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ABSTRACT

Background and aims: Metformin, the most prescribed drug for the treatment of type 2 diabetes mellitus, has been recently reported to promote weight loss by upregulating the anorectic cytokine growth differentiation factor 15 (GDF15). Since the antidiabetic effects of metformin are mostly mediated by the activation of AMPK, a key metabolic sensor in energy homeostasis, we examined whether the activation of this kinase by metformin was dependent on GDF15.
Methods: Cultured hepatocytes and myotubes, and wild-type and *Gdf15*$^{-/-}$ mice were utilized in a series of studies to investigate the involvement of GDF15 in the activation of AMPK by metformin.
Results: A low dose of metformin increased GDF15 levels without significantly reducing body weight or food intake, but it ameliorated glucose intolerance and activated AMPK in the liver and skeletal muscle of wild-type mice but not *Gdf15*$^{-/-}$ mice fed a high-fat diet. Cultured hepatocytes and myotubes treated with metformin showed AMPK-mediated increases in GDF15 levels independently of its central receptor GFRAL, while *Gdf15* knockdown blunted the effect of metformin on AMPK activation, suggesting that AMPK is required for the metformin-mediated increase in GDF15, which in turn is needed to sustain the full activation of this kinase independently of the CNS.
Conclusion: Overall, these findings uncover a novel mechanism through which GDF15 upregulation by metformin is involved in achieving and sustaining full AMPK activation by this drug independently of the CNS.

1. Introduction

Metformin is the most prescribed drug for the treatment of type 2 diabetes mellitus, due to its hypoglycemic effect, which occurs primarily through the inhibition of hepatic glucose production [1]. However, its precise mechanism of action remains only partially known. Metformin is not metabolized and, consequently, its target tissues are identified by the cellular uptake and accumulation of the drug [2]. In mice

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administered therapeutic doses of metformin orally (100–300 mg/kg/day), the drug is absorbed and delivered by the portal vein to the liver, where it accumulates, reaching up to 1 mM concentrations [2–6]. In the liver, metformin attenuates hepatic glucose production by inhibiting mitochondrial glycerol-3-phosphate dehydrogenase, and by activating AMP-activated protein kinase (AMPK), which inhibits the expression of gluconeogenic genes [1,7,8]. While pharmacological concentrations of metformin directly activate AMPK in the liver by promoting the formation of the heterotrimeric complex of this kinase and through Thr^{172} phosphorylation of AMPKα, supra-pharmacological concentrations (~5 mM) activate AMPK indirectly by the inhibition of complex I of the mitochondrial electron transport chain [1,9].

Although the liver is considered the primary target organ for the glucose-lowering effects of metformin, the beneficial effects of this antidiabetic drug have been extended to other organs, including the intestines, kidney and muscle [2,9,10]. Skeletal muscle, which accounts for most of the insulin-stimulated glucose use [11], also takes up and accumulates metformin [2]. This is consistent with the reported activation of AMPK by metformin in the skeletal muscle of patients with type 2 diabetes [12].

In 2017, a clinical trial revealed that metformin increased the serum levels of growth differentiation factor 15 (GDF15), a divergent member of the transforming growth factor β (TGFβ) superfamily, in a dose-dependent manner, making it a suitable biomarker for the response to this drug [13]. More recently, two studies reported that the increase in GDF15 levels caused by metformin was mediated by the upregulation of C/EBP homologous protein (CHOP), providing a new mechanism by which metformin lowers body weight [10,14]. In fact, GDF15 is a stress response cytokine that regulates appetite and metabolism, thereby impacting obesity, diabetes and non-alcoholic fatty liver disease (NAFLD) [15]. Initially, it was proposed that TGFβ receptors were mediating GDF15 effects, but the lack of binding of this cytokine to these receptors was then documented, which led to explore other receptors. The presence of TGFβ contamination in the first purified GDF15 batches from mammalian cell culture might have affected the interpretation of some in vitro studies [16]. More recent studies have demonstrated that GDF15 signals via the glial cell line-derived neurotrophic factor (GDNF) family receptor α-like (GFRAL) in the hindbrain [17–19]. Metformin-induced increase in GDF15 suppresses appetite and promotes weight loss through GFRAL [10,14]. However, some recent studies indicate that the anorectic effect of exogenous administered GDF15 via GFRAL occurs only at high pharmacological doses, while physiological increases (2–3-fold) of endogenous GDF15 levels (basal values of ~50 pg/mL in mice) do not suppress acute food intake [20]. In fact, a GDF15 dose of 20 μg/kg resulted in serum levels of this cytokine of ~1500 pg/mL that reduced food intake, whereas a lower dose of GDF15 of 2 μg/kg had no effect on food intake at any of the time points studied [21]. In addition, we have recently reported that GDF15 activates AMPK in cultured myotubes and isolated skeletal muscle that do not express GFRAL [22], thereby suggesting GFRAL independent GDF15 signaling in muscle cells. In this work, we examined whether the increase in GDF15 caused by metformin contributes to the activation of AMPK independently of GFRAL.

2. Materials and methods

2.1. Mice and in vivo studies

Male *Gdf15⁻/⁻* and wild-type (WT) mice (10–12 weeks old, C57BL/6/129/SvJ background), obtained from the Johns Hopkins University School of Medicine, were randomly distributed into three experimental groups: standard diet plus one daily oral gavage of vehicle (0.5% w/v carboxymethylcellulose); Western-type high-fat diet (HFD, 45% kcal from fat, product D12451, Research Diets Inc.) plus one daily oral gavage of vehicle; and HFD plus one daily oral dose of 100 mg/kg of metformin dissolved in the vehicle. At the end of the 3-week treatment,

the mice were sacrificed, and skeletal muscle (gastrocnemius) and liver samples were frozen in liquid nitrogen and then stored at − 80ºC.

For the glucose tolerance test (GTT), animals fasted for 6 h received 2 g/kg of body weight of glucose by an intraperitoneal injection and blood was collected from the tail vein after 0, 15, 30, 60 and 120 min. Animal experimentation complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition: National Academies Press; 2011). All procedures were approved by the Bioethics Committee of the University of Barcelona, as stated in Law 5/21 July 1995 passed by the Generalitat de Catalunya. The animals were treated humanely, and all efforts were made to minimize both animal numbers and suffering.

2.2. Serum GDF15 levels

Serum GDF15 levels were analyzed using an ELISA kit, according to the manufacturer's instructions (Biorbyt catalog orb391081).

2.3. Liver histology

For histological staining studies, liver sections were collected in 4% paraformaldehyde in PBS (for hematoxylin and eosin staining) or embedded in a cryomold containing optimal temperature cutting (OCT, Tissue-Tek) compound for cryopreservation and later cryosectioning to perform Oil Red O (ORO) staining. Microscopy images of ORO stained histological sections were quantified using the IHC profiler plugin of the Image J software.

2.4. Cell culture

Mouse C2C12 myoblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 units/mL of penicillin and 50 mg/mL of streptomycin. When cells reached confluence, the medium was switched to the differentiation medium containing DMEM and 2% horse serum, which was changed every other day. After 4 more days, the differentiated C2C12 cells had fused into myotubes. These were incubated in serum-free DMEM in either the absence (control cells) or presence of metformin, A769662 (60 μM) (Tocris Bioscience, catalog 3336), compound C (30 μM) (Santa Cruz Biotechnology Inc., catalog sc-200689) or E. coli-derived mouse recombinant GDF15 protein (100 ng/mL) (R&D Systems, catalog 8944-GD). This E. coli derived-GDF15 does not increase p-SMAD2 [16], indicating the absence of TGFβ contamination in contrast to some batches of GDF15 derived from mammalian cell culture which have been reported to be contaminated with TGFβ resulting in increased p-SMAD2 [16].

Differentiated myotubes were transiently transfected with 70 nM of siRNA against AMPK1/2 (Santa Cruz Biotechnology Inc., catalog sc-45313), GDF15 (Santa Cruz Biotechnology Inc., catalog sc-39799), p53 (Santa Cruz Biotechnology Inc., catalog sc-29436), ATF3 (Santa Cruz Biotechnology Inc., catalog sc-29758), or control siRNA (Santa Cruz Biotechnology Inc., catalog sc-37007) in Opti-MEM medium (Thermo Fisher, MA), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) (7 μl per 1.5-mL well) according to the manufacturer's instructions. Compounds were tested after 24 h of transfection.

Lipid-containing media were prepared by conjugation of palmitic acid with fatty acid-free bovine serum albumin, as previously described [23].

Human Huh-7 hepatoma cells (kindly donated by Dr. Mayka Sanchez from the Josep Carreras Leukemia Research Institute, Barcelona) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, at 37 °C under 5% CO₂.

Primary mouse hepatocytes were isolated from non-fasting male C57BL/6 mice (10–12 weeks old) by perfusion with collagenase as described elsewhere [24]. siRNA transfections were performed with Lipofectamine 2000 as mentioned above.

2.5. Reverse Transcriptase-Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction

Isolated RNA was reverse transcribed to obtain 1 μg of complementary DNA (cDNA) using Random Hexamers (Thermo Scientific), 10 mM deoxynucleotide (dNTP) mix and the reverse transcriptase enzyme derived from the Moloney murine leukemia virus (MMLV, Thermo Fisher). The protocol was run in a thermocycler (BioRad) and consisted in a program with different steps and temperatures: 65 °C for 5 min, 4 °C for 5 min, 37 °C for 2 min, 25 °C for 10 min, 37 °C for 50 min and 70 °C for 15 min. The relative levels of specific mRNAs were assessed by real-time RT-PCR technique in a Mini-48 well T100™ thermal cycler (Bio-Rad) using SYBR Green Master Mix (Applied Biosystems), as previously described [23]. Briefly, samples contained a final volume of 20 μl, with 25 ng of total cDNA, 0.9 μM of primer mix and 10 μl of 2x SYBR Green master mix. The thermal cycler protocol for real time PCR included a first step of denaturation at 95 °C for 10 min and 40 repetitive cycles with three steps for denaturation, primer annealing and amplification: 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Primers sequences were designed using the Primer-BLAST tool (NCBI), based on the full mRNA sequences to find optimal primers for amplification and evaluated with the Oligo-Analyzer tool (Integrated DNA Technologies) to ensure optimal melting temperature (Tm) and avoid the formation of homo/hetero-dimers or nonspecific structures that can interfere with the interpretation of the results. The primer sequences were designed specifically spanning the junction between exons. The primer sequences used are provided in the Supplementary Table 1. Values were normalized to glyceraldehyde phosphate dehydrogenase (*Gapdh*) or adenine phosphoribosyltransferase (*Aprt*) expression levels, and measurements were done in triplicate. All expression changes were normalized to the untreated control.

2.6. Immunoblotting

Isolation of total protein extracts was performed as described elsewhere [25]. Immunoblotting was performed with antibodies against β-Actin (Sigma A5441), Akt (Cell Signaling Technology 9272), phosphorylated (p) Akt^{S473} (Cell Signaling Technology 9272), AMPK (Cell Signaling Technology 2532), pAMPK^{T172} (Cell Signaling Technology 2531), CHOP (Cell Signaling Technology 2895), GAPDH (MilliporeSigma MAB374), GDF15 (Santa Cruz Biotechnology Inc. sc-515675), IκBα (Santa Cruz Biotechnology Inc. sc-371), insulin receptor β (Cell Signaling Technology 3025), IRS-2 (Cell Signaling Technology 4502), lamin B (Santa Cruz Biotechnology Inc. sc-6216), NF-κB p65 (Santa Cruz Biotechnology Inc. sc-109), PGC-1α (Abcam ab54481), PPARα (Santa Cruz Biotechnology sc-1985), SOCS3 (Santa Cruz Biotechnology sc-9023), pStat3^{S727} (Cell Signaling Technology 9134), TBP(Santa Cruz Biotechnology Inc. sc-74596), α-tubulin (Sigma, T6074). Signal acquisition was performed using the Bio-Rad ChemiDoc apparatus and quantification of the immunoblot signal was performed with the Bio-Rad Image Lab software. The results for protein quantification were normalized to the levels of a control protein (GAPDH, α-tubulin or TBP) to avoid unwanted sources of variation.

2.7. Statistics

Results are expressed as the mean ± SEM. Significant differences were assessed by either Student's-t test or one-way and two-way ANOVA, according to the number of groups compared, using the GraphPad Prism program (version 9.0.2) (GraphPad Software Inc., San Diego, CA). When significant variations were found by ANOVA, the Tukey-Kramer post-hoc test for multiple comparisons was performed only if F achieved a p value < 0.05. Differences were considered significant at p < 0.05.

3. Results

3.1. Gdf15-knockout mice present an attenuated response to metformin

To examine whether the antidiabetic effects of metformin were dependent on GDF15, we used *Gdf15*-knockout (*Gdf15$^{-/-}$*) mice and their WT littermates as controls. The mice of both genotypes were fed a standard diet or a HFD and treated with metformin at 100 mg/kg/day or vehicle as control. This dose of metformin was chosen to be close to that given in human treatments/studies and was lower than that used in previous mouse studies, which causes weight loss through GFRAL signaling [10,14]. WT mice on the HFD showed a significantly increased body weight, but the low dose of metformin did not cause a significant reduction of body weight (Fig. 1a). In *Gdf15$^{-/-}$* mice, no significant differences in body weight were observed compared to WT mice (Fig. 1a). WT mice fed the HFD displayed increased epididymal white adipose tissue (eWAT) and this was attenuated by metformin treatment, whereas no changes in eWAT were observed in *Gdf15$^{-/-}$* mice (Fig. 1b). Likewise, metformin treatment did not affect cumulative caloric intake, which was higher for mice fed the HFD compared with mice fed the standard diet. For *Gdf15$^{-/-}$* mice fed the standard diet, there was a trend towards higher caloric intake compared with WT mice fed the same diet, which became significant at the 3-week time point (Fig. 1c). As expected, metformin ameliorated glucose intolerance in WT mice, but this effect was abolished in *Gdf15$^{-/-}$* mice (Fig. 1d). Interestingly, WT mice fed the HFD and treated with metformin had significantly higher levels of serum GDF15 than those fed the HFD and treated with vehicle (Fig. 1e).

In the liver, hematoxylin-eosin and Oil Red O (ORO) staining showed that the HFD caused significant hepatic lipid accumulation that was reduced by metformin treatment (Fig. 2a). Of note, hepatic steatosis was much higher in the HFD-fed *Gdf15$^{-/-}$* mice, and metformin treatment did not ameliorate it. In WT mice, metformin treatment increased the hepatic expression and protein levels of GDF15 (Fig. 2b-c). Metformin also increased the hepatic expression of genes involved in fatty acid β-oxidation (*Cpt1a*, *Acadm* and *Acox*), and reduced the expression of the pro-inflammatory cytokine gene *Il6* in WT mice. By contrast, these effects of metformin were attenuated or abolished in *Gdf15$^{-/-}$* mice (Fig. 2c). The HFD decreased hepatic phospho-AMPK levels in WT mice, but this reduction was prevented by metformin treatment (Fig. 2d). Remarkably, phospho-AMPK levels in the liver of *Gdf15$^{-/-}$* mice were lower than in the livers of their WT littermates, which is in line with the regulation of the activity of this kinase by GDF15. Accordingly, metformin did not increase the phosphorylated levels of AMPK in the liver of *Gdf15$^{-/-}$* mice.

Next, we examined the effects of the HFD and metformin on suppressor of cytokine signaling 3 (SOCS3), which is under the control of signal transducer and activator of transcription 3 (STAT3), and inhibits insulin signaling [26]. WT mice fed the HFD showed increased hepatic levels of phosphorylated STAT3 at Ser^{727}, indicating increased activity of this transcription factor, as confirmed by the higher protein levels of its target gene *Socs3* (Fig. 2d). These changes were prevented by metformin treatment in WT mice, but not in *Gdf15$^{-/-}$* mice. Consistent with the increase in the levels of SOCS3, which inhibits insulin signaling through several mechanisms, such as the degradation of insulin receptor substrate (IRS) [26], WT mice fed the HFD exhibited a reduction in hepatic IRS2 protein levels. Conversely, HFD-fed mice treated with metformin showed a marked increase in IRS2 protein levels, presenting higher values than control mice (Fig. 2d). Again, this beneficial effect of metformin was absent in *Gdf15$^{-/-}$* mice. Since CHOP is a key transcription factor upregulating GDF15 expression [27] and given that metformin has been reported to induce GDF15 by increasing CHOP levels [10,14], we examined the protein levels of this factor. WT mice fed the HFD showed increased hepatic CHOP protein levels, which is consistent with the presence of lipid-induced ER stress (Fig. 2d). However, metformin treatment reduced hepatic CHOP protein levels in WT mice fed the HFD, thereby suggesting that the increase in CHOP was not responsible for the
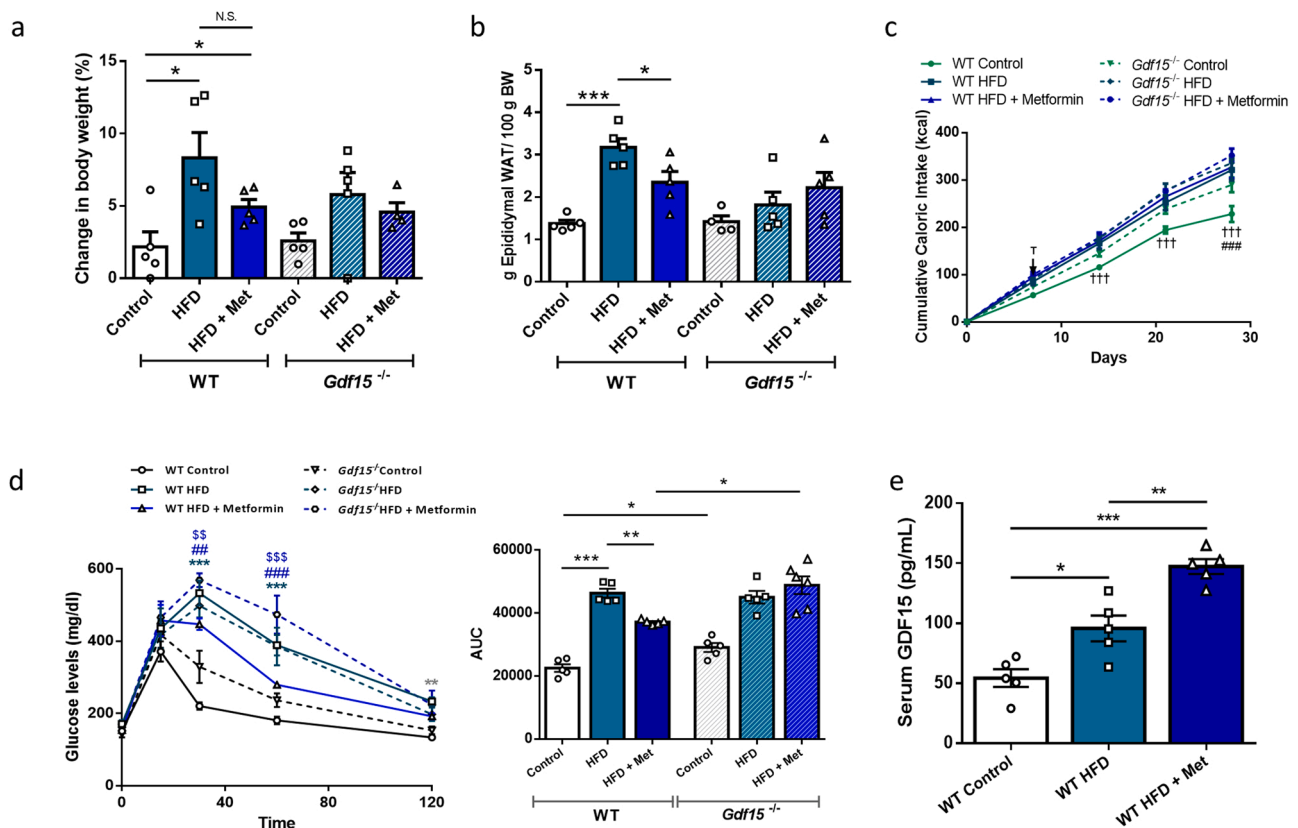
Fig. 1. Metformin antidiabetic effect is abolished in *Gdf15*^{-/-} mice. (**a**) Final body weight in wild-type (WT) and *Gdf15*^{-/-} mice fed a standard diet (control) or a high-fat diet (HFD) and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. (**b**) Epididymal adipose tissue. (**c**) Cumulative caloric intake. T: start of metformin treatment. [††]$p < 0.01$ and [†††]$p < 0.001$ for WT control vs all HDF fed groups of mice. [##]$p < 0.01$ for *Gdf15*^{-/-} control vs WT control. (**d**) Glucose tolerance test and area under the curve (AUC). (**e**) Serum GDF15 levels. n = 5 per group. Data are presented as the mean ± SEM. Significant differences were established by ANOVA. *$p < 0.05$, * *$p < 0.01$ and * * *$p < 0.001$.

upregulation of GDF15. This agrees with the findings of previous reports showing that AMPK activation [28,29] and metformin [30,31] decrease ER stress. A different trend was observed in *Gdf15*^{-/-} mice, in which metformin did not reduce CHOP protein levels. Overall, these findings indicate that metformin can increase hepatic levels of GDF15 independently of CHOP upregulation.

In the skeletal muscle of WT mice, metformin treatment increased *Gdf15* expression (Fig. 3a) and protein levels (Fig. 3c). This was accompanied by increased mRNA levels of genes involved in fatty acid β-oxidation, including *Cpt1b*, *Ppara* and *Acox* in WT mice. This effect of metformin was mitigated in *Gdf15*^{-/-} mice (Fig. 3a). As expected, the HFD increased the skeletal muscle expression of markers of both ER stress (*Bip/Grp78* and *Chop,* also known as *Ddit3*) and inflammation (*Mcp1,* also known as *Ccl2*), with metformin abrogating these increases in WT mice, but being ineffective in *Gdf15*^{-/-} mice (Fig. 3b). Remarkably, the HFD caused a larger increase in *Mcp1* mRNA levels in *Gdf15*-deficient mice than in WT mice (Fig. 3b). In the skeletal muscle of WT mice, metformin treatment increased the protein levels of phospho-AMPK, whereas this effect was absent in *Gdf15*^{-/-} mice (Fig. 3d). Similarly, the protein levels of PGC-1α and PPARα, which enhance energy metabolism, were increased by metformin only in WT mice (Fig. 3e). In line with the changes in the expression of the NF-κB target gene *Mcp1*, WT mice fed the HFD showed reduced IκBα protein levels, but increased levels of the p65 subunit of NF-κB (Fig. 3e). These changes were prevented by metformin in WT mice, but not in *Gdf15*^{-/-} mice. Metformin also increased the protein levels of IRβ in WT mice, whereas no such increase was observed in *Gdf15*^{-/-} mice (Fig. 3e). Finally, CHOP protein levels were increased by the HFD in WT mice, but metformin restored basal levels, indicating that the increased levels of this transcription

factor were not responsible for the increase in GDF15 levels caused by metformin in skeletal muscle (Fig. 3e). This effect of metformin on CHOP was absent in mice lacking *Gdf15* (Fig. 3e). Altogether, these findings suggest that the increase in GDF15 levels caused by metformin in the liver and skeletal muscle does not depend on CHOP upregulation. Furthermore, the effects of metformin on AMPK activation, fatty acid oxidation, ER stress, inflammation and insulin signaling require the presence of GDF15.

3.2. Metformin increases GDF15 levels through AMPK in cultured myotubes and hepatocytes

To establish the pathway involved in GDF15 upregulation by metformin and the role of this cytokine in AMPK activation, C2C12 myotubes were treated with different concentrations of metformin. *Gdf15* mRNA levels were significantly increased at metformin concentrations of 1 mM and 2 mM, and GDF15 secretion into the culture media at 2 mM (Fig. 4a-b). This is consistent with the fact that approximately 2 mM metformin is required to maximally elevate the AMP/ATP ratio for AMPK activation [7]. However, this concentration is lower than the 5 mM required to inhibit mitochondrial respiratory chain complex I in vitro [1]. In agreement with the uptake and accumulation of metformin by the skeletal muscle [2], myotubes exposed to 0.5 mM metformin for 3 days showed increased *Gdf15* expression (Fig. 4c). Moreover, knockdown of the AMPKα1/α2 subunits by siRNA transfection in C2C12 myotubes (Supplementary Fig. 1a and b), completely abolished the increase in *Gdf15* mRNA levels caused by metformin, indicating that this effect was mediated by AMPK (Fig. 4d). p53 is another transcription factor known to control GDF15 expression [22,29]. However, it is
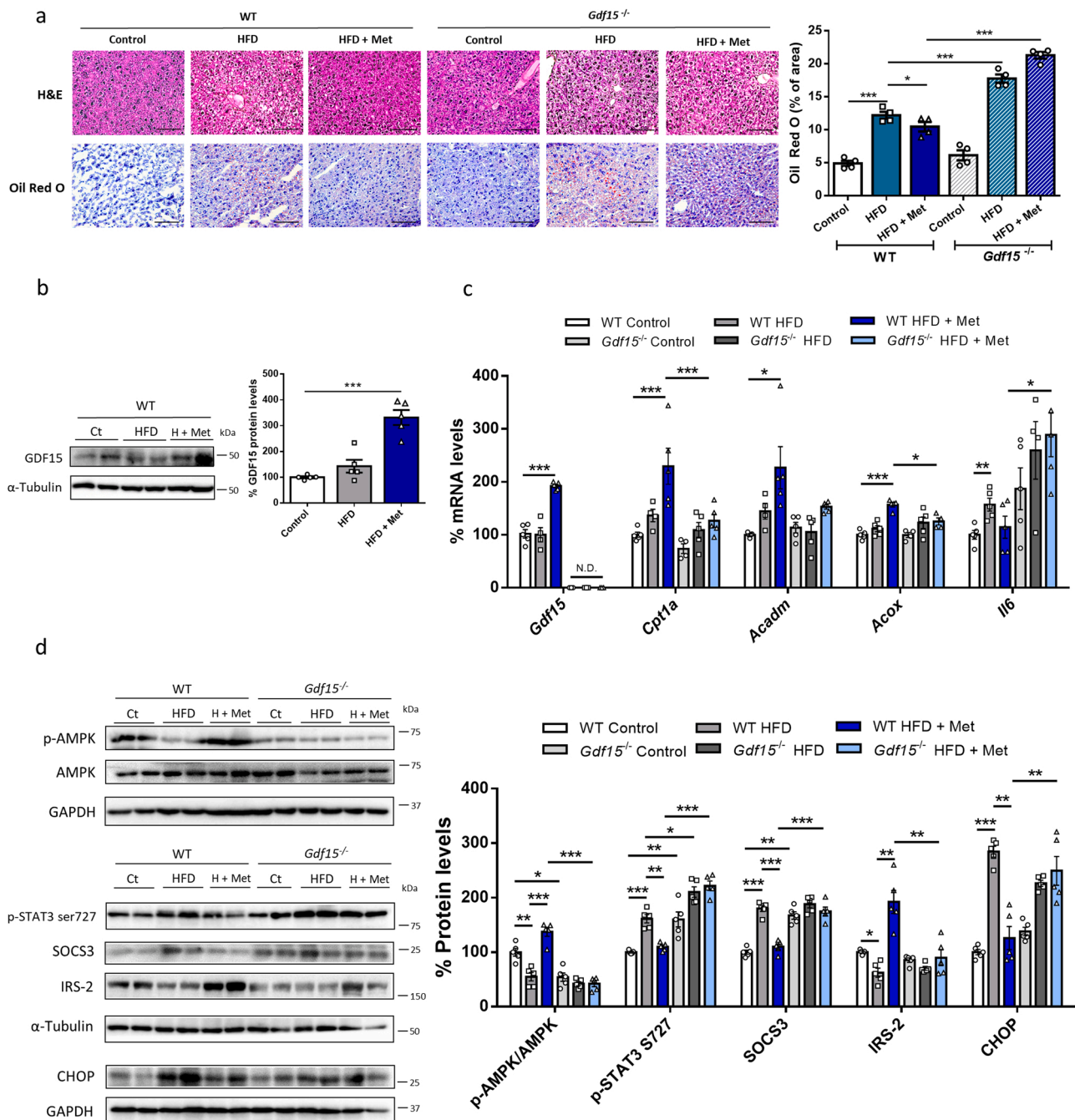
**Fig. 2.** Metformin activates AMPK in the livers of wild-type mice, but not in those of *Gdf15*⁻/⁻ mice. (**a**) Hematoxylin–eosin (H&E) and Oil Red O (ORO) staining of liver sections and quantification of ORO staining. Scale bar: 100 μm. (**b**) Immunoblot analysis of GDF15 in the liver of WT and *Gdf15*⁻/⁻ mice fed a standard diet (control) or a high-fat diet (HFD) and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. (**c**) mRNA levels of *Gdf15, Cpt1a*, *Acadm*, *Acox* and *Il6* in the liver. (**d**) Immunoblot analysis of total and phospho-AMPK, total and phospho-STAT3 (Ser⁷²⁷), SOCS3, IRS2, and CHOP in the liver. (a-d) n = 5 per group. Data are presented as the mean ± SEM. *p < 0.05, * *p < 0.01 and * **p < 0.001.

unlikely to be involved in the observed increase in *Gdf15* expression since knockdown of p53 did not affect metformin-induced stimulation of *Gdf15* (Supplementary Fig. 1c and d). Activation transcription factor 3 (ATF3) has been reported to be necessary for the upregulation of *Gdf15* by green tea polyphenols that are also AMPK activators [32], via an ATF3 binding site in its promoter [33]. Since metformin also increases ATF3 levels [30], we next explored the possible involvement of this transcription factor in metformin-mediated *Gdf15* upregulation. *Atf3* knockdown in myotubes (Supplementary Fig. 1e and f) significantly attenuated the increase caused by metformin in *Gdf15* expression, confirming the participation of ATF3 in the regulation of *Gdf15* (Fig. 4e).

In addition, metformin increased *Atf3* mRNA at 2 mM concentration in myotubes (Fig. 4f) and this increase was attenuated by knockdown of the AMPKα1/α2 subunits (Fig. 4 g; Supplementary Fig. 1a and b). A concentration-response study in the Huh-7 human hepatoma cell line showed that metformin also increased *GDF15* expression in cells of liver origin (Fig. 4h). Consistent with the increase of *GDF15* expression in Huh-7 cells caused by metformin at 2 mM, this drug also increased the expression of *ATF3* at this concentration (Fig. 4i). In a mouse primary culture of hepatocytes, metformin also increased *Gdf15* expression (Fig. 4j), whereas AMPKα1/α2 subunits knockdown (Supplementary Fig. 1g) decreased *Gdf15* mRNA levels in vehicle exposed cells and
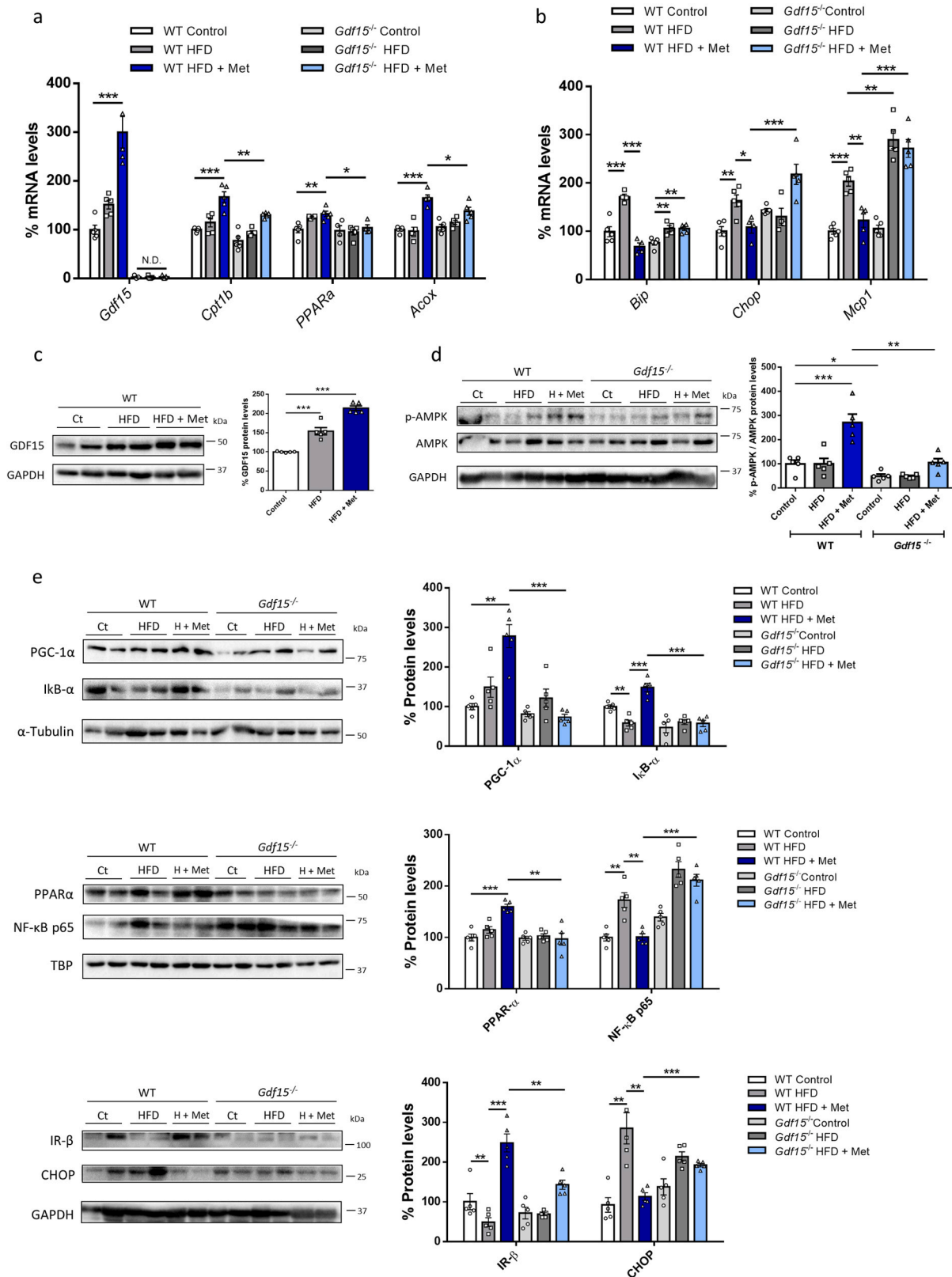
Fig. 3. Metformin increases *Gdf15* levels independently of CHOP and activates AMPK in the skeletal muscle of WT mice, but not *Gdf15−/−* mice. (**a**) mRNA levels of *Gdf15, Cpt1b, Ppara and Acox* in the skeletal muscle of wild-type (WT) and *Gdf15−/−* mice fed a standard diet (control) or a high-fat diet (HFD) and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. (**b**) mRNA levels of *Bip, Chop* and *Mcp1* in skeletal muscle. (**c**) Immunoblot analysis of GDF15 in skeletal muscle. (**d**) Immunoblot analysis of total and phospho-AMPK in skeletal muscle. (**e**) Immunoblot analysis of PGC-1α, IκBα, PPARα, the p65 subunit of NF-κB, IRβ and CHOP in skeletal muscle. (a-e) n = 5 per group. Data are presented as the mean ± SEM. *p < 0.05, * *p < 0.01 and * **p < 0.001.
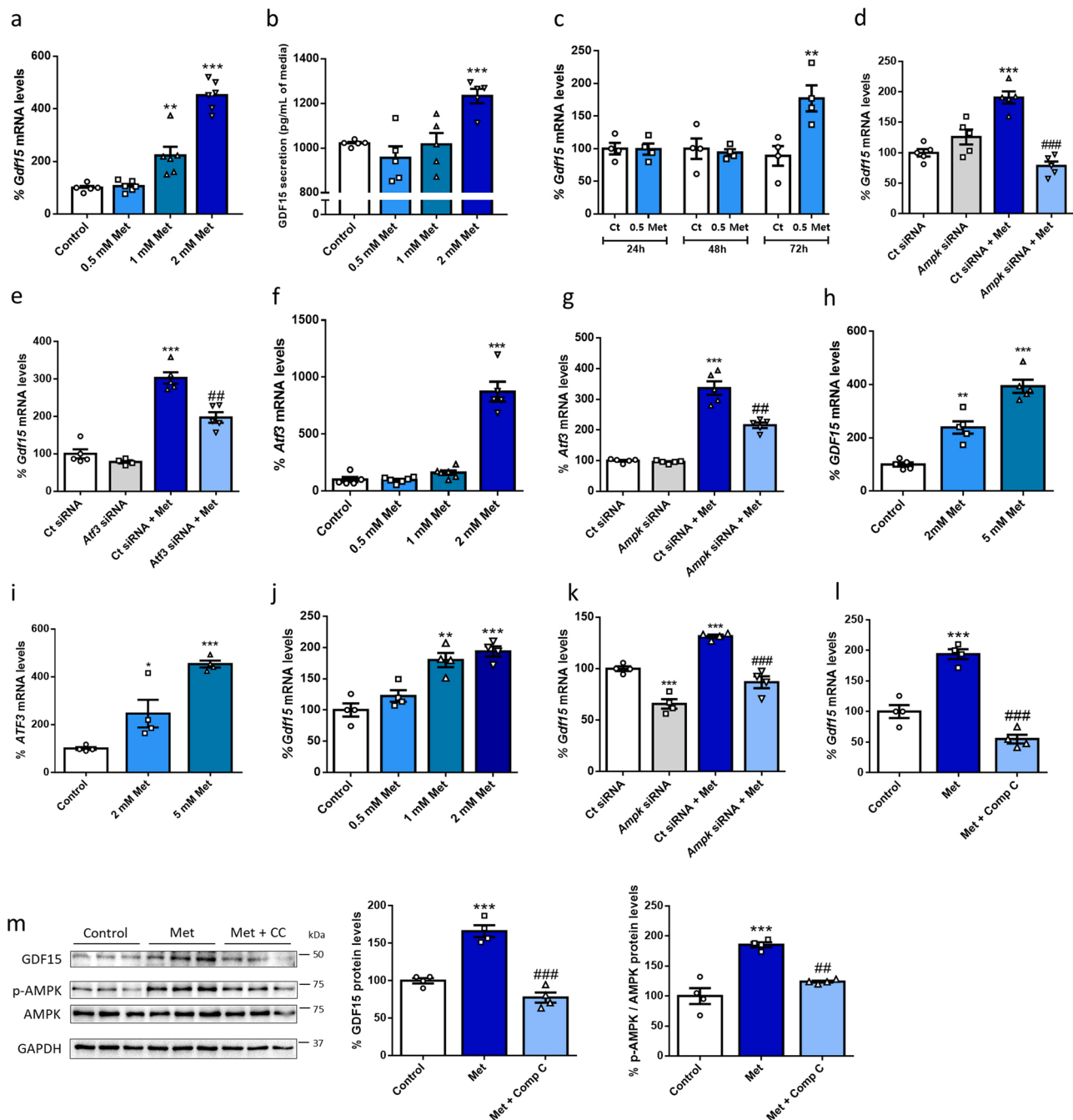
Fig. 4. Metformin increases GDF15 levels via AMPK activation. (**a**) *Gdf15* mRNA levels, and (**b**) GDF15 secreted into the culture medium in C2C12 myotubes exposed to different concentrations of metformin (Met) for 24 h. (**c**) *Gdf15* mRNA levels in C2C12 myotubes exposed to 0.5 mM metformin (Met) for 24, 48 or 72 h or (**d**) transfected with control (Ct) (scrambled) siRNA or AMPKα1/α2 siRNA and treated with 2 mM metformin for 24 h. (**e**) *Gdf15* mRNA levels in C2C12 myotubes transfected with control (Ct) (scrambled) siRNA or ATF3 siRNA and treated with 2 mM metformin for 24 h. (**f**) *Atf3* mRNA levels in C2C12 myotubes exposed to different concentrations of metformin (Met) for 24 h or (**g**) transfected with control (Ct) (scrambled) siRNA or AMPKα1/α2 siRNA and treated with 2 mM metformin for 24 h. (**h**) *GDF15* mRNA levels in Huh-7 human hepatoma cells exposed to different concentrations of metformin (Met) for 24 h. (**i**) *ATF3* mRNA levels in Huh-7 cells exposed to different concentrations of metformin (Met) for 24 h. (**j**) *Gdf15* mRNA levels in mouse primary hepatocytes exposed to different concentrations of metformin (Met) for 24 h or (**k**) transfected with control (Ct) (scrambled) siRNA or AMPKα1/α2 siRNA and treated with 2 mM metformin for 24 h. (**l**) *Gdf15* mRNA levels in mouse primary culture of hepatocytes exposed to 2 mM metformin (Met) for 24 h in the presence or absence of 30 μM compound C (Comp C). (**m**) Immunoblot analysis of GDF15, and total and phospho-AMPK in C2C12 in mouse primary culture of hepatocytes exposed to 2 mM metformin (Met) for 24 h in the presence or absence of 30 μM compound C (Comp C). (a,b,d-h) n = 5 per group. (c,i-l) n = 4 per group. Data are presented as the mean ± SEM. * *p < 0.01 and * **p < 0.001 vs. control. ###p < 0.001 vs. Ct siRNA + Met or Met.

prevented *Gdf15* upregulation in cells exposed to metformin (Fig. 4k). Likewise, the AMPK inhibitor compound C prevented the increase in *Gdf15* mRNA levels caused by metformin in mouse primary hepatocytes (Fig. 4l). In fact, compound C blocked both the phosphorylation of AMPK and the increase in GDF15 protein levels caused by metformin in these primary hepatocytes (Fig. 4m). On the contrary, treatment of primary hepatocytes with the AMPK activator A769662 increased the mRNA levels of GDF15. This effect was blocked by co-incubating the

cells with compound C (Supplementary Fig. 1h). Overall, these findings confirm that metformin increases GDF15 levels through AMPK phosphorylation by a mechanism involving *ATF3* upregulation.

3.3. Gdf15 knockdown attenuates metformin-mediated AMPK activation in the absence of GFRAL

A previous study has reported that recombinant GDF15 upregulates the expression of genes involved in fatty acid β-oxidation, including *Pgc1a, Ppara, Cpt1a/b* and *Acadm* in the liver and skeletal muscle of obese mice [34]. To demonstrate that GDF15 regulates the expression of these genes in the absence of the central receptor GFRAL, whose expression is limited to hindbrain neurons [35], we treated cultured myotubes, in which *Gfral* expression was virtually absent [22], with recombinant GDF15. This cytokine increased the expression of *Cpt1b, Acadm* and *Acox*, whereas *Pgc1a* mRNA levels were not altered (Fig. 5a). In addition, recombinant GDF15 reduced the mRNA levels of the pro-inflammatory cytokines *Tnfa* and *Il6*, but no changes were observed in the endoplasmic reticulum (ER) stress marker *Trb3* (Fig. 5a). GDF15 also increased the protein levels of PGC-1α, PPARα, and the β unit of the
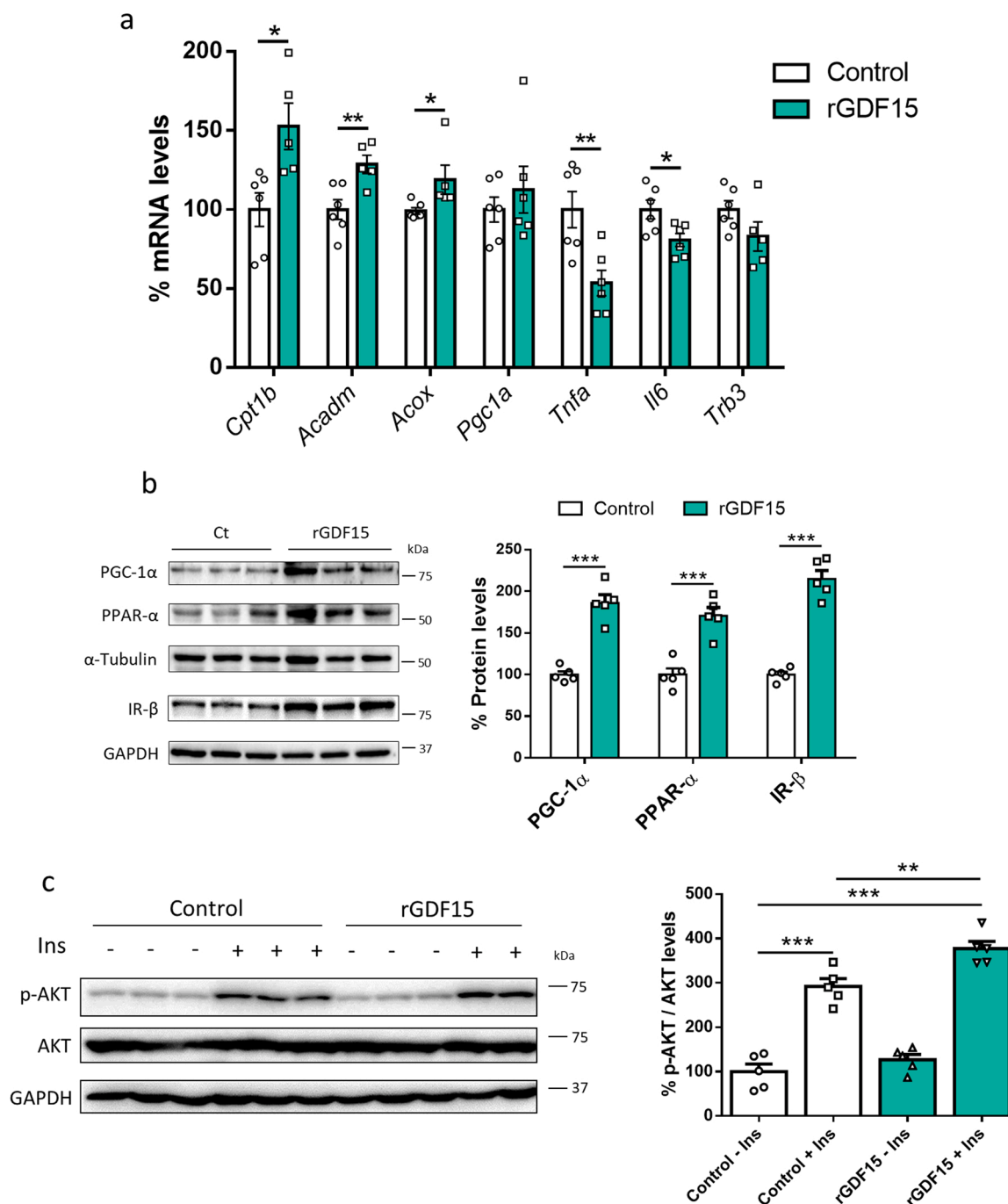
Fig. 5. GDF15 stimulates the insulin signaling pathway in cultured myotubes. (**a**) mRNA levels of *Cpt1b, Acadm, Acox, Pgc1a, Tnfa, Il6* and *Trb3* in C2C12 myotubes exposed to 100 ng/mL of recombinant GDF15 (rGDF15) for 24 h. (**b**) Immunoblot analysis of PGC-1α, PPARα, and IRβ in C2C12 myotubes exposed to 100 ng/mL of recombinant GDF15 (rGDF15) for 24 h. (**c**) Immunoblot analysis of total and phospho-Akt (Ser473) in C2C12 myotubes exposed to 100 ng/mL of recombinant GDF15 (rGDF15) for 24 h. Where indicated, cells were incubated with 100 nM of insulin (Ins) for the last 10 min n ≥ 5 per group. Data are presented as the mean ± SEM. *p < 0.05, * *p < 0.01 and * **p < 0.001.

insulin receptor (IRβ), thereby suggesting that it can influence insulin signaling in addition to fatty acid oxidation (Fig. 5b). In fact, GDF15 treatment significantly increased insulin-stimulated Akt phosphorylation in myotubes compared to non-exposed cells (Fig. 5c). Collectively, these data indicate that GDF15 has peripheral effects on the levels of proteins involved in fatty acid β-oxidation and in insulin signaling in cultured myotubes, and these effects are not dependent on GFRAL signaling, but the signaling pathway involved remains to be identified.

Next, we evaluated whether metformin contributed to AMPK activation and the regulation of proteins involved in lipid metabolism and insulin signaling in cultured myotubes, which, as detailed above, lack GFRAL. First, *Gdf15* was knocked down by siRNA transfection (Supplementary Fig. 2a), which blunted the phosphorylation of AMPK by metformin, indicating that GDF15 is required for metformin-mediated AMPK activation (Fig. 6a). Similarly, *Gdf15* knockdown partially prevented the increase in *Pgc1α* expression and abrogated the increase in *Acox* expression and the decrease in *Mcp1* expression caused by metformin (Fig. 6b). Second, it is well established that the saturated fatty acid palmitate lowers PGC-1α and phospho-AMPK levels and increases inflammation in myotubes and skeletal muscle [36]. In line with this, incubation of myotubes with palmitate decreased *Pgc1α* mRNA levels, but co-incubation with metformin caused a notable increase in the expression of this gene, which was attenuated by *Gdf15* knockdown (Fig. 6c, Supplementary Fig. 2b). The induction of *Il6* expression caused by palmitate was mitigated by co-treatment with metformin, whereas *Gdf15* knockdown significantly increased the expression of *Il6* in cells exposed to palmitate and in those co-incubated with metformin (Fig. 6c). Palmitate also reduced phospho-AMPK levels, whereas treatment with metformin caused a large increase in the phosphorylated levels of this kinase that was attenuated by *Gdf15* knockdown (Fig. 6d, Supplementary Fig. 2c). The increase in the protein levels of PPARα, IRβ and the NF-κB inhibitor IκBα caused by metformin was also prevented by *Gdf15* knockdown (Fig. 6d). Finally, the reduction in PGC-1α protein levels caused by palmitate was prevented by metformin treatment, but this effect of metformin was abolished by *Gdf15* knockdown (Fig. 6d). These findings indicate that GDF15 upregulation by metformin contributes to AMPK activation and impacts the levels of proteins involved in fatty acid oxidation, inflammation, and insulin signaling.

4. Discussion

AMPK is a central regulator of multiple metabolic pathways and is of therapeutic interest for the treatment of insulin resistance, type 2 diabetes mellitus, NAFLD, and cardiovascular disease [37]. This kinase is a target for metformin, the first-line drug in the treatment of type 2 diabetes mellitus. How metformin activates this kinase remains only partially understood [1]. Here, we show that AMPK activation by metformin increases the levels of GDF15, which in turn is required to promote the activation of AMPK through a positive feedback loop. *Gdf15*⁻/⁻ mice showed reduced hepatic phospho-AMPK levels and the enhanced phosphorylated levels of this kinase caused by metformin in WT mice was not observed in *Gdf15*⁻/⁻ mice. In addition, many of the AMPK-mediated effects of metformin on the levels of proteins involved in fatty acid oxidation, inflammation, ER stress and insulin signaling were attenuated or abolished by *Gdf15* knockdown.

The involvement of AMPK in the metformin-mediated increase in GDF15 levels provides a new mechanism in addition to those recently reported in studies showing that metformin upregulates GDF15 by increasing CHOP levels [10,14]. Since these later studies used high doses of metformin (250–600 mg/kg/day) that can inhibit complex I of the mitochondrial electron transport chain resulting in mitochondrial stress, which increases GDF15 levels through CHOP [38], we speculated that an alternative mechanism may exist by which lower doses of metformin could increase GDF15 levels. To follow up on our previous study [22] showing that PPARβ/δ ligands increased GDF15 via AMPK, we focused on possible upregulation of GDF15 by metformin via AMPK. We also

used a low dose of metformin (100 mg/kg/day), which is closer to the dose utilized in human studies (~35 mg/kg/day) [39].

Previous studies [10,14] have reported that the reduction in body weight promoted by metformin occurred via an increase in GDF15 levels and the subsequent activation of GFRAL, a receptor solely expressed in the hindbrain that suppresses appetite. Consistent with this, a reduction in food intake was observed in mice treated with metformin (250–600 mg/kg/day) [10,14]. In our current study, the administration of metformin at a dose of 100 mg/kg/day to WT mice did not affect cumulative food intake, which agrees with a recent previous study [40], suggesting that GFRAL was not involved in the effects we observed. The observed differences in the effects of metformin on food intake might result from disparate levels of serum GDF15 achieved according to the doses of metformin administered. In accordance with this proposal, Coll et al. (2020) reported that 300 mg/kg/day metformin resulted in serum GDF15 levels of ~750 pg/mL and reduced food intake [10], while in our study metformin at 100 mg/kg/day led to serum GDF15 levels of ~150 pg/mL with no change in food intake. Consistent with these observations, it has been suggested that the anorectic action of GDF15 requires a threshold of circulating levels of this cytokine of ≥ 400 pg/mL [41]. Interestingly, and in contrast to previous studies [10,14], a recent report showed that the increase in GDF15 caused by metformin reduced body weight independently of the GFRAL pathway [40], while in another study, a 2.5-fold increase in plasma GDF15 levels caused by the antitumor drug camptothecin lowered food intake and body weight through GFRAL [42]. The reasons for the apparently different pathways of GDF15-mediated anorectic effects are unknown, but several factors may contribute, including the primary source of circulating GDF15, the type of HFD feeding, which influences the effect of metformin on plasma GDF15 levels, the time point of the light phase selected for the administration of the drug, and, given the short-life of GDF15, the time elapsed between the last drug administration and the analysis of plasma GDF15.

Although in our study metformin did not affect food intake, it elicited an important improvement in metabolic parameters and reduced the amount of epididymal fat. This reduction in fat mass caused by metformin is consistent with the increase in the expression of genes involved in fatty acid oxidation in the liver and skeletal muscle, which may reduce the amounts of fatty acids for storage, as suggested by the reduction in hepatic triglyceride levels. In fact, our experiments using isolated cells, without the influence of central GFRAL, suggest that both metformin and GDF15 increase the expression of the above-mentioned genes by signaling through peripheral receptors whose nature remains to be determined (see below). These peripheral receptors would mediate autocrine and paracrine effects induced by modest increases in GDF15 expression caused by metformin in different tissues, including skeletal muscle (this manuscript), kidney and duodenum [40] with only modest increases in plasma GDF15. This process would allow GDF15 to affect energy balance independently of GFRAL. It is important to note that feeding a HFD for 18 weeks increased the expression of *Gdf15* in the liver, brown and epididymal adipose tissue, but not in skeletal muscle, kidney or subcutaneous adipose tissue [27]. In the same study, feeding mice with a HFD for 16 weeks increased plasma GDF15 levels from 100 to approximately 300 pg/mL, but this increase was not sufficient to prevent the development of obesity and glucose intolerance [27]. In contrast, administration of metformin increased *Gdf15* expression in the liver, in skeletal muscle (this manuscript), gut and kidney [40]. As already mentioned above, this suggests that the drug-induced upregulation of GDF15 in some tissues could affect metabolism via autocrine/paracrine signaling through peripheral receptors. Since we have not examined the effects of metformin in the gut and kidney, we cannot dismiss that a possible upregulation of GDF15 in these organs may also contribute to the effects of metformin.

In this study, we have observed that the beneficial effect of 100 mg/kg/day metformin on plasma glucose levels measured in the GTT in wild-type mice was abolished in *Gdf15*⁻/⁻ mice. However, other studies have reported that metformin doses of 300 and 400 mg/kg/day, but not
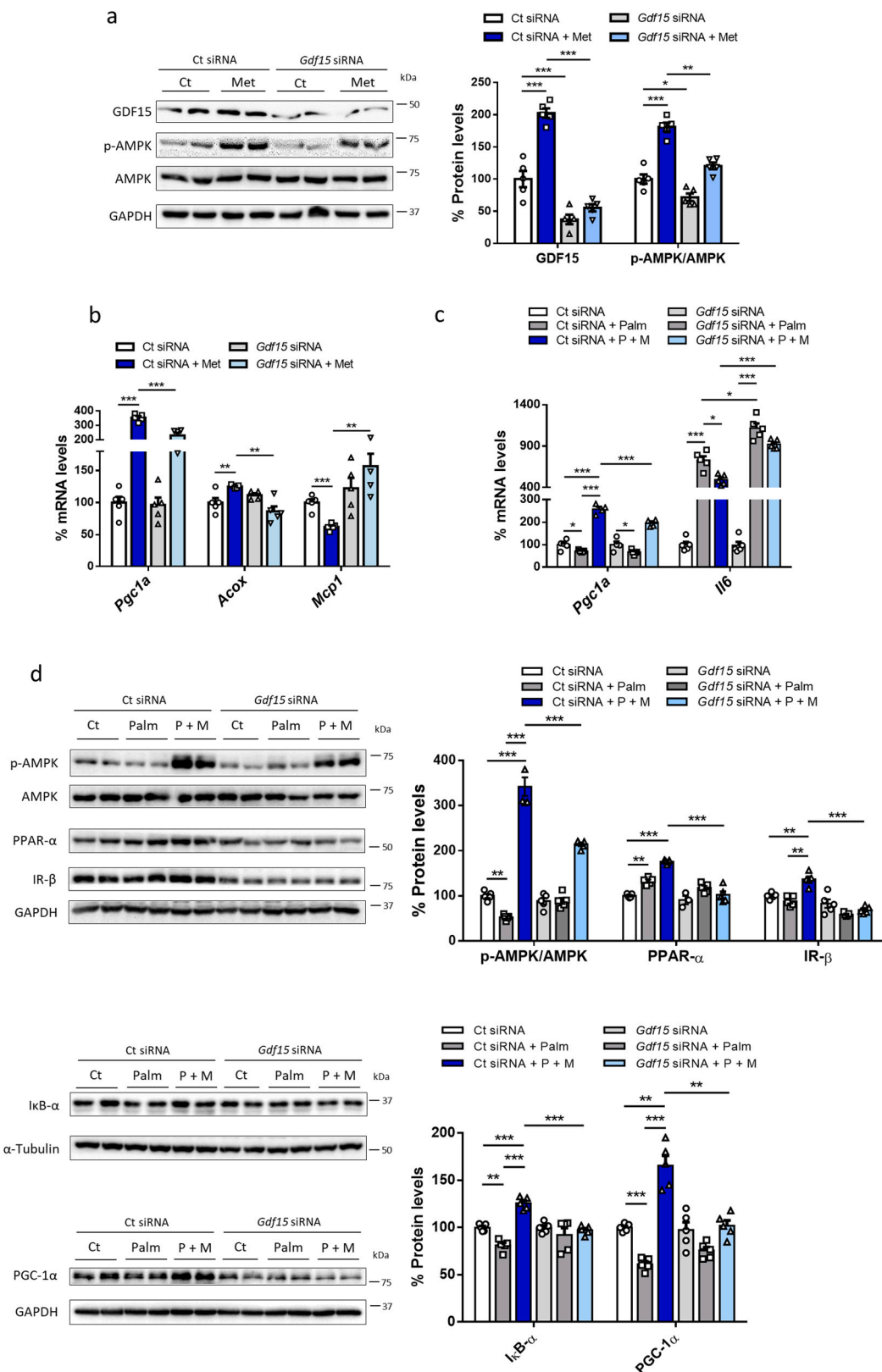
Fig. 6. *Gdf15* knockdown prevents metformin-mediated AMPK activation. (**a**) Immunoblot analysis of GDF15, and total and phospho-AMPK in C2C12 myotubes transfected with control (Ct) (scrambled) siRNA or GDF15 siRNA and treated with 2 mM metformin for 24 h. (**b**) mRNA levels of *Pgc1a, Acox* and *Mcp1* in C2C12 myotubes transfected with control (Ct) (scrambled) siRNA or GDF15 siRNA and treated with 2 mM metformin for 24 h. (**c**) mRNA levels of *Pgc1a* and *Il6* in C2C12 myotubes transfected with control (Ct) (scrambled) siRNA or GDF15 siRNA and exposed to 0.5 mM palmitate or palmitate plus 2 mM metformin for 24 h. (**d**) Immunoblot analysis of total and phospho-AMPK, PPARα, IRβ, IκBα and PGC-1α in C2C12 myotubes transfected with control (Ct) (scrambled) siRNA or GDF15 siRNA and exposed to 0.5 mM palmitate or palmitate plus 2 mM metformin for 24 h. (a,b) n = 5 per group. (c,d) n = 4–5 per group. Data are presented as the mean ± SEM. *p < 0.05, * *p < 0.01 and * **p < 0.001.

a 200 mg/kg/day dose, improved glucose levels in both wild-type and *Gdf15*$^{-/-}$ mice [40]. Together these findings indicate that metformin influences glucose levels through GDF15-dependent and independent mechanisms. The GDF15-independent effects are likely to rely on direct or indirect activations of AMPK by metformin. Further studies are warranted to clarify the points and discrepancies addressed above.

Day et al. (2019) showed that metformin increased GDF15 secretion in primary hepatocytes from AMPKβ1$^{-/-}$ mice, suggesting that AMPK activation by metformin was not responsible for the increase in GDF15 levels. However, in our study, knockdown of the AMPKα subunit in primary hepatocytes and myotubes prevented the increase in GDF15 levels caused by metformin, suggesting that this drug increases GDF15 levels through AMPK. Again, we do not know the reasons for these discrepancies. However, in support of the proposition that GDF15 is regulated by AMPK, a recent study showed that activation of AMPK by A769662 increased hepatic GDF15 levels independently of CHOP [43]. In the same study, pharmacological activation of AMPK ß1-containing complexes induced GDF15 levels, whereas liver *Gdf15* expression and serum GDF15 levels were attenuated in mice lacking AMPK ß1-containing complexes [43]. Likewise, we have recently reported that agonists of PPARβ/δ increase GDF15 by activating AMPK [22]. Furthermore, we observed that phospho-AMPK levels were reduced in the liver and skeletal muscle of mice lacking *Gdf15*, confirming the activation of AMPK by GDF15.

Although the increase in *Gdf15* expression caused by metformin has been reported to be dependent on CHOP [10,14], administration in our study of a lower dose of metformin to WT mice did not increase CHOP protein levels as caused by the HFD in the liver and skeletal muscle. These observations make unlikely that, in our conditions, CHOP upregulation is involved in the increase in GDF15, in line with the fact that gut organoids from *Chop* knockout mice still secrete GDF15 in response to metformin stimulation [10].

Although it has been reported that AMPK activation upregulates p53 [44], a transcription factor reported to regulate *Gdf15* expression [45], the present findings discard the involvement of p53 in metformin-mediated upregulation of GDF15, establishing a distinction with the pathway used by PPARβ/δ agonists that increase GDF15 via the AMPK-p53 axis [22]. By contrast, our current findings suggest that AMPK activation increases the expression levels of *Atf3*, which in turn upregulates the expression of *Gdf15*. In fact, ATF3 has been involved in the upregulation of GDF15 by non-steroidal anti-inflammatory drugs and natural compounds, such as resveratrol, green tea catechins and capsaicin [30,39,41,46].

Our current study also uncovers an important and to date unrecognized role of skeletal muscle in the action of metformin. Although the liver, the organ with the highest expression of GDF15 [27], is central for the effect of metformin, other tissues may also contribute to the effects of metformin with increased *Gdf15* expression. In fact, the lower small intestine and colon [10] and the kidney [40] have also been reported to be major sites of metformin-induced *Gdf15* expression. Despite the effects of metformin in skeletal muscle being previously disregarded [47], metformin activates AMPK in skeletal muscle [12] and increases insulin-stimulated glucose uptake in this tissue in patients with type 2 diabetes [48,49]. We show here that a low dose of metformin increased *Gdf15* expression in skeletal muscle, which was accompanied by an increased expression of the genes involved in fatty acid oxidation, and a decreased expression of ER stress and inflammatory markers. These autocrine and paracrine effects of GDF15 in skeletal muscle may contribute to the improvement of glucose intolerance and other metabolic disorders. This is further supported by the findings obtained in myotubes exposed to palmitate, where the beneficial effects of metformin were attenuated by *Gdf15* knockdown. In addition, a potential systemic effect of muscle derived GDF15, as previously reported [41], remains possible. Muscle derived GDF15 might result in autocrine and paracrine effects, which is consistent with our previous data [22], and implies that some of the peripheral metabolic effects of metformin

require the upregulation of *Gdf15* in this tissue. In agreement with this, *Gdf15* knockdown in myotubes attenuated the effects of metformin on AMPK activation as well as its effects on the proteins involved in fatty acid oxidation and inflammation.

A limitation of this work is that we cannot totally exclude that the activation of AMPK caused by metformin in vivo via GDF15 might involve GFRAL. Studies using neutralizing antibodies against GFRAL or knockout mice for this receptor will be needed to dismiss this possibility, given that in vivo studies provide a wider integrated biological system than in vitro studies. However, the in vitro findings of this study demonstrate that GDF15 induction by metformin contributes to the activation of AMPK and its metabolic effects in cultured myotubes, where *Gfral* expression is absent [22]. In addition, new studies are needed to uncover the new potential receptor responsible for the autocrine/paracrine effects of GDF15 in skeletal muscle. Likewise, the use of a single dose of metformin is also a limitation, since the use of higher doses may result in different effects, especially considering that high doses of metformin can inhibit the complex I of the mitochondrial electron transport chain [1,9].

The concentration of recombinant GDF15 used in in vitro experiments in this study is similar to that reported in previous studies [50]. Doses or concentrations of exogenous GDF15 used in many studies are 50- to 100-fold higher compared to endogenous GDF15 levels [51]. It has been speculated that endogenous GDF15 might be more potent, that it may act in concert with FGF21 [52] or it may function through alternative receptors [22,53], which remains to be elucidated.

Overall, the most remarkable and unexpected finding of this study is that metformin requires GDF15 to achieve full AMPK activation. Consistent with this, metformin increased phospho-AMPK levels in WT mice, but not in *Gdf15*$^{-/-}$ mice. In addition, *Gdf15* knockdown in myotubes also attenuated the increase in AMPK phosphorylation caused by metformin, while phospho-AMPK levels were reduced in *Gdf15*$^{-/-}$ mice compared to WT mice. Two mechanisms have been reported to date to explain the activation of AMPK by metformin: pharmacological concentrations of metformin directly activate AMPK, whereas supra-pharmacological concentrations (approximately 5 mM in in vitro studies) or high doses in vivo act indirectly by inhibiting the mitochondrial respiratory chain complex I, causing an increase in the AMP/ATP ratio, that in turn activates AMPK [1]. However, this inhibition of mitochondrial respiration by supra-pharmacological doses of metformin can result in mitochondrial stress and the subsequent activation of CHOP upregulation, leading to increased *Gdf15* expression. As illustrated in Fig. 7, our findings now suggest that AMPK activation by metformin increases the levels of GDF15, which in turn is involved in achieving and sustaining full AMPK activation through a positive feedback loop. This effect of GDF15 might be mediated through a yet to be determined peripheral receptor [22]. Interestingly, a recent study has reported that GDF15 acts in T cells through a previously unrecognized receptor, CD48, which is mainly expressed in lymphoid cells [54], but is not expressed in the cells and organs used in this study (data not shown). In addition, the anti-inflammatory effects of colchicine have been reported to be mediated by GDF15 through a new and unknown receptor on myeloid cells that requires further characterization [53]. These findings extend the signaling of GDF15 beyond GFRAL and indicate the presence of peripheral receptors, which remain to be discovered, contributing to mediate the effects of this cytokine.

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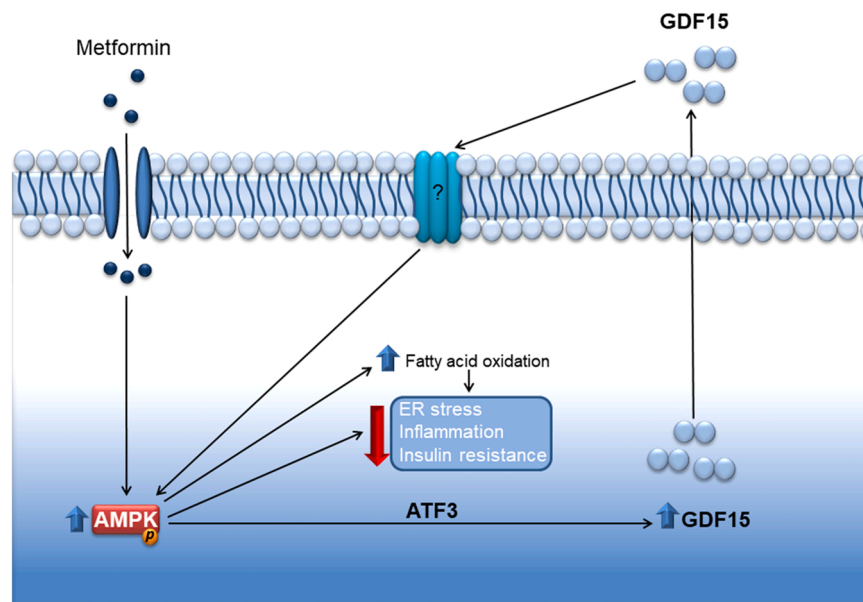
Fig. 7. GDF15 is required for the full activation of AMPK by metformin. AMPK activation by metformin increases the levels of GDF15, which in turn is involved in achieving and sustaining full AMPK activation through a positive feedback loop.

CRediT authorship contribution statement

DAR, EB, MZ, PR, JPD, LP, XP, AMV and MVC performed the experiments; DAR, XP, WW and MVC analyzed the data and reviewed the results; DAR and MVC designed the experiments and reviewed the results. MVC is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Declarations of interest

None

Data Availability

Data will be made available on request.

Acknowledgements

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106578.

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