Ffar1*$^{R258W/R258W}$ mice (black bars). Liver fat droplets (red staining) in (C) wild-type and (D) *Ffar1*$^{R258W/R258W}$ liver tissue sections counterstained with hematoxylin. Scale bars, 100 μm. (E) Mean hepatic TG of HFD fed wild-type (WT, white bar) and *Ffar1*$^{R258W/R258W}$ (HO, black bar) mice. (F) Plasma glucose concentrations before (0 min) and 15 and 30 min after an oral glucose load (n = 6–8). Data are expressed as means ± SEM. *** denotes significance to the respective plasma concentration of CD fed mice; * significant to respective 0 min.

suggesting that the *in vitro* observation may not translate to the *in vivo* situation and, consequently, does not link to HFD-induced glucose intolerance. Chronic stimulation of G-protein coupled receptors, including FFAR1, is known to induce a downregulation of receptors and receptor function [26]. The exposure of wild-type islets to palmitate for 24 h was not sufficient for a significant reduction of *Ffar1* mRNA levels. Nevertheless, after 8 weeks HFD feeding *Ffar1* mRNA levels were reduced 5-fold indicating that chronic stimulation may attenuate receptor function.

The improved glucose tolerance of *Ffar1*$^{R258W/R258W}$ mice on HFD was unexpected. In combination with similar fasting blood glucose levels, the significant lower fasting plasma insulin of mutant mice compared to wild-type mice is indicative of improved insulin sensitivity. However, peripheral insulin resistance assessed with ipITT was not different between wild-type and mutant mice. The degree of liver steatosis was also independent of the expression of a functional FFAR1. Indeed, any change of liver steatosis and insulin resistance can only be attributed to an indirect effect of FFAR1, since the receptor is not expressed in rodent liver, muscle and adipose tissue (Refs. [1,27]; data not shown). The lower basal plasma insulin levels of *Ffar1*$^{R258W/R258W}$ compared to wild-type mice could be attributed to FFAR1 deficiency, because fatty acids are increased after overnight fasting and blood glucose levels were elevated, i.e. at 6 mM. In view of similar HFD-induced insulin resistance and liver steatosis, the significantly higher glucose excursions in wild-type mice during ipGTT cannot be explained by β-cell dysfunction only. It is more likely that additional, insulin-independent factors regulating blood glucose levels, e.g. via the regulation of hepatic glucose production, account for differences in glucose tolerance between wild-type and FFAR1 mutant mice.

An increased sympathetic tone and the hormone glucagon are the main glucose mobilizing factors [28,29]. Single-cell transcriptome analysis of human islet cells suggests the expression of *Ffar1* not only in β-cells but also in α-cells [30]. Moreover, analysis of rat α-cells indicates that FFAR1 expression is under the control of PAX6 [31]. At least in rodents, long chain fatty acids stimulate glucagon secretion at low glucose, i.e. under hypoglycemic condition [32]. However, there was no significant difference in plasma glucagon levels of CD-fed mutant and wild-type mice under fasting conditions. In HFD-fed mice, glucagon levels were much lower than in CD-fed mice and unfortunately under the detection level. In view of stable glucagon levels in humans during FFAR1-agonist administration and the lack of FFAR1-dependent stimulation of glucagon secretion in isolated human

and rat islets at high glucose, it seems unlikely that FFAR1-dependent glucagon secretion inducing hepatic glucose mobilization accounts for higher glucose levels during a glucose load [33,34]. Recently, evidence was presented that FFAR1 deficient mice display higher noradrenaline levels in brain [35]. The effects of changes in sympathetic nervous function during fat-rich feeding on glucose homeostasis in FFAR1-deficient mice require further studies.

During ipGTT, plasma insulin concentrations increased to a similar level in wild-type and *Ffar1*$^{R258W/R258W}$ mice, reflecting a β-cell glucose-responsiveness independent of FFAR1 function. Indeed, during ipGTT, plasma fatty acid concentrations decline and, therefore, it is unlikely that FFAR1 contributes to insulin secretion during ipGTT [36]. Glucose homeostasis is further regulated by incretins, and FFAR1-agonists increase incretin release in rodents [12,37,38]. In contrast to the significantly different plasma glucose levels at 30 min after ip glucose administration, 30 min after an oral glucose load, plasma glucose levels were not significantly different between wild-type and *Ffar1*$^{R258W/R258W}$ mice. GLP-1 secretion is stimulated by FFAR1 from the vascular but not from the luminal site, making it unlikely that FFAR1 is activated and augments incretin secretion during an oral glucose load when plasma fatty acids decline [36,38]. Plasma glucose homeostasis is maintained via an interaction of many organs, which generate a large variety of metabolic regulators. Only a detailed analysis of the individual players and the reciprocal influences will give an explanation why *Ffar1*$^{R258W/R258W}$ mice are protected against diet induced glucose intolerance.

This study introduces a mouse model carrying the point mutation R258W in *Ffar1*, which abolishes the stimulation of insulin secretion in response to long chain fatty acids. The minimal genetic alteration mirrors the human situation and has the advantage over conventional knockout/congenic mouse models. It also circumvents side effects generated by viral constructs, the removal of additional non-coding regions within the deleted gene, and changes in protein—protein interactions such as receptor G-protein coupling due to complete abrogation of a receptor protein.

DISCLOSURE STATEMENT

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AUTHOR CONTRIBUTIONS

GKHP, HUH, MHA, and SU designed the study, SS, SM, and GKHP established the mouse models, SS, GK, FG, TS, and SM generated and analyzed the mouse strains and performed and analyzed the *in vivo* experiments. GK, FG, MH, ELG, MP, and SU performed the *in vitro* experiments, analyzed data, and wrote the manuscript. All authors approved the final version.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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