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Targeting ERK3/MK5 complex for treatment of obesity and diabetes

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ABSTRACT

Kinases represent one of the largest druggable families of proteins. Importantly, many kinases are aberrantly activated/de-activated in multiple organs during obesity, which contributes to the development of diabetes and associated diseases. Previous results indicate that the complex between Extracellular-regulated kinase 3 (ERK3) and Mitogen-Activated Protein Kinase (MAPK)-activated protein kinase 5 (MK5) suppresses energy dissipation and promotes fatty acids (FAs) output in adipose tissue and, therefore promotes obesity and diabetes. However, the therapeutic potential of targeting this complex at the systemic level has not been fully explored. Here we applied a translational approach to target the ERK3/MK5 complex in mice. Importantly, deletion of ERK3 in the whole body or administration of MK5-specific inhibitor protects against obesity and promotes insulin sensitivity. Finally, we show that the expression of ERK3 and MK5 correlates with the degree of obesity and that ERK3/MK5 complex regulates energy dissipation in human adipocytes. Altogether, we demonstrate that ERK3/MK5 complex can be targeted *in vivo* to preserve metabolic health and combat obesity and diabetes.

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1. Introduction

Positive energy balance induces increased adiposity and a shift of the metabolic processes in the adipocytes favoring storage of the excessive energy in the form of triglycerides (TG). This causes adipocyte hypertrophy and hyperplasia and is often associated with the induction of systemic low-grade inflammation. Consequently, increased levels of lipids and inflammatory mediators-induced peripheral insulin resistance which in the long term can lead to type 2 diabetes (T2D). Impaired adipocyte function is one of the primary causes for the development of obesity and T2D [1]. At least three functionally different types of adipocytes have been described: white, beige, and brown adipocytes. The primary feature distinguishing brown and beige adipocytes from white adipocytes is their ability to dissipate energy in the form of heat [1]. Brown and beige adipocytes present also high mitochondrial content, activity, and dynamics, which allow them to efficiently burn fatty acids and glucose to produce energy. The primary mechanism utilized by both cell types to dissipate energy involves uncoupling protein 1 (UCP1), which dissociates the proton gradient generated during respiration from ATP synthesis and therefore promotes heat production [2]. Importantly, white and beige adipocytes can transdifferentiate into each other [2]. White adipocytes, despite their endocrine function, are primarily designated to store large quantities of energy in the form of TGs. At the time when food is available in excess, adipocytes convert glucose into fatty acids (FAs) in the process of lipogenesis and absorb FAs from the circulation to store them in lipid droplets in the form of TGs. Thus, strategies to increase the energy dissipation by adipose tissue and limit fat deposition in white adipocytes can potentially ameliorate obesity and improve glycemia in diabetic patients [3].

Classical members of Mitogen-Activated Protein Kinase (MAPK

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family (c-Jun N-terminal kinase 1, 2, 3, p38α, β, γ, δ as well as Extracellular-regulated kinase 1 and 2) have been broadly discussed in the context of metabolism regulation as well as in the development of obesity, diabetes and associated diseases [4,5]. Until recently, the role of atypical members of the MAPK family in metabolic homeostasis has been neglected [5]. In a high-throughput screen, we identified ERK3, also known as MAPK6, as a central regulator of lipolysis in adipocytes [6]. ERK3 is a constitutively active kinase; therefore, its abundance determines the rate of substrates phosphorylation [7,8]. In quiescent cells, ERK3 is subject to rapid proteasome-mediated degradation [7,8]. We proved that stabilization of ERK3 by β-adrenergic stimulation is indispensable for lipolysis. Mechanistically, in adipocytes ERK3 stabilization requires protein kinase A (PKA)-dependent translocation of the cofactor MAPK-activated protein kinase 5 (MK5), also known as PRAK, from the nucleus to the cytoplasm to regulate gene expression. Consistently, the elimination of ERK3/MK5 in adipocytes of mice inhibits lipolysis. Moreover, suppression of ERK3/MK5 action in adipocytes elevates energy dissipation [6]. These results indicate that ERK/MK5 complex might be potentially targeted to increase energy expenditure and ameliorate obesity. However, up to now, the impact of systemic inhibition or genetic ablation of components of ERK3/MK5 complex has not been explored.

Here we show that, inducible deletion of ERK3 in all tissues results in lower adiposity and improved metabolic parameters. In addition, targeting ERK3/MK5 complex with MK5 inhibitor (GLPG0259) in mice reduces adiposity and promotes insulin sensitivity, without causing any obvious side effects. Finally, targeting ERK3/MK5 complex in human adipocytes results in similar alterations as observed in murine adipocytes. Taken together, our data indicate that ERK3/MK5 complex can be targeted to preserve metabolic health, reduce adiposity and improve insulin sensitivity.

2. Results

2.1. Systemic deletion of ERK3 in mice protects against obesity and diabetes

Previous work indicate that systemic targeting ERK3/MK5-dependent signaling might protect against body weight gain [6]. To test this hypothesis, we generated mice with tamoxifen-inducible deletion of *Erk3* in all types of tissue (*Erk3*$^{global\ ind.\ \Delta/\Delta}$) [6,9] and fed these animals with a high-fat diet (HFD) to induce obesity. Of note, administration of tamoxifen resulted in the deletion of ERK3 in all the tissues tested and the mice depleted from ERK3 were viable and did not show any obvious phenotype (Supplementary Figs. 1A and B). At first, we induced deletion of *Erk3* directly at the start of the HFD (at six weeks of age). *Erk3*$^{global\ ind.\ \Delta/\Delta}$ mice gained less weight than corresponding control animals when fed HFD (Fig. 1A). Moreover, mice deficient in ERK3 presented better glucose and insulin tolerance, indicating that deletion for ERK3 protects not only against obesity but also against diet-induced diabetes (Supplementary Fig. 1C). To test if deletion of ERK3 can attenuate the course of already established obesity, we induced deletion of ERK3 in mice that were fed HFD for 8 weeks. Also in this case deletion of ERK3 resulted in decreased body weight gain and improved insulin sensitivity (Fig. 1B and C). Further analysis revealed that deletion of ERK3 results in attenuated fat deposition in the mice and reduced weight of different depots of adipose tissue (Fig. 1D and E). Consistently, the average size of the adipocytes was smaller in the absence of ERK3 (Fig. 1F–H). Moreover, in the mice deficient for ERK3 fewer lipid droplets were observed in the liver, indicating a reduced lipid deposition in this organ (Fig. 1I).

Taken together, these data indicate that ablation of ERK3-dependent signaling protects against obesity and diabetes by decreasing lipid accumulation. Taken together, we show that genetic or pharmacological targeting of ERK3/MK5 complex reduces lipid accumulation in and promotes energy dissipation protecting against obesity and diabetes.

2.2. Inhibition of MK5 suppresses lipid accumulation and promotes energy dissipation by adipocytes

The previous study [6] and our current results established that ERK3 promotes lipolysis. At the same time, previous studies indicate that ERK3 suppresses energy dissipation by these cells [6]. To test if the interaction partner of ERK3, MK5 also regulates these processes, and if this pathway could be targeted pharmacologically, we utilized the MK5-specific inhibitor, GLPG0259. Treatment of differentiated adipocytes with MK5 inhibitor for 16 h resulted in a dose-dependent attenuation of free fatty acids (FFAs) secretion, indicating a reduced lipolysis rate (Fig. 2A). Of note, the doses of inhibitor used in this experiment did not affect cell viability (Supplementary Fig. 2A). Moreover, treatment of adipocytes with GLPG0259 enhanced basal respiration of adipocytes and energy dissipation (Fig. 2B and C). Altogether, these results indicate that inhibition of MK5 in adipocytes recapitulates the phenotype observed in the absence of ERK3.

2.3. Administration of MK5-specific inhibitor (GLPG0259) protects against obesity and increases energy expenditure in mice

Our data indicate that inhibition of the activity of the ERK3/MK5 complex might protect against obesity. Up to date, a specific ERK3 inhibitor was not tested in living organisms. However, an inhibitor of MK5 has been proven to work effectively and safely in different clinical trials in humans [10]. We treated obese mice (in which obesity was induced by feeding with HFD), with GLPG0259 for seven weeks. This resulted in reduction of the phosphorylation of the MK5 downstream target, Heat shock protein 27 (HSP27) on serine 82 [11] in the white adipose tissue, confirming effective inhibition of this kinase (Fig. 3A). Importantly, animals treated with MK5 inhibitor gained significantly less weight than corresponding control animals (Fig. 3B) and presented improved insulin sensitivity (Fig. 3C). Similarly to mice deficient for ERK3 in adipose tissue [6], pharmacological inhibition of MK5 resulted in increased energy expenditure while food intake and mice activity were not altered by this inhibitor (Fig. 3D–F). Of note, treatment of mice with GLPG0259 inhibitor resulted in decreased adiposity, smaller adipocytes, and reduced accumulation of fat in adipocytes and in the liver (Fig. 3G-L). Taken together, we show that genetic or pharmacological targeting of ERK3/MK5 complex reduces lipid accumulation in s and promotes energy dissipation protecting against obesity and diabetes.

2.4. Elevation of ERK3/MK5 levels during obesity contributes to the suppression of energy expenditure in human adipocytes

To test if the function of the ERK3/MK5 complex is conserved in human adipocytes, we utilized the Simpson Golabi Behmel Syndrome (SGBS) cell strain [12]. Silencing of ERK3 in differentiated SGBS cells resulted not only in the reduction of ERK3 levels, but also in the decreased abundance of MK5 (Fig. 4A). These data indicate that similarly to the mouse adipocytes [6], formation of the complex between ERK3 and MK5 is also required for the stability of these proteins in human adipocytes. Of note, silencing of ERK3 in differentiated SGBS cells resulted in elevated OCR at the basal condition as well as OCR associated with proton leak and ATP production (Fig. 4B–D).

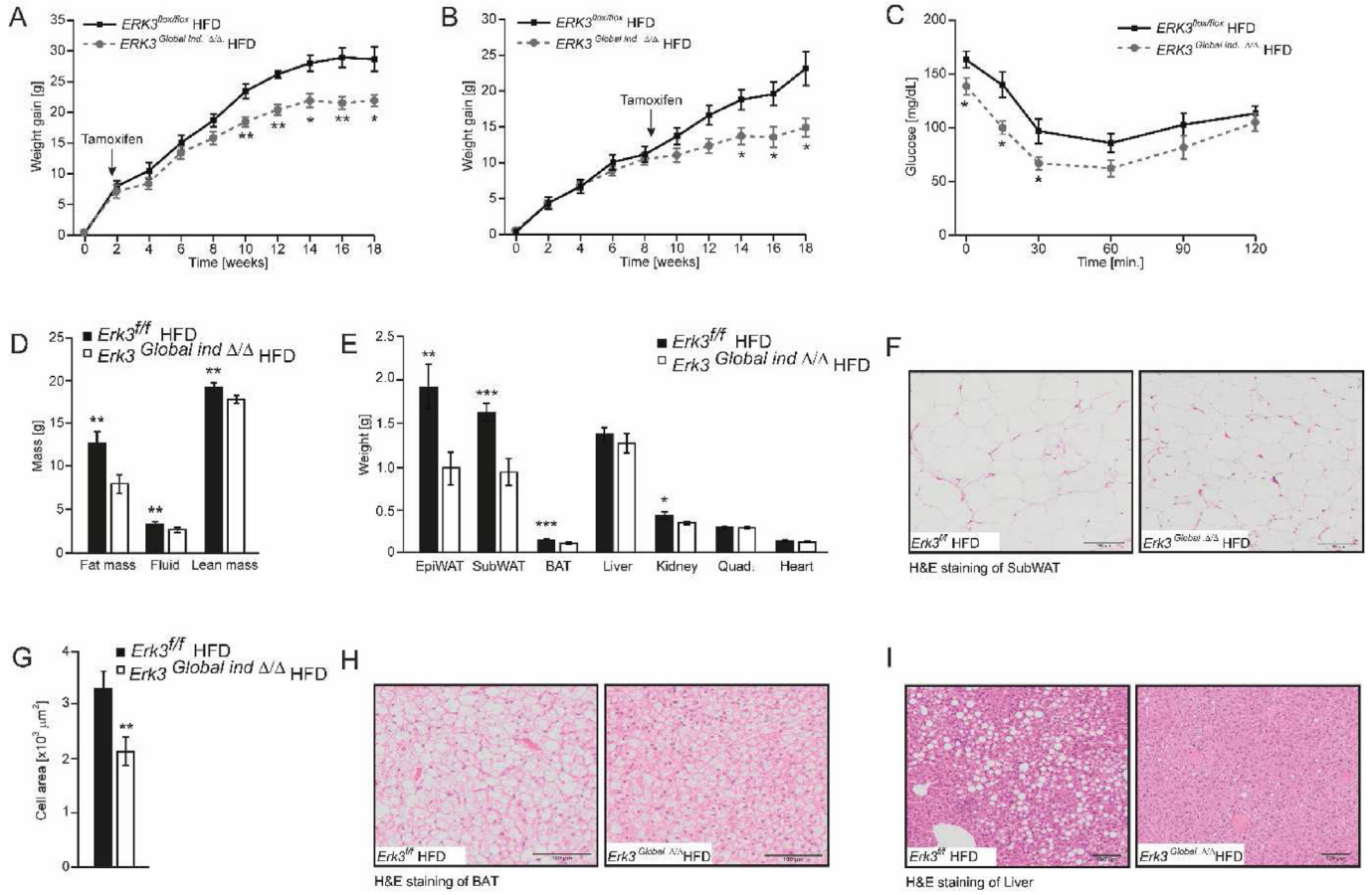

Fig. 1. Systemic inactivation of ERK3 prevents adiposity and preserves metabolic health. A) Body weight gain of mice with the indicated genotypes and gavage with tamoxifen 2 weeks after the beginning of the HFD (n = 4). B) Body weight gain of mice with the specified genotypes and gavage 8 weeks after the beginning of the HFD (n = 5). C) Insulin tolerance test of $Erk3^{f/f}$ and $Erk3^{global\ ind.\ \Delta/\Delta}$ mice after 18 weeks in HFD. D) Quantification of fat, Free fluid, and lean mas by nuclear magnetic resonance (NMR) of mice in HFD. E) Organ weight (in g) of different fat depots, liver, kidney, quadriceps and heart of $Erk3^{f/f}$ and $Erk3^{global\ ind.\ \Delta/\Delta}$ Organ weight in HFD. F) Representative pictures of H&E staining of Subwat of mice of the indicated genotypes fed HFD. G) Quantification of the average adipocyte area in Subwat of mice of the indicated genotypes fed HFD. H) Representative H&E staining of brown adipose tissue (BAT) on the indicated mice fed a HFD. I) Representative H&E staining of the liver on the indicated mice fed a HFD. Data presented as average ± SEM. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Significances were assessed by using a two-tailed Student's *t*-test for independent groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

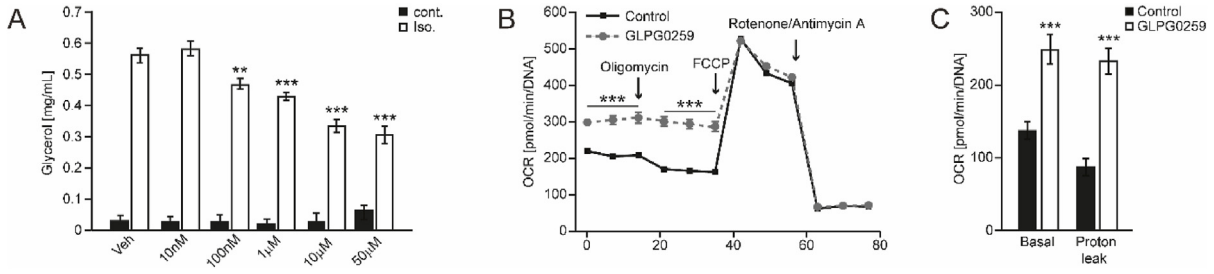

Fig. 2. MK5 inhibitor (GLPG0259) impairs lipolysis and enhances energy dissipation. A) Isoproterenol (Iso.) induced release of glycerol from adipocytes-derived from SVC of white adipose tissue treated with indicated doses of Mk5 inhibitor (GLPG0259). Oxygen consumption in control or GLPG0259 treated SVC-derived adipocytes in response to the indicated substances (B) and indicated cellular processes (C). Data presented as average ± SEM. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Significances were assessed by using a two-tailed Student's *t*-test for independent groups.

Finally, the expression of ERK3 and MK5 was elevated in human visceral adipose tissue isolated from obese patients compared to the age and sex-matched lean controls (Fig. 4E and F). Altogether, these data suggest that functions of the ERK3/MK5 complex are conserved in humans and that this complex could be targeted to increase energy expenditure in adipose tissue of obese patients to combat obesity and associated diseases.

3. Discussion

Decades of studies established classical members of the MAPK family as central regulators of glucose and lipid metabolism [4,5]. However, the roles of atypical members of the MAPK family in the regulation of the processes defining metabolic homeostasis only start to be explored [5,6]. Our previous study established ERK3/

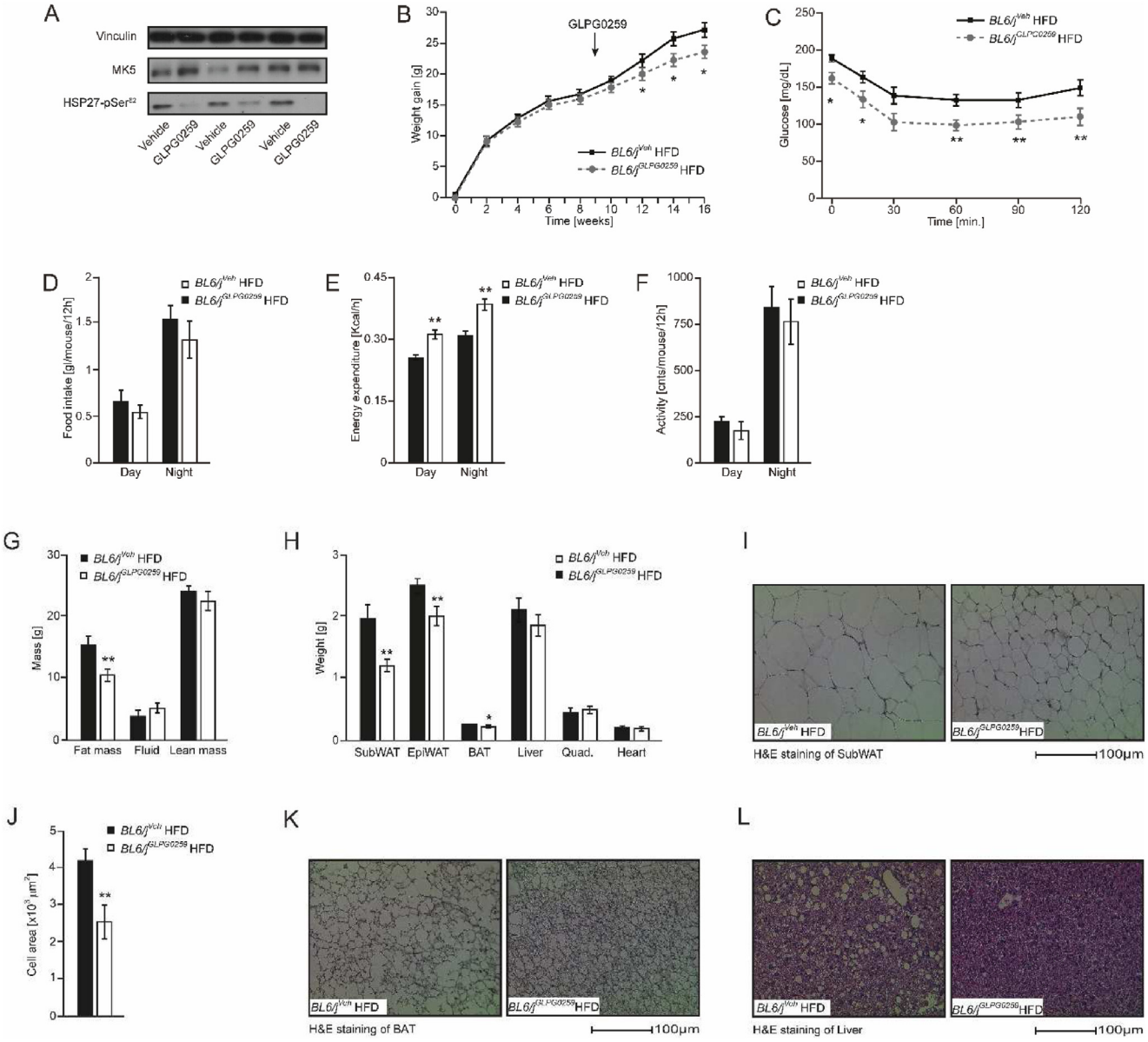

Fig. 3. Targeting MK5 in vivo prevents body weight gain and increases energy dissipation in mice. A) Western blot analysis of phosphorylated HSP27 (S82) and total MK5 levels in subcutaneous adipose tissue of C57BL/6 male mice fed a HFD and treated with vehicle or MK5 inhibitor (n = 3). B) body weight gain of mice fed a HFD and treated with vehicle or an MK5 inhibitor (n = 15). C) Insulin tolerance test of mice treated with vehicle or MK5 inhibitor fed with HFD (n = 15). D) Food intake of mice treated with vehicle or MK5 inhibitor in HFD (n = 4). E) Energy expenditure of mice treated with vehicle or MK5 inhibitor (n = 4) in HFD. F) motor activity of mice of mice treated with vehicle or MK5 inhibitor in HFD (n = 4). G) Quantification of fat, free fluid, and lean mass by nuclear magnetic resonance (NMR) of mice treated with vehicle or MK5 inhibitor in HFD. H) Organ weight (in G) of different fat depots, liver, quadriceps and heart. I) Representative pictures of H&E staining of Subwat of mice treated with vehicle or MK5 inhibitor fed with HFD. J) Quantification of the average adipocyte area in Subwat of mice treated with vehicle or MK5 inhibitor fed with HFD. K) Representative pictures of H&E staining of BAT of mice treated with vehicle or MK5 inhibitor fed with HFD. L) Representative pictures of H&E staining of liver of mice treated with vehicle or MK5 inhibitor maintained on HFD. Data presented as average ± SEM. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Significances were assessed by using a two-tailed Student's *t*-test for independent groups.

MK5 complex as a central factor in β-adrenergic induced lipolysis [6]. Initial data indicated that deletion of ERK3 in the whole body results in postnatal lethality due to the malfunction of the respiratory system linked to an abnormal development of the diaphragm [13]. Recent studies proved that whole body deletion of ERK3 does not impair lung function and indicate that previously observed malfunction of the lung was caused by the accidental deletion of other genes during the process of ERK3 targeting [14]. In line with these findings, our results show that inducible deletion of ERK3 in the whole body does not lead to any obvious abnormalities. Moreover, mice deficient for ERK3 in the whole body are protected from diet-induced obesity and insulin resistance. Altogether, these data indicate that ERK3/MK5 complex might be safely targeted for the treatment of metabolic diseases. An inhibitor of MK5 was developed (GLPG0259) and has been safely used in humans [10]. Originally, GLPG0259 was tested for the treatment of rheumatoid arthritis, but it failed to outperform the existing therapies. Nevertheless, GLPG0259 effectively inhibits MK5 action as indicated by reduced phosphorylation of the target of this kinase - HSP27. Also, other research groups proved the efficiency of this inhibitor *in vivo* as a negative regulator of tumor cell invasion [15]. Our data indicate that application of GLPG0259 reduces body weight, adiposity and improves insulin sensitivity. Moreover, our results prove that the function of ERK3/MK5 complex in the regulation of adipose tissue

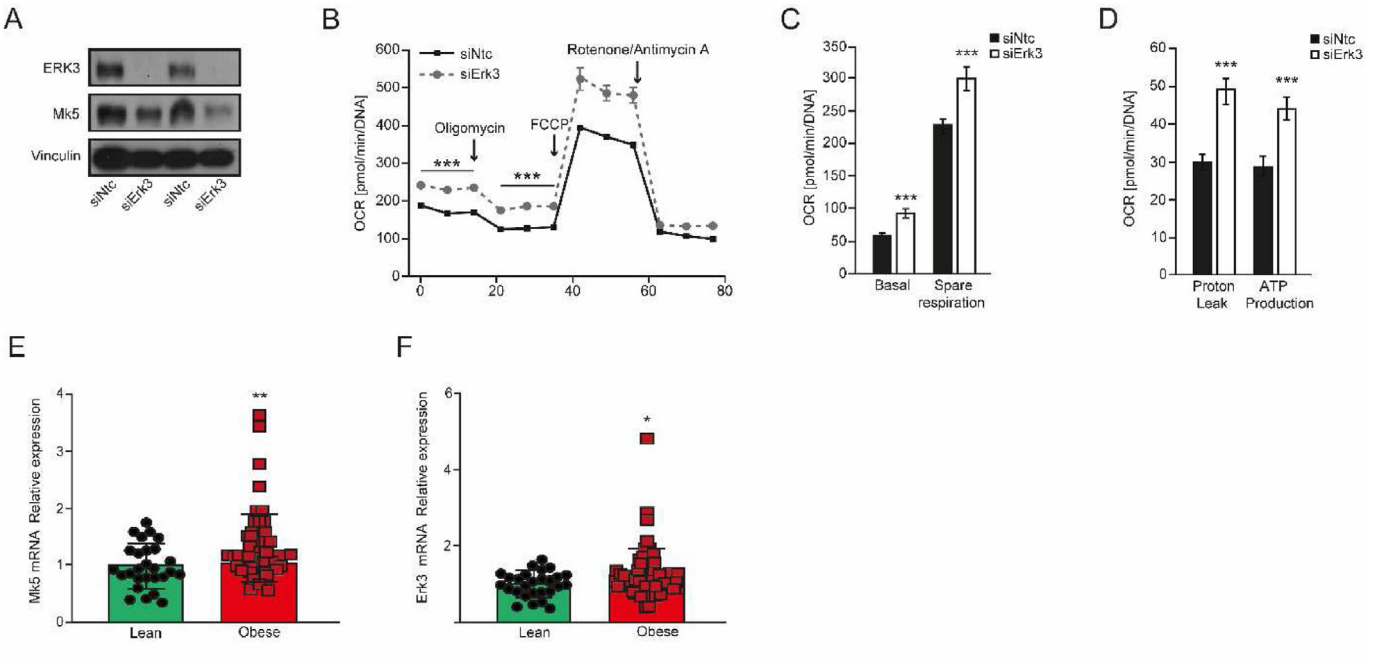

Fig. 4. ERK3 drives energy dissipation in human adipocytes. A) Western blot analysis of ERK3 and MK5 during differentiation of SGBS human pre-adipocytes. Oxygen consumption rate (OCR) in response to indicated substances in SGBS adipocytes. D) OCR values for basal and spare respiration in SGBS adipocytes transfected with siRNAs against non-targeting control and ERK3. D) OCR values for proton leak and ATP production in SGBS adipocytes transfected with siRNAs against non-targeting control and ERK3. E) MK5 mRNA levels in subcutaneous adipose tissue in healthy lean subjects obese subjects. F) ERK3 mRNA levels in subcutaneous adipose tissue in healthy lean subjects obese subjects. Data presented as average ± SEM. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Significances were assessed by using a two-tailed Student's *t*-test for independent groups.

metabolism is conserved in humans. In line with our findings, a recent study identified two single nucleotide polymorphisms (SNPs) in the MAPK6/ERK3 gene that correlate nominally with plasmatic FFA [16]. Altogether, previous findings and our results indicate that ERK3/MK5 pathway can be safely and effectively targeted to ameliorate obesity as well as insulin resistance.

4. Materials and methods

4.1. Generation of mouse models

Erk3 floxed mice (Erk3 fl/fl) were genetically engineered on C57BL/6 background as previously reported [6]. Briefly, for the generation of targeted deletion of Erk3 in the whole body Erk3 f/f mice were cross-bred with ubiquitin promoter-driven Cre-ERT2 mice [9]. For the Cre-ERT2 activation, 100 mg/kg of Tamoxifen (Sigma-aldrich T5648) was given orally by using a feeding needle for five consecutive days.

4.2. Oral administration of MK5 inhibitor

C57BL/6 mice were fed with a High-fat diet (HFD) for 10 weeks. Afterward, the mice were administered orally with an MK5 inhibitor (MK5i) (GLPG0259; Medkoo 25986) 10 mg/kg of body weight for 6 weeks.

4.3. Animal experiments

Mice ($Erk3^{global\ ind.\ \Delta/\Delta}$) and mice treated with MK5i were fed a High-fat diet (HFD) for 20 and 18 weeks respectively. Indirect calorimetry analysis using the Phenomaster system (TSE) as well as glucose and insulin tolerance test was performed at the end of the dietary intervention as described previously [17,18].

4.4. Cell culture

Stromal vascular cells (SVCs) containing pre-adipocytes were isolated, cultured and differentiated as previously described [19].

SGBS cells (P36-40) were cultured and maintained as described before [20]. Cells were grown and maintained in DMEM-F12 (Invitrogen) supplemented with 33 μM biotin (Sigma), 17 μM pantothenic acid (Sigma), 40 μg/mL gentamycin, and 10% FBS (Gibco) at 37 °C in 5% CO_2. Upon confluency adipose differentiation was induced by using a serum-free medium supplemented with 33 μM biotin, 17 μM pantothenic acid, 2 μM rosiglitazone (Sigma), 10 μg/ml human apo-transferrin (Sigma), 20 nM human insulin (Sigma), 25 nM dexamethasone (Sigma), 500 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), 100 nM cortisol (Sigma) and 200pM triiodothyronine (Sigma). On day 4, the medium was changed, rosiglitazone, IBMX, and dexamethasone were removed. The medium was changed every second day. Transfection of differentiated SGBS was carried out on day 10, adipocytes were detached with accutase solution for 10 min, spun down at 1500 rpm, and counted. Subsequently, cells were added to a mix containing the Indicated siRNAs (non-targeting) and human ERK3 and Dharmafect-Duo transfection reagent (Dharmacon) were diluted in Opti-MEM I reduced serum medium separately before being mixed by pipetting. The mix of siRNA and transfection reagent was incubated for 30 min at room temperature. The final concentrations of Duofect, siRNA, and cell number were adjusted per surface area in a ratio of 2.1 μL/cm2 duofect. The mixture of the cells was plated in a 96 well plate for seahorse analysis precoated with 0.5% Matrigel. Lipolysis assay was performed as described previously [6]. Mitochondrial respiration of adipocytes either derived from the stromal-vascular fraction or the cell line SGBSS was determined by measuring oxygen consumption rate (OCR) by using the seahorse XF Cell mito stress test (Agilent technologies) in the seahorse Xfe 96 Analyzer as described previously [19]. For the

experiment in SVF derived adipocytes, cells were pretreated for 16 h with MK5 inhibitor.

5. Histology

Histological analyses of different depots of adipose tissue and liver were performed as described previously [19].

6. Adipose size quantification

Images from H&E-stained sections of Subcutaneous adipose tissue sWAT from mice fed a (HFD) were taken with 20 × magnification under the microscope. Four representative pictures were analyzed from each sample by manually measuring the area of each cell in a total area of 0.6 mm^2. ImageJ was utilized for this measurement by using the tool, which allows manually encircling of each cell, and automatic measurement of the area.

7. Western blot

Cells and tissues were lysed by a standard RIPA buffer. The following antibodies were used: (rabbit anti-ERK3 (Abcam #ab53277), rabbit anti-MAPKAPK-5 (Cell signaling #ab7419), (Cell signaling #ab5568), rabbit anti-VINCULIN (Cell signaling #13901), rabbit anti phospo HSP27 Ser 82.

8. Real-time PCR

Total RNA was extracted from cells and tissues using the RNeasy kit according to the manufacturer's protocol. Reverse transcription (RT) was performed using 1 μg of RNA and first-strand cDNA synthesis kit (Thermo-Scientific). Quantitative PCR was performed using 10 ng of cDNA, power-up SYBR Green and the respective pair of primer sequences. The genes and sequences used were as follows:

9. Human primers

Erk3 (forward: 5′ - TCGATGAGTCGGAGAAGTCC -3′; reverse: 5′ - GAAGATGTCTTTTGTTAGTGATCAGGT -3′),
Mk5 (forward: 5′ - AAAAACTCCGAGATGTGATTGC -3′; reverse: 5′ - GAGTTTGCATTCCCGGTTATAC-3′),
Gapdh (forward: 5′ - CAAGGTCATCCATGACAACTTTG-3′; reverse: 5′ -TCCACCACCCTGTTGCTGTAG-3′),

10. Human samples

The human studies were approved by the Ethics Committee of the University Hospital of Salamanca, and all subjects provided written informed consent to undergo subcutaneous fat biopsy under direct vision during surgery. The study population included adults who underwent elective bariatric surgery at the University Hospital of Salamanca. Patients were excluded if they had a history of alcohol abuse or excessive alcohol consumption (>30 g/day in men and >20 g/day in women), or chronic hepatitis C or B or diabetes. Data were collected on demographic information (age, sex, and ethnicity), anthropomorphic measurements (BMI), smoking and alcohol history, coexisting medical conditions, and medication use. Fasting venous blood samples were collected, and sWAT was obtained during surgery and frozen for posterior analysis.

10.1. Statistical analyses

All data, unless otherwise indicated, are presented as mean values ± standard error of the mean (SEM). Determination of significances between two independent groups was determined with an unpaired, two-tailed Student's *t*-test. *P*-values at the level of 0.05 were considered statistically significant.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.070.

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