Repression of the iron exporter ferroportin may contribute to hepatocyte iron overload in individuals with type 2 diabetes

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ABSTRACT

Objective: Hyperferremia and hyperferritinemia are observed in patients and disease models of type 2 diabetes mellitus (T2DM). Likewise, patients with genetic iron overload diseases develop diabetes, suggesting a tight link between iron metabolism and diabetes. The liver controls systemic iron homeostasis and is a central organ for T2DM. Here, we investigate how the control of iron metabolism in hepatocytes is affected by T2DM.

Methods: Perls Prussian blue staining was applied to analyze iron distribution in liver biopsies of T2DM patients. To identify molecular mechanisms underlying hepatocyte iron accumulation we established cellular models of insulin resistance by treatment with palmitate and insulin.

Results: We show that a subset of T2DM patients accumulates iron in hepatocytes, a finding mirrored in a hepatocyte model of insulin resistance. Iron accumulation can be explained by the repression of the iron exporter ferroportin upon palmitate and/or insulin treatment. While during palmitate treatment the activation of the iron regulatory hormone hepcidin may contribute to reducing ferroportin protein levels in a cell-autonomous manner, insulin treatment decreases ferroportin transcription via the PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways.

Conclusion: Repression of ferroportin at the transcriptional and post-transcriptional level may contribute to iron accumulation in hepatocytes observed in a subset of patients with T2DM.

Keywords T2DM; Iron overload; Hepatocytes; Insulin resistance

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder hallmarked by the presence of hyperglycemia caused by insulin resistance. According to the report of the International Diabetes Federation (IDF) in 2021, 537 million adults were diagnosed with diabetes with a rise to 783 million predicted by 2045 (https://www.idf.org/). Worldwide, T2DM causes a large burden on our societies in terms of health, disability and finance [1].

T2DM is hallmarked by insulin resistance, a state whereby organs and cells become less responsive to the actions of insulin. Insulin resistance in hepatocytes causes increased hepatic glucose production via glycogenolysis and gluconeogenesis [2]. Mechanisms triggering the development of hepatic insulin resistance include the accumulation of diacylglycerol (DAG), triglycerides and ceramide [3], overproduction of reactive oxygen species (ROS) [4,5] and systemic inflammation [6].

To compensate for insulin resistance, insulin production is increased, which in turn reduces receptor affinity, and decreases surface receptor numbers and receptor’s kinase activity [7,8], thus enhancing insulin resistance.

Globally, more than 70% of T2DM patients show nonalcoholic fatty liver disease (NAFLD) [9,10]. The prevalence of NAFLD in individuals...
with T2DM is five-fold increased compared to individuals without T2DM [11], whereby insulin resistance was a predictive factor for NAFLD in both obese and lean subjects [12]. Furthermore, insulin resistance was associated with the progression of NAFLD (e.g. ballooning and hepatic fibrosis) [13,14]. This suggests a vicious circle, in which insulin resistance links T2DM and NAFLD. Both, T2DM and NAFLD show imbalances of iron homeostasis. For example, elevated serum ferritin concentrations and altered hepatic iron levels were reported to be associated with an increased risk of T2DM [15–17]. In turn, T2DM and obesity can affect body iron homeostasis [16,18–20]. Hepatocytes are central for maintaining metabolic activities as well as for iron storage and regulation. Whether or not hepatocytes accumulate iron in T2DM or NAFLD remains controversial. Some studies showed that individuals with NAFLD [21] or high-fat-diet fed rats [22] have an increased hepatic iron content. On the contrary, db/db mice showed lower iron deficiency [18]. Free iron (Fe°2+) participates in the Fenton and Haber–Weiss reactions to generate highly reactive superoxide anion (O2°−) and hydroxyl radicals (OH−) [23]. These free radicals can generate oxidative stress that damages a wide range of macromolecules, such as nucleic acids, proteins, carbohydrates and lipid membranes resulting in cell damage [24]. Iron-induced oxidative stress in the liver impairs insulin signaling [25], causing enhanced insulin resistance [26]. Iron further contributes to liver injury by generating endoplasmic reticulum (ER) stress [27], increasing liver cholesterol synthesis [28] and hepatic inflammation [29]. In Kupffer cells, iron overload activates nuclear factor-κB (NF-κB) and induces tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) [29]. Iron fluxes in mammals are controlled by Ferroportin (FPN), the only known iron exporter [30]. Its surface expression is regulated at multiple levels: (1) post-translationally FPN is controlled by the iron-regulated hepatic hormone hepcidin which triggers its internalization and degradation [31]; (2) post-transcriptionally FPN is controlled by the iron-responsive element/iron-regulatory protein (IRE/IRP) system in response to iron [32] and microRNAs [33,34]; (3) transcriptionally HIF-2α and nuclear factor erythroid 2-related factor 2 (NRF2) control FPN levels [36]. Besides, it can be reduced by activation of TLR2 and TLR6 in response to inflammation [37]. In this study, we show that a subset of patients with T2DM accumulates iron in hepatocytes. Iron accumulation may be explained by transcriptional and post-transcriptional repression of Fpn expression involving the PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways.

2. MATERIALS AND METHODS

2.1. Clinical participants

Human tissue sections and clinical data used in the study were provided by the Tissue Bank of the National Center for Tumor Diseases (NCT) Heidelberg, Germany in accordance with the regulations of the tissue bank and the approval of the ethics committee of Heidelberg University (S-272/2018 and S-284/2018). Sample donors (23 individuals with T2DM) have undergone liver surgery or biopsy because of liver diseases such as primary or metastatic tumors. The areas of the liver that were free of cancer (confirmed by pathologists via H&E staining) were used for histological staining and analysis in our study.

2.2. Mice

Mice were housed in EMBL’s Laboratory Animal Resources (LAR) under constant light–dark cycle and maintained on a standard mouse diet containing 192 ppm iron (Altobrim 1318 Fortified with ad libitum access to food and water. All mouse breeding and experiments were approved by and conducted in compliance with the guidelines of the EMBL Institutional Animal Care and Use Committee (Project NR 2018-01-02_MH).

2.3. Chemicals

Insulin, ActinomycinD and Wortmannin (Sigma—Aldrich). MK2206, GSK690693, CCT128930, Sorafenib, Raf265, U0126, PD98059, SCH772984 and Ulixertinib (Biomol).

2.4. Preparation of palmitate

Palmitate (Sigma—Aldrich) was first dissolved in 70% ethanol at a concentration of 100 mM at 65 °C, then the solution was diluted with pre-warmed 10% fatty acid-free BSA (Sigma—Aldrich) to 5 mM and incubated at 37 °C for 6 h. The same concentration of ethanol mixed BSA (10%) was administered as vehicle control. The stock solution was sterile filtered (pore: 0.2 μm) and stored at −20 °C.

2.5. Cell culture and establishment of insulin resistance models

Hepa 1–6 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with high glucose (Invitrogen) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma—Aldrich), 10% (v/v) FBS (Gibco). Primary hepatocytes were isolated from C57BL/6 J mice (9 weeks old, males) following a previously described protocol [38]. Primary hepatocytes were plated in a density of 5 × 10^4 cells/cm² and cultured in William’s E medium (Life Technologies) supplemented with 4% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C humid air with 5% CO₂. For the establishment of insulin resistance, Hepa 1–6 cells were seeded in 6-well plates (5 × 10^5 cells/well) one day before treatment. The following day, cells were treated for 16 h with palmitate and/or insulin at a final concentration of 200 μM and 100 nM, respectively. Primary murine hepatocytes were treated 6 h after plating with palmitate (500 μM) and/or insulin (100 nM) for 24 h. Fatty acid-free BSA was used as control.

2.6. RNA interference

siRNAs were transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer’s instructions. siRNAs (Ambion, Life Technologies) used in this study are listed in Table S3. 48 h after silencing, cells were treated with palmitate or insulin. The knock-down efficiency was confirmed by Western blotting and/or qRT-PCR.

2.7. Protein extraction and western blotting analysis

Total protein lysates were obtained by homogenizing cellular pellets in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (cOmplete tablets EASYpack, Roche) and phosphatase inhibitors (PhosSTOP, Roche). Protein concentration was determined using the BCA protein assay (Life Technologies). 50 μg of total protein extracts were separated by 10% or 15% SDS-PAGE and subjected to Western blotting analysis with the antibodies listed in Table S4. Western blotting images were quantitatively acquired with the Vilber Lourmat Fusion-FX Chemiluminescence system. β-Actin and Vinculin were used as loading controls whenever appropriate.

2.8. Oil red O staining

Hepa 1–6 cells were fixed with 4% paraformaldehyde (w/v) (Life Technologies), and then incubated with 60% isopropanol for 5 min and Oil Red O solution (Sigma—Aldrich) for 20 min. Mayer’s Hematoxylin was used as a counterstain. For semi-quantitation, stained cells were treated with palmitate or insulin. The knock-down efficiency was confirmed by Western blotting and/or qRT-PCR.
2.9 Histology
Formalin-fixed paraffin-embedded liver biopsies from T2DM patients were cut with a thickness of 3 μm. After rehydration, the slides were processed for iron staining or CD68 immunohistochemistry.

Standard Perls' Prussian blue staining was performed by using the Iron Stain Kit (Sigma–Aldrich). Briefly, the slides were incubated for 15 min in iron stain solution (prepared by mixing equal volumes of 4% w/v potassium ferrocyanide and 1.2 mM hydrochloric acid). Nuclear Fast Red was used as counterstain.

CD68 expression was analyzed by CD68 antibody staining (Dako; Ref.- Nr.: M0876, Lot-Nr.: 00094,759; clone PG-M1) on the Ventana Benchmark Ultra (Roche) according to the manufacturer's instructions. Microscopy slides containing the tissue were preincubated with the EDTA-containing CC1 solution (Roche) for 16 min after which the slides were incubated with a 1:100 dilution of the CD68 antibody for 24 min. The stained slides were digitally scanned with the Aperio AT2 (Leica).

2.10 Total RNA extraction, reverse transcription and quantitative RT-PCR
Total RNA extraction from cells was performed with RNA-Mini kits following the manufacturer’s instructions (Bio&Sell) and reverse transcribed using random hexamers and the RevertAid H Minus Reverse Transcriptase (Life Technologies). SYBR-Green qRT-PCRs were run on a StepOnePlus Real-Time PCR System with the primers shown in Table S5. Relative mRNA expression was normalized to ribosomal protein L19 (Rpl19) mRNA levels. Data were analyzed by using the 2^−ΔΔCt method [39].

2.11 Measurement of intracellular ROS accumulation
ROS accumulation in Hepa 1–6 cells was detected by using the CellROX® Green Flow Cytometry Assay Kit (Life Technologies) according to the manufacturer’s protocol. Briefly, after stimulation, cells were incubated with 800 nM of CellROX® Green for 30 min at 37 °C under 5% CO2 atmosphere. Then, cells were washed with Hanks’ balanced salt solution (HBSS) and continually incubated with 5 nM of SYTOX Red Dead Cell stain solution for 5 min. Fluorescence intensity and the percentage of ROS-positive cells (dead cells excluded) were measured using flow cytometry (BD Accuri™ C6 Pluses). H2O2 (1 mM) and N-acetyl-cysteine (5 mM) were used as positive and negative controls, respectively.

2.12 Atomic absorption spectrometry
Iron content in Hepa 1–6 cells was measured by graphite furnace atomic absorption spectrometry (AAS) as described before [40].

2.13 Statistics analysis
Data are presented as mean ± SEM. Statistical analyses were performed using Prism v8 (GraphPad Software). The two-tailed, Student’s t-test, one-way or two-way ANOVA were used where appropriate. Significance levels were showed as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Logistic regression analysis was performed with the R v4.1.2 statistical software.

3. RESULTS

3.1 In a subgroup of T2DM patients iron accumulates in hepatocytes
Our previous work showed that T2DM patients display a biomarker profile that indicates systemic iron accumulation: plasma iron and ferritin levels are increased and hepcidin levels are reduced [18]. An elevated iron state in T2DM may enhance oxidative stress and contribute to insulin resistance [4,23].

We now explored whether the human diabetic liver indeed accumulates iron. We analyzed liver biopsies from 23 individuals with T2DM. Table S1 summarizes the clinical parameters available in these patients. Perls Prussian blue staining of the biopsies detected iron deposition in both hepatocytes and CD68 positive Kupffer cells. However, the proportion of cells with detectable iron accumulation differed profoundly between samples (Figure 1). Among the 23 samples analyzed, 4 (17.4%) biopsies were only positive for iron staining in hepatocytes and other 4 (17.4%) samples in Kupffer cells. Additional 6 (26.1%) samples were iron positive in both Kupffer cells and hepatocytes. In the remaining samples (39.1%) iron was not detectable. Overall, these results indicate that around 45% of patients affected by T2DM show hepatocyte iron accumulation. Logistic regression analysis showed that T2DM patients with detectable iron in hepatocytes showed a tendency to higher glucose levels, while patients with Kupffer cells iron accumulation more likely displayed steatosis, abnormal liver function, inflammation, and an increased number of hypochromic erythrocytes indicative of iron deficiency (Table S2).

3.2 Iron accumulation occurs in cellular models of insulin resistance
To understand the underlying molecular mechanisms causing iron accumulation in hepatocytes, we established and characterized cellular models of insulin resistance. Insulin resistance can be induced by the treatment of cells with free fatty acid (FFA) (e.g. palmitate (C16:0) the most abundant dietary and plasma fatty acid) [41] and insulin, which can induce a loss of insulin sensitivity at high concentrations [18,42]. Hepatocytes (Hepa 1–6) were exposed to palmitate (200 μM) and/or long-term (16 h) insulin (100 nM) treatment. The induction of insulin resistance was confirmed by (1) higher basal levels of phosphorylated AKT and a blunted response to short-term (15 min) insulin treatment; (2) prevention of short-term insulin-stimulated FOXO1 phosphorylation and (3) impairment of ERK1/2 phosphorylation (Figure 2A). Of note, the combination treatment of palmitate and insulin induced more severe insulin resistance compared to either agent alone (Figure 2A). In line with the results obtained in the Hepa 1–6 hepatoma cell line, incubation of primary murine hepatocytes with palmitate (500 μM) and long-term insulin (100 nM) also induced insulin resistance, as shown by the impaired acute insulin-induced AKT and ERK1/2 phosphorylation (Figure 2B). To investigate whether insulin resistance was induced at the level of the receptor, we measured the protein expression of the insulin receptors (IR). We show that long-term insulin treatment caused decreased IRα levels in both Hepa 1–6 cells and primary hepatocytes (Figure 2A–B). However, palmitate-mediated IRβ downregulation was observed only in primary hepatocytes (Figure 2B). In addition, palmitate treatment caused severe lipid accumulation, which can be ameliorated by insulin treatment (Figure 2C) and increased de novo lipogenesis, as indicated by elevated diacylglycerol O-acyltransferase 2 (Dgat2), Stearoyl-CoA desaturase-1 (Scd1), ATP citrate synthase/ATP citrate lyase (Acly) and Lpin1 mRNA expression (Figure 2D).

Oxidative stress and inflammation are important hallmarks of insulin resistance [4,6]. Consistently, in response to palmitate treatment, the percentage of ROS-positive cells as well as ROS production is increased (Figure 2E). Accordingly, mRNA expression of NRF2 target genes was increased in all three models of insulin resistance (Figure 2F). The degree of activation of markers for oxidative stress was lower in cells treated with insulin compared to those treated with palmitate (Figure 2F). Elevated mRNA expression of the suppressor of cytokine signalling 3 (SOCS3) and serum amyloid A1 (SAA1) following palmitate treatment further indicates inflammation. However, insulin...
per se did not induce oxidative stress or inflammation (Figure 2E–F). These data indicate that steatosis may be a driver for oxidative stress and a pro-inflammatory state, leading to insulin resistance.

Following the establishment of insulin-resistant models, we next assessed how insulin resistance influences iron homeostasis. Similar to observations in patients, insulin-resistant hepatocytes (i.e. treated with palmitate, insulin or a combination) accumulated more iron (Figure 3A) and accordingly, increased ferritin levels (Figure 3B, C), whereby the combination of palmitate and insulin treatment resulted in a higher degree of iron accumulation compared to palmitate or insulin alone (Figure 3A, B). Additional treatment of the insulin-resistant hepatocytes with an exogenous iron source (ferric ammonium citrate; FAC) further increased iron retention (Figure 3D). Cellular iron accumulation can be caused by increased iron uptake mediated by the iron transporters transferrin receptor 1 (TfR1), ZRT/IRT-like protein-14 (ZIP14) and SLC11A2 or decreased iron export, which is controlled by FPN [43].

TfR1 protein levels were reduced after palmitate and palmitate + insulin treatment, both in Hepa 1–6 cells and in primary hepatocytes (Figure 3B, C). This is consistent with a cellular response to iron overload mediated by the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory system. However, TIR1 levels remained unchanged following the single insulin treatment (Figure 3B, C) [32]. By contrast, Zip14 mRNA expression was elevated in insulin-resistant Hepa 1–6 cells in conditions that mimic hyperinsulinemia (PA + Ins or Ins), suggesting that non-transferrin-bound iron (NTBI) uptake may contribute to iron accumulation (Figure 3E). Due to the lack of a specific and sensitive antibody for ZIP14, the protein levels could not be assessed. The mRNA expression of the iron importer Slc11a2 was not affected by insulin resistance (Figure S1).

An interesting finding is that Fpn mRNA is repressed upon palmitate and/or insulin treatment, with the most pronounced effect observed in insulin and palmitate-treated cells (Figure 3E). To reach sufficient sensitivity to detect FPN protein levels in Hepa 1–6 cells, we exposed the cells to FAC, which increases its expression. In line with Fpn mRNA expression, FPN protein levels were also reduced by palmitate and insulin treatment in both Hepa 1–6 cells and primary hepatocytes (Figure 3F, G). These results suggested that decreased FPN levels may at least in part explain the increased iron content in both palmitate and/or insulin-induced insulin resistance models. Palmitate treatment additionally increased hepcidin mRNA expression (Figure 3E), suggesting that decreased FPN protein levels in response to palmitate may be a consequence of reducing Fpn mRNA expression and hepcidin-mediated FPN degradation.

3.3. Palmitate regulates FPN expression at the post-transcriptional level in an hepcidin-independent manner

To understand the mechanisms involved in the suppression of FPN expression in response to palmitate treatment, we analyzed FPN protein and mRNA expression with increasing concentrations of palmitate, at different time points. We found that palmitate reduced
Figure 2: Cellular models of palmitate and/or insulin-induced insulin resistance show dysregulated lipid metabolism and increased oxidative stress. (A) Hepa 1–6 hepatocytes were incubated with palmitate (200 μM), insulin (100 nM) or the combination for 16 h to establish insulin resistance. Western blot of phospho-AKT (Ser473), AKT, phospho-FOXO1 (Ser256), FOXO1, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, IRα and IRβ with or without short-term insulin treatment (100 nM for 15 min). β-Actin is shown as a loading control. (B) Primary hepatocytes were incubated with palmitate (500 μM), insulin (100 nM) or the combination for 24 h. Western blot of phospho-AKT (Ser473), AKT, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, IRα and IRβ with or without short-term insulin treatment (100 nM for 15 min). β-Actin is shown as a loading control. (C–F) Hepa 1–6 hepatocytes were incubated with palmitate (200 μM), insulin (100 nM) or the combination for 16 h. Oil red O staining to detect lipid accumulation (C). De novo lipogenesis was assessed by measuring mRNA expression of Dgat2, Scd1, Acly, and Lpin1 (D). Measurement of ROS content and the percentage of ROS positive cells by flow cytometry. H2O2 (1 mM) and NAC (5 mM) were used as positive and negative controls, respectively (E). Oxidative stress and inflammation were analyzed by mRNA expression of Nqo1, Ho1, Socs3 and Saa1 (F). The data in (D) and (F) are qRT-PCR data, normalized to the housekeeping gene Rpl19 and presented as fold-change compared to the vehicle control. Data are represented as mean ± SEM and shown as representative of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA or two-way ANOVA. Ins, insulin; IR, insulin receptor; NAC, N-acetyl-cysteine; PA, palmitate.
Figure 3: Palmitate and/or insulin treatment dysregulate iron homeostasis. (A–B) Hepa 1–6 hepatocytes were incubated with palmitate (200 μM), insulin (100 nM) or the combination for 16 h. The cellular iron content was analyzed by atomic absorption spectrometry (AAS) and normalized to the protein content (A). Western blot and quantitative analysis of TR1, Fth and Flt (B). (C) Primary hepatocytes were treated with palmitate (500 μM), insulin (100 nM) or the combination for 24 h. Western blot and quantitative analysis of TR1, Fth and Flt. (D–F) Hepa 1–6 hepatocytes were incubated with palmitate (200 μM), insulin (100 nM) or the combination for 16 h. Western blot analysis of Fth and Flt in the presence of FAC (100 μM) (D). Fpn, Hepcidin and Zip14 mRNA expression was analyzed by qRT-PCR and normalized to the housekeeping gene Rpl19. Data are shown as fold-change compared to the vehicle control (E). Western blot and quantitative analysis of FPN with and without FAC treatment (100 μM) (F). (G) Western blot and quantitative analysis of FPN in primary hepatocytes treated with palmitate (500 μM), insulin (100 nM) or the combination for 24 h. Data are represented as mean ± SEM and representative of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA or two-way ANOVA. FAC, ferric ammonium citrate; Fth, ferritin heavy chain; Flt, ferritin light chain; Ins, insulin; PA, palmitate; TR1, transferrin receptor.
FPN expression at both the protein and mRNA levels in a dose-dependent manner (Figure 4A,B), and decreased Fpn mRNA expression in a time-dependent manner (Figure S2A). In addition to the regulation of FPN expression, palmitate decreased TfR1 mRNA levels, but had no impact on the mRNA levels of Zip14 and Slc11a2 (Figure S2B). Hepcidin mRNA expression can be activated by iron overload, inflammation and oxidative stress [44]. In addition, CREBH, an endoplasmic reticulum stress response factor, controls hepcidin mRNA expression in response to gluconeogenesis during starvation [45]. Here we show that hepcidin mRNA expression is significantly increased by palmitate treatment in a dose- and time-dependent manner (Figure 4C and Figure S1B). In addition, mRNA expression of Ho1, Socs3 and CrebH responds to palmitate treatment (Figure 4D,E and 4F), suggesting that multiple signals such as iron content (Figure 3A), oxidative stress, inflammation and CrebH may increase hepcidin mRNA levels.

**Figure 4:** Palmitate reduces FPN expression at the post-transcriptional level in an hepcidin-independent manner. (A–F) Protein expression of FPN analyzed by western blot (A) with or without FAC (100 μM) and mRNA expression of Fpn (B), hepcidin (C), Ho1 (B), Socs3 (G) and CrebH (F) analyzed by qRT-PCR in Hepa 1–6 hepatocytes treated with vehicle, 50 μM, 100 μM, 150 μM, 200 μM and 250 μM of palmitate for 16 h. (G) Western blot and quantitative analysis of FPN protein expression in Hepa 1–6 hepatocytes transfected with a scramble (unrelated control siRNA) or siHamp for 36 h following 16 h of palmitate (200 μM) or vehicle (control) treatment in the presence of FAC (100 μM). (H) Fpn mRNA expression in the cells treated with DMSO or 1 μg/ml actinomycin D in the presence or absence of palmitate for 2 h, 4 h, 6 h and 8 h. The data in (B, C, D, E, F and H) were analyzed by qRT-PCR, normalized to the housekeeping gene Rpl19 and are presented as fold-change compared to the vehicle control. Data are represented as mean ± SEM and representative of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA. ActD, actinomycin D; FAC, ferric ammonium citrate; ns, no significant difference; PA, palmitate.
RNAi-mediated silencing of hepcidin, which reduced hepcidin mRNA expression by 55% (Figure S2D) was unable to derepress FPN protein expression suggesting that hepcidin may play a limited role in controlling iron export following palmitate treatment in this setting (Figure 4G). Fpn may rather be affected by mRNA degradation in response to palmitate treatment (Figure 4H). Of note, there was no significant difference in palmitate mediated Fpn responses upon DMSO and actinomycin D treatment (Figure S2E), suggesting that transcriptional regulation is unlikely to be involved.

3.4. Insulin regulates FPN expression at the transcriptional level

We next focused on the control of FPN expression in response to insulin. In Hepa 1–6 cells, insulin treatment (5 nM) induced AKT and FOXO1 phosphorylation (Figure 5A) and repressed FPN protein and mRNA levels (Figure 5B,C), while hepcidin mRNA expression remained unchanged (Figure 5D). We show that the rate of mRNA decay is comparable between insulin-treated and untreated cells upon actinomycin D treatment (Figure 5E), which suggests that insulin treatment promotes a significant decrease in Fpn mRNA transcription after 4 h. Additionally, insulin treatment increased Zip14 mRNA levels (Figure 5F). However, while silencing of Zip14 significantly decreased Zip14 mRNA expression (Figure S3A), it did not prevent insulin-regulated FTL elevation, suggesting that ZIP14 unlikely is involved in insulin-controlled iron accumulation (Figure 5G). Additionally, mRNA expression of Slc11a2 and TfR1 was not altered upon insulin treatment (Figure S3B).

3.5. Insulin regulates FPN mRNA expression through the PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways

Signal transduction in response to insulin in hepatocytes has been extensively studied. As shown in Figure S4, insulin binding to IR triggers the phosphorylation of the insulin receptor substrate (IRS) causing activation of the PI3K/AKT signaling pathway. Activated AKT can phosphorylate its downstream targets and control glucose and lipid metabolism. In addition, activated IRS also activates the Ras/Raf/MEK/ERK cascade, which is involved in cell growth, differentiation and protein synthesis [46].

We next sought to investigate which mechanism(s) are involved in insulin-mediated FPN repression. In a time course experiment we...
Figure 6: Insulin controls Fpn mRNA expression via PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways. (A) Western blot of phospho-FOXO1 (Ser256), FOXO1, phospho-AKT (Ser473), AKT, phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 in Hepa 1–6 hepatocytes treated with 100 nM insulin for 5 min, 15 min, 30 min, 2 h, 4 h, 6 h, 8 h and 16 h. (B–C) Western blot of phospho-FOXO1 (Ser256), FOXO1, phospho-AKT (Ser473) and AKT (B) or Fpn mRNA expression (C) in Hepa 1–6 hepatocytes pre-treated with 1 μM of wortmannin or DMSO for 1 h following 2 h or 16 h of insulin (100 nM) treatment, respectively. (D–E) Western blot of phospho-FOXO1 (Ser256), FOXO1, phospho-AKT (Ser473) and AKT (D) or Fpn mRNA expression (E) in Hepa 1–6 hepatocytes pre-treated with DMG0, MK2206 (2.5 μM), GSK690693 (5.0 μM) or CCT128930 (5.0 μM) for 1 h following 2 h or 16 h of insulin (100 nM) treatment, respectively. (F–G) Western blot of phospho-FOXO1 (Ser256), FOXO1, phospho-AKT (Ser473) and AKT (F) or Fpn mRNA expression (G) in Hepa 1–6 hepatocytes transfected with scramble, siAkt1, siAkt2 or siAkt1 + siAkt2 for 48 h following 2 h or 16 h of insulin (100 nM) treatment, respectively. (H–I) Western blot of phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 (H) or Fpn mRNA expression (I) in the cells pre-treated with DMSO, Sorafenib (10 μM), Raf265 (10 μM), U0126 (10 μM), PD98059 (50 μM), SCH772984 (10 μM) or Ulixertinib (5 μM) for 1 h following 2 h or 16 h of insulin (100 nM) treatment, respectively. (J–K) Western blot of phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 (J) or Fpn mRNA expression (K) in the cells transfected with scramble, siErk1, siErk2 or siErk1 + siErk2 for 48 h following 2 h or 16 h of insulin (100 nM) treatment, respectively. For the data in (A, B, D, F, H and J), β-actin or Vinculin was used as loading control. The data in (C, E, G, I and K) were analyzed by qRT-PCR, normalized to the housekeeping gene Rpl19 and are presented as fold-change compared to the vehicle or scramble control. Data are represented as mean ± SEM and representative of at least three independent experiments. ***p < 0.01, ****p < 0.001, *****p < 0.0001, one-way ANOVA or two-way ANOVA. ns, no significant difference.
show that Hepa 1–6 cells treated with 100 nM insulin increased phosphorylation of AKT, FOXO1 and ERK1/2 after 5 min, a response that peaked at 2 h and gradually decreased until 16 h (Figure 5A). To understand which signaling branches affect insulin-mediated FPN mRNA repression we inhibited the activity of PI3K with wortmannin (1 μM). Wortmannin successfully blocked the insulin-induced phosphorylation of AKT and FOXO1, downstream of PI3K (Figure 6B) as well as attenuating insulin-mediated Fpn mRNA downregulation (Figure 6C) in the absence of hepcidin responses (Figure S5A). These results indicate that PI3K is involved in insulin-mediated Fpn mRNA downregulation. Once activated, PI3K phosphorylates AKT which consists of the 3 isoforms AKT1, AKT2 and AKT3, whereby AKT1 and AKT2 are abundant in the liver (www.proteinatlas.org). To clarify whether AKTs are required for insulin-mediated Fpn mRNA repression, we treated cells with three AKT inhibitors: MK2206 and GSK690693 are pan-AKT inhibitors and CCT128930 specifically affects AKT2. We show that all AKT inhibitors reduce AKT kinase activity, as demonstrated by reduced phosphorylation of FOXO1, which acts downstream of AKT (Figure 6D). In addition, AKT inhibition prevented insulin-induced Srebp1c mRNA expression and suppression of Srebp1c steady-state levels (Figure S5B). SREBP1c is one of downstream of AKT and controls de novo lipogenesis. AKT inhibition did not affect the Ras/Raf/MEK/ERK signalling branch, as levels of ERK1/2 and phosphorylated ERK1/2 levels were not affected. (Figure S5C). Importantly, treatment with MK2206 and GSK690693, but not CCT128930, completely blocked insulin-mediated Fpn mRNA repression (Figure 6E) without affecting hepcidin mRNA expression (Figure S5D). We additionally observed that AKT inhibition increases steady-state Fpn mRNA expression (Figure 6E).

Consistent with pharmacological AKT inhibition the combined silencing of Akt1 and Akt2 (Figure S5E) most effectively inhibited insulin signalling in Hepa 1–6 cells, as indicated by reduced phosphorylation of AKT and FOXO1 following insulin treatment (Figure 6F). Because the knockdown of Akt1 but not Akt2 reduced total AKT protein expression, AKT1 seems to be the most abundant isoform in Hepa 1–6 cells (Figure 6F). In addition, combined silencing of Akt1 and Akt2 attenuated insulin-mediated Srebp1c upregulation (Figure S5F). Importantly, Akt1 and Akt2 silencing attenuated Fpn mRNA repression in response to insulin treatment in a hepcidin-independent manner (Figure 6G, S5G) and also seems to control steady-state Fpn mRNA levels in Hepa 1–6 cells (Figure 6G).

We next targeted the Ras/Raf/MEK/ERK signalling branch and assessed insulin-mediated Fpn mRNA responses. The following inhibitors were applied to target Raf (Sorafenib and Raf265), MEK1/2 (U0126 and PD98059) and ERK1/2 (SCH772984 and Ulixertinib). All pharmacological inhibitors significantly reduced ERK1/2 phosphorylation (except Ulixertinib which led to elevated phosphorylated ERK1/2 levels) (Figure 6H) and the target gene cyclin D1 (Cnd1) (Figure S6A) and did not affect insulin-mediated AKT phosphorylation (Figure S6B). Interestingly, all the inhibitors efficiently prevented insulin-mediated Fpn repression and increased steady-state Fpn mRNA expression (Figure 6I). Surprisingly, ERK1/2 inhibitors induced hepcidin mRNA expression, a response attenuated by treatment with insulin (Figure S6C).

In addition, RNAi-mediated silencing of Erk1 and Erk2 (Figure 6J) significantly decreased mRNA expression of the ERK1/2 target gene Cnd1 (Figure S6D), blocked insulin-mediated Fpn mRNA repression and increased steady-state Fpn mRNA expression level (Figure 6K), and no effect on AKT phosphorylation (Figure S6E). In contrast to applying pharmacological inhibitors, silencing of Erk1 and/or Erk2 did not alter hepcidin mRNA expression (Figure S6F), suggesting that the hepcidin response to the pharmacological inhibitors may be non-specific. In conclusion, these results suggest that both the PI3K/AKT and the Ras/Raf/MEK/ERK signaling pathways are involved in insulin-mediated Fpn mRNA downregulation.

### 4. DISCUSSION

Increased serum iron and ferritin levels were observed in patients and mouse models of T2DM [18,20]. These biomarkers are frequently linked to iron accumulation in the liver [47,48] that may contribute to the progression of hepatocyte insulin resistance, lipid accumulation and liver damage [26,48]. We show that a subset of patients with T2DM stains positive for iron in hepatocytes (Figure 1). Our results obtained in hepatocytes exposed to palmitate and/or insulin suggest that repression of the iron exporter FPN induced by transcriptional and post-transcriptional mechanisms may contribute to iron retention in hepatocytes. Specifically, insulin treatment decreases FPN transcription through the PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways (Figure 7).

The first important finding of our study was that iron can be detected in the liver of 60.9% of patients with T2DM either in hepatocytes (17.4%), Kupffer cells (17.4%), or both cell types (26.1%) (Figure 1). Iron deposition involving these three patterns was previously observed by Nelson et al. in liver biopsies of NAFLD patients, however, the relative number of samples with detectable iron was overall lower in this study (7.4%, 10.7% and 16.4% in hepatocytes, Kupffer cells or both cell types, respectively) [49]. Differences may be explained by the fact that, in addition to liver disease, elevated liver iron levels are subject to gender, age, red meat intake and pharmacological treatments [50]. Previous work has shown that iron accumulation in Kupffer cells is associated with advanced disease, e.g. fibrosis, portal inflammation and steatosis [49]. Our data are consistent with this observation as patients with increased iron levels in Kupffer cells showed a tendency towards higher GGT levels and steatosis (Table S2). However, due to the limited number of available hepatic biopsies in this study, these data require validation in a larger cohort. Elevated liver iron content was further observed in individuals with NAFLD [21] and in a cohort of 9108 individuals with increased liver adiposity [50]. All patients in our study displayed normal liver function as suggested by normal AST, ALT, Bilirubin, and CHE (Table S1). However, some participants showed elevated GGT levels consistent with a previous study of T2DM patients [18]. Besides, increased GGT levels may also be associated with cancer of digestive organs and liver [51,52] as is the case in our patients. This may offer an additional explanation for the disruption of iron homeostasis.

In T2DM, insulin resistance can be caused by multiple mechanisms involving high FFA accumulation and hyperinsulinemia. In this study, we established a cell-based assay of insulin resistance by treating Hepa 1–6 cells and primary hepatocytes with palmitate and/or insulin. Insulin resistance and downstream consequences were demonstrated by (1) impaired insulin signalling (2) severe lipid accumulation in response to external lipid treatment and de novo lipogenesis and (3) increased oxidative stress (Figure 2). Thus, this model reflects well upon features of insulin resistance observed in vivo [53].

Analysis of these cellular models showed that insulin resistance causes iron accumulation (Figure 3A,B and 3C), similar to observations in T2DM patients (Figure 1) that coincide with reduced mRNA and protein levels of the iron exporter FPN. Decreased FPN mRNA and protein levels were observed in liver biopsies of patients with NAFLD [54]. A previous study has implicated TIR1 in increasing iron uptake in palmitate-treated skeletal muscle cells [55], while another study...
showed that in Chinese hamster ovary cells, palmitoylation of TfR1 inhibits its endocytosis [56]. In this study, palmitate treatment decreased TfR1 protein levels in primary hepatocytes and Hepa 1–6 cells (Figure 3B,C) suggesting that the control of TfR1 expression in response to palmitate treatment may be cell-type specific. In addition, we observed increased levels of oxidative stress (Figure 2E,F) and inflammation (Figure 2F), suggesting that this enhances insulin resistance [4,6]. These results suggest a vicious cycle between insulin resistance and iron overload, which is linked by oxidative stress and inflammation (Figure 7).

The established cellular models of insulin resistance allowed us to investigate the molecular mechanisms of FPN repression. We show that palmitate treatment post-transcriptionally represses $Fpn$ mRNA (Figure 4H). However, the underlying mechanism remains unclear. Future work will have to investigate whether miRNAs (e.g. miR-20a [33] and miR-485 [34]) may be involved in controlling $Fpn$ mRNA levels. By contrast, insulin treatment of Hepa 1–6 cells represses $Fpn$ transcription (Figure 5E) involving the PI3K/AKT and Ras/Raf/MEK/ERK signalling pathway. The PI3K/AKT pathway was previously demonstrated to contribute to inflammation-mediated FPN downregulation in macrophages [57]. Furthermore, in U373MG cells and mouse spleen, ERK1/2 activation induced FPN expression [58,59]. The latter observation contradicts the observations reported here, emphasizing that the control of FPN levels may vary between cell types. Of note, $Fpn$ transcription can be induced by ROS via NRF2 [36]. We expect this pathway to be active in our experimental settings, as the mRNA expression of the Nr2 target genes $Ho1$ and $Nqo1$ are increased in response to insulin treatment (Figure 2F). However, this effect may be blunted by the activation of PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways following insulin treatment.

In conclusion, our results reveal a previously unidentified role for palmitate and insulin in mediating FPN repression in hepatocytes possibly contributing to iron accumulation in the diabetic liver. We demonstrated for the first time that PI3K/AKT and Ras/Raf/MEK/ERK signaling pathways are involved in insulin-mediated $Fpn$ downregulation. This finding may have clinical implications, as several PI3K/AKT and Ras/Raf/MEK/ERK inhibitors have been approved by the Food and Drug Administration (e.g., idelalisib and sorafenib) (www.fda.gov) or are under clinical trials (e.g., MK2206 [60] and Ulixertinib [61]) for anti-cancer treatment. Monitoring the systemic and liver iron level in individuals treated with these inhibitors, especially those with T2DM, may provide a better understanding of iron homeostasis, and thus benefits patients.

Author’s contribution
R.Q., K.A. and O.M. designed the experiments, R.Q. performed the experiments. K.A. carried out the Perls Prussian blue staining. N.V. provided human liver sections, collect patients’ characteristics and performed CD68 staining. C.M. participated in the measurement of iron content by using AAS method. A.A analyzed the clinical data. R.Palmitic
and S-S helped with experiments during the review. R.Q., S.A. and M.U.M. drafted the manuscript. M.U.M. and S.A. supervised the project. All authors read and reviewed the manuscript.

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DATA AVAILABILITY

Data will be made available on request.

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CONFLICTS OF INTEREST

The authors have no conflicts to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101644.

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