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Small extracellular vesicle targeting of hypothalamic AMPKα1 promotes weight loss in leptin receptor deficient mice

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ABSTRACT

Background and aims: Leptin receptor (LEPR) deficiency promotes severe obesity and metabolic disorders. However, the current therapeutic options against this syndrome are scarce.
Methods: db/db mice and their wildtypes were systemically treated with neuronal-targeted small extracellular vesicles (sEVs) harboring a plasmid encoding a dominant negative mutant of AMP-activated protein kinase alpha 1 (AMPKα1-DN) driven by steroidogenic factor 1 (SF1) promoter; this approach allowed to modulate AMPK activity, specifically in SF1 cells of the ventromedial nucleus of the hypothalamus (VMH). Animals were metabolically phenotyped.
Results: db/db mice intravenously injected with SF1-AMPKα1-DN loaded sEVs showed a marked feeding-independent weight loss and decreased adiposity, associated with increased sympathetic tone, brown adipose tissue (BAT) thermogenesis and browning of white adipose tissue (WAT).
Conclusion: Overall, this evidence indicates that specific modulation of hypothalamic AMPK using a sEV-based technology may be a suitable strategy against genetic forms of obesity, such as LEPR deficiency.

1. Introduction

Genetic obesity falls in two categories: monogenic obesity, which is inherited in a Mendelian pattern, is typically rare, early-onset and severe; and polygenic obesity, which is the result of hundreds of polymorphisms, each having small effects [1–3]. In the case of monogenetic obesity, the therapeutic options are scarce [1–3]. If the mutation involves the lack of ligand, for example a hormone (leading to a hypohormonal syndrome), one possibility could be a replacement therapy. That strategy has given different degrees of efficiency, depending on the factor; for instance, for leptin or proopiomelanocortin (POMC) deficiencies [1–3]. However, when the mutation occurs on a receptor, such as leptin receptor (LEPR), or melanocortin 4 receptor (MC4R), the therapeutic options are more limited, and even bariatric surgery may fail [1,2,4–7].

Current evidence has shown that LEPR-deficient patients treated with the MC4R agonist setmelanotide (also known as RM-493 or BIM-22493) resulted in significant and durable decrease in hyperphagia

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and weight loss [4,5,7]. Similar data have been obtained after intraperitoneal injections of setmelanotide in leptin receptor-deficient (*db/db*) mice, resulting in profound reductions of feeding and body weight [4]. This suggests that targeting LEPR downstream pathways modulating feeding could be a suitable strategy for the treatment of this syndrome [2,4–7].

Hypothalamic AMP-activated protein kinase (AMPK) is a canonical regulator of energy balance and metabolism at the whole-body level [8,9]. AMPK is a key downstream factor for both leptin's anorectic effect and possibly leptin's brown adipose tissue (BAT) thermogenic actions [10–12]. We have recently used small extracellular vesicles (sEVs; formerly exosomes) as cargos of DNA sequences, to inhibit hypothalamic AMPKα1 (using a dominant negative mutant, AMPKα1-DN) specifically in steroidogenic factor 1 (SF1) neurons of the ventromedial nucleus of the hypothalamus (VMH), a key population regulating BAT thermogenic activity [13,14]. Remarkably, when diet-induced obese (DIO) mice were intravenously injected with SF1-AMPKα1-DN loaded sEVs, they displayed a marked feeding-independent weight loss associated with sympathetic nerve activation and increased uncoupling protein 1 (UCP1)-dependent thermogenesis in BAT [14]. Importantly, no metabolic, endocrine, or cardiovascular inflammatory reactions or other adverse effects were developed [14].

The aim of this study has been to use sEVs harboring SF1-AMPKα1-DN in *db/db* mice to address whether: i) our strategy was valid for the treatment of genetic forms of obesity and ii) the targeting central mechanism modulating BAT thermogenesis could offer a new therapeutic option for the treatment of LEPR-deficiency.

2. Materials and methods

2.1. Animals

Male null LEPR (*db/db*) and wildtype (WT) littermate mice (C57/BL/6J; 8-week-old; Janvier Labs) were used for the experiments. Mice were individually housed with an artificial 12-hour light (8:00 to 20:00)/12-hour dark cycle, under controlled temperature (22 ± 1 °C) and humidity (50 ± 5 %). They were allowed free access to water and standard laboratory diet (Scientific-Animal-Food-Engineering; Newmark, DE, USA). Seven to 8 mice/group were used. The experiments were performed in agreement with the International Law on Animal Experimentation and were approved by the USC Ethical Committee (15012/2020/010).

2.2. Generation, validation, and treatment with sEVs

To confer neuronal targeting capacities to sEVs, and to limit host immune reactions, we used immature dendritic cells which were genetically modified to express a fusion protein of lysosome-associated membrane protein 2b (Lamp2b, a protein highly expressed in sEV membranes) fused to a specific glycoprotein derived from the neurotrophic rabies virus (RVG), which enables the blood-brain-barrier (BBB) crossing *via* its binding to the nicotinic acetylcholine receptor (nAChR), as shown [14,15]. sEVs were deeply characterized using electron microscopy, nanoparticle tracking analysis (NTA) membrane protein markers, as previously demonstrated [14]. The sEVs were loaded with a plasmid encoding a AMPKα1-DN mutant expressed under the control of SF1 promoter (SF1-AMPKα1-DN), to limit their actions to SF1 cells in the VMH [14].

2.3. Systemic treatment with sEVs

One hundred μg of non-loaded or SF1-AMPKα1-DN loaded sEVs were injected in the tail vein of the mice every 3 days for 2 weeks, as shown [14]. Body weight and food intake were daily measured.

2.4. Temperature measurements

Skin temperature surrounding BAT was recorded (days 1, 2, 4, 5, 6, 8, 9, 11, 12 and 14) with an infrared camera (B335: Compact-Infrared-Thermal-Imaging-Camera; FLIR; West Malling, Kent, UK) and analyzed with a specific software package (FLIR-Tools-Software, FLIR; West Malling, Kent, UK), as previously shown [13,14,16–20].

2.5. Insulin tolerance tests

Glycaemia was measured with a glucometer (Accucheck; Roche; Barcelona, Spain), after an intraperitoneal injection of 0.75 U/kg of insulin (Actrapid, Novonordisk; Bagsvaerd, Denmark) for insulin tolerance test (ITT), as previously shown [13,21]. Animals were fasted 6 h before the procedure.

2.6. Blood biochemistry

Serum triglycerides (#1001314, Spinreact; Girona, Spain) were analyzed by spectrophotometry in a Multiskan GO spectrophotometer (Invitrogen-Thermofisher; Hennigsdorf, Germany) [14].

2.7. Nuclear magnetic resonance

For the measurement of body composition, we used nuclear magnetic resonance (NMR; Whole Body Composition Analyzer; EchoMRI; Houston, TX, USA) [13,17,18].

2.8. Sample processing

Mice were killed by cervical dislocation. From each animal, we dissected the VMH (from the whole hypothalamus, for western blotting), the BAT (for western blotting, immunohistochemistry and HPLC analysis), the subcutaneous white adipose tissue (sWAT; for immunohistochemistry and HPLC analysis), and the non-neuronal SF1-expressing tissues (pituitary, adrenals, and testes, for western blotting). Each harvested tissue was immediately homogenized on ice to preserve phosphorylated proteins. Samples were stored at −80 °C until further processing. Dissection of the VMH was performed by micropunches under the microscope, as before [13,14].

2.9. High-performance liquid chromatography

BAT and sWAT were dissected on an ice-cold plate, immediately frozen on dry ice, and stored at −80 °C until analysis. Tissue was homogenized, sonicated, and subsequently centrifuged (14,000*g* for 20 min at 4 °C). The remaining supernatant fraction was filtered and injected (20 μl/injection) into the high-performance liquid chromatography (HPLC) system (Shimadzu LC Prominence; Shimadzu Corporation; Kyoto, Japan) [18,22]. Norepinephrine (NE), dopamine (DA) and serotonin (5-HT) were separated using a reverse phase analytical column (Waters Symmetry 300C18; Waters, Milford, MA, USA). The mobile phase consisted of a 10 % MeOH solution (pH = 4) containing 70 mM KH_2PO_4, 1 mM octanesulfonic acid and 1 mM EDTA and was delivered at a rate of 1 ml/min. Detection was performed with a coulometric electrochemical detector (ESA Coulochem III; Thermo Scientific; Waltham, MA, USA). The first and the second electrode of the analytical cell were set at +50 mV and +350 mV, respectively, and the guard cell was set at −100 mV. Data were acquired and processed with the Shimadzu LC solution software (Shimadzu Corporation; Kyoto, Japan) and the concentrations of the neurotransmitters were expressed as pg/mg of wet tissue.

2.10. Western blotting

Protein lysates from the VMH were subjected to SDS-PAGE,

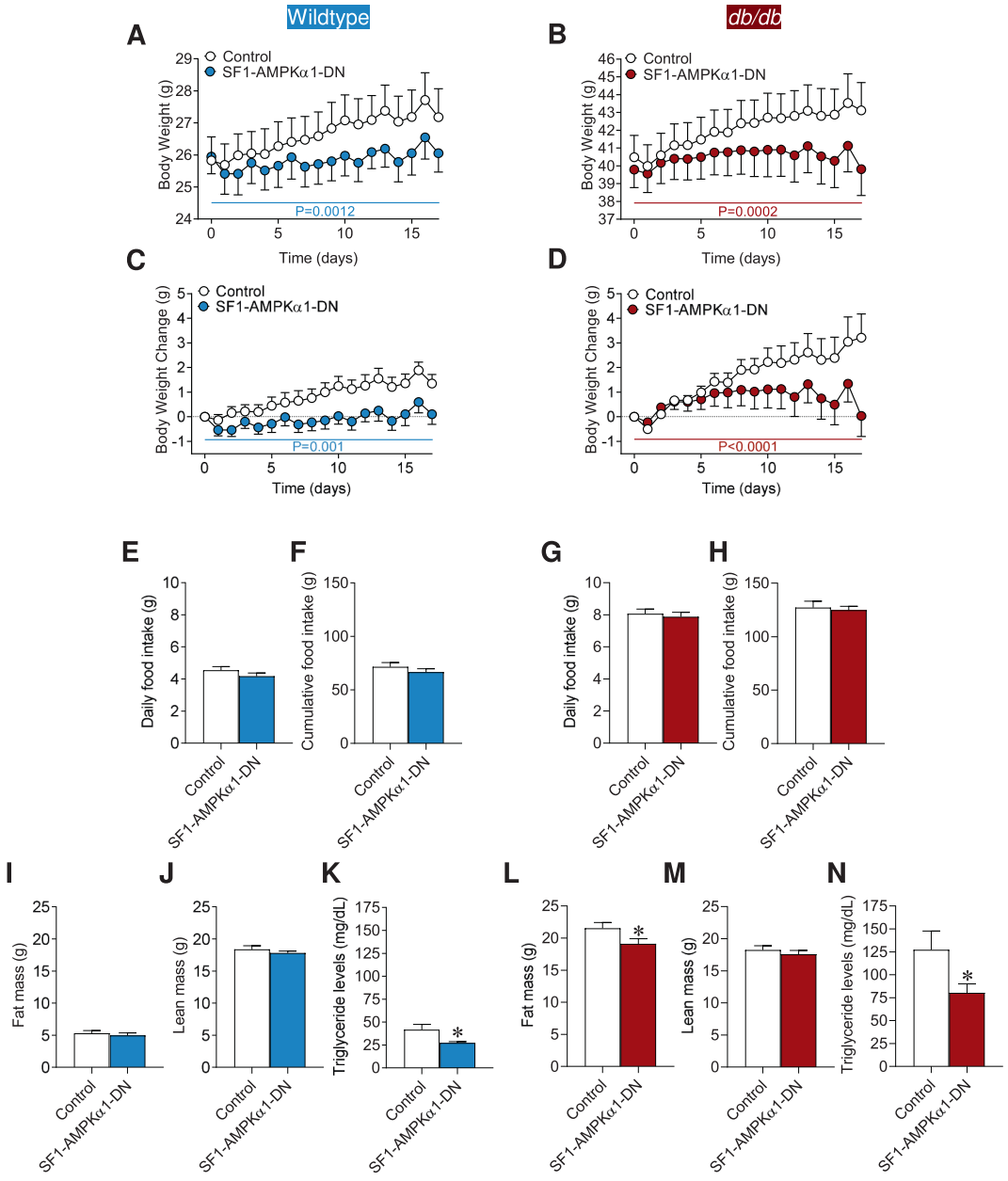

Fig. 1. Effect of systemic treatment with SF1-AMPKα1-DN sEVs on energy balance in *db/db* mice.
(A–B) Body weight; (C–D) body weight change; (E–H) daily and cumulative food intake; (I–J and L–M) lean and fat mass and (K and N) serum triglyceride levels of wildtype and *db/db* mice intravenously treated with control (non-loaded; n = 8 wildtype mice; n = 8 *db/db* mice) or SF1-AMPKα1-DN loaded (n = 7 wildtype; n = 7 *db/db* mice) sEVs. Data are expressed as mean ± SEM. Statistical significance was determined by Mixed effect analysis (A–D) or Student's *t*-test (E–L; one-sided in L). *P < 0.05 *vs.* control. For the Mixed effect analyses the P values are as follows: A) Time P < 0.0001; Treatment P = 0.4254; Time × Treatment P = 0.0012 (shown in the graph); B) Time P < 0.0069; Treatment P = 0.4222; Time × Treatment P = 0.0002 (shown in the graph); C) Time P < 0.001; Treatment P = 0.05; Time × Treatment: P = 0.001 (shown in the graph); D) Time P < 0.004; Treatment P = 0.2506; Time × Treatment: P < 0.0001 (shown in the graph).

electrotransferred to polyvinylidene difluoride membranes (PVDF; Merck Millipore; Billerica, MA, USA) with a semidry blotter and probed with antibodies against β-actin (1:5000; A5316), α-tubulin (1:5000; T5168) (Sigma; St Louis, MO, USA); pACCα-Ser^{79} (1:1000; 3661; Cell Signaling; Danvers; MA, USA); ACCα (1:1000; 04-322; Merck Millipore; Billerica, MA, USA) [13,14,16–19]. Autoradiographic films (Fujifilm; Tokyo, Japan) were scanned and the band signals were quantified by densitometry using ImageJ-1.44 software (National Institutes of Health; Bethesda, MD, USA) [13,14,16–19]. Values were expressed in relation to α-tubulin (BAT) or ACCα (VMH). Representative images for all proteins are shown with all bands for each picture derived from the same gel, although they may be spliced for clarity; a black line was inserted when samples were not loaded side by side.

2.11. Immunohistochemistry

Adipose tissue depots were fixed in 10 % buffered formaldehyde and paraffin embedded. For the hematoxylin-eosin processing, the BAT and sWAT sections were first stained with hematoxylin for 5 min, washed and stained again with eosin for 1 min. Detection of UCP1 in sWAT was performed using anti-UCP1 antibody (1:500; ab10983; Abcam, Cambridge, UK) [14,16,18,19]. Images were taken with a digital camera Olympus XC50 (Olympus Corporation; Tokyo, Japan) at 20×. Digital images for BAT and sWAT were quantified with ImageJ 1.44 software

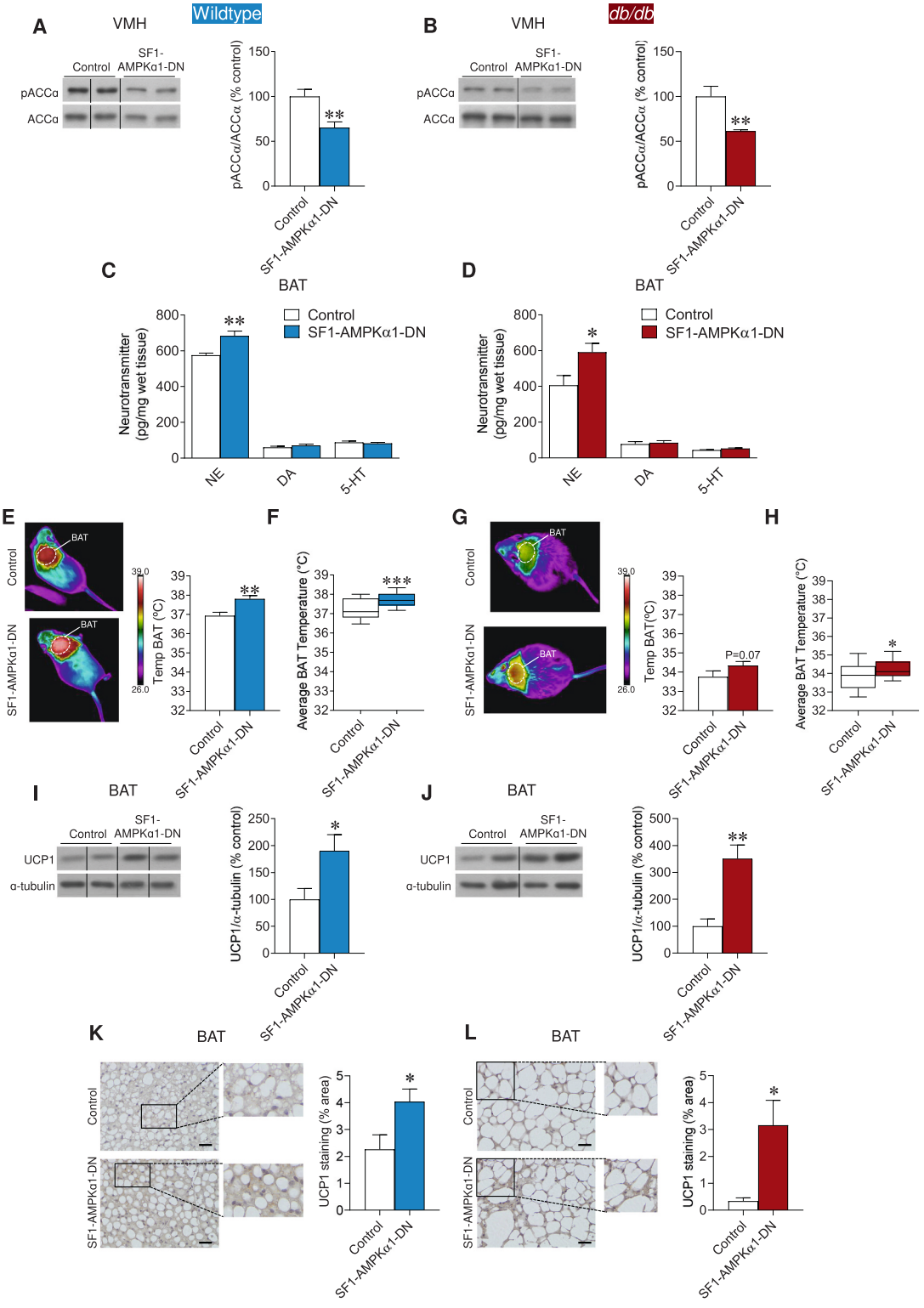

Fig. 2. Effect of systemic treatment with SF1-AMPKα1-DN sEVs on BAT thermogenesis.
(A–B) Representative pACCα and ACCα western blot images and levels in the VMH (n = 7 mice/group); (C–D) BAT neurotransmitter levels (n = 6 mice/group); (E–H) representative BAT thermographic images and BAT temperature [E and G right panels: at day 14; n = 7–8 mice/group; F and H: at days 5–12; n = 28–30 individual values/group; box plot indicate median (middle line), 25th, 75th percentile (box) and 10th–90th percentiles]; (I–J) representative UCP1 western blot images and levels in the BAT (n = 6 mice/group); and (K–L) representative BAT UCP1 staining and levels (n = 7–8 mice/group) of wildtype and *db/db* mice intravenously injected with control or SF1-AMPKα1-DN loaded sEVs. In the western blot analyses (A–B and I–J) β-actin (in the VMH; not shown) and α-tubulin (in the BAT) were used as controls of protein loading. A black line was inserted on the immunoblots when samples were loaded on the same gel, but not side by side. Data are expressed as mean ± SEM. Statistical significance was determined by Student's *t*-test (one-sided in G). *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* control.

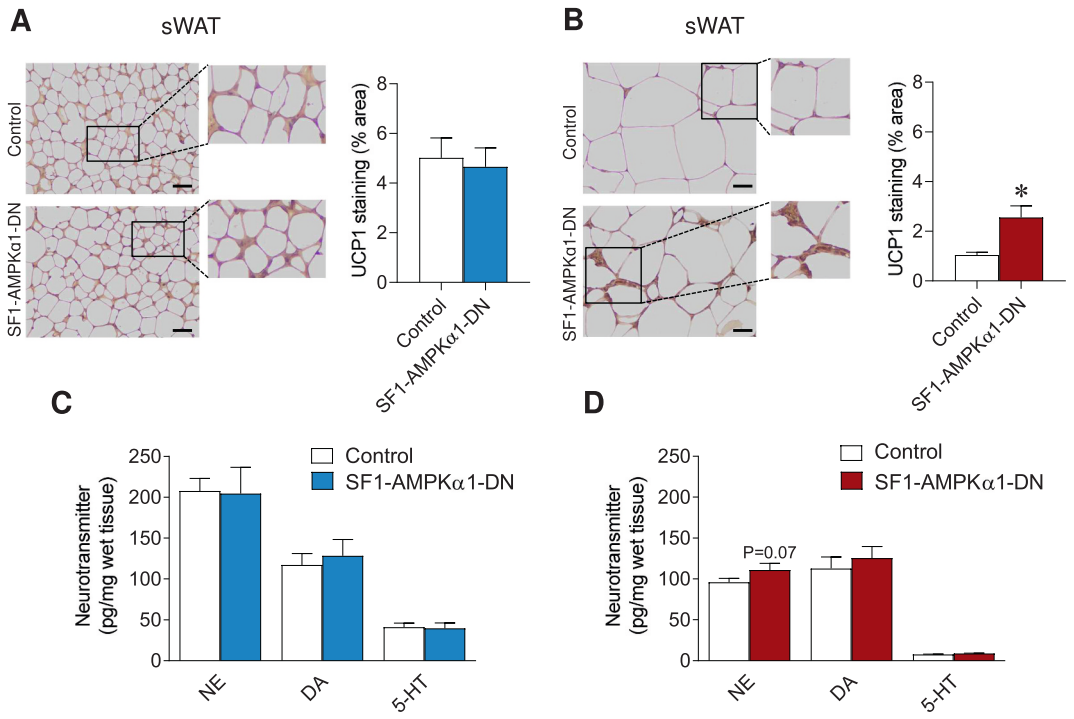

Fig. 3. Effect of systemic treatment with SF1-AMPKα1-DN sEVs on WAT browning in *db/db* mice.
(A–B) Representative sWAT UCP1 staining and levels (n = 7–8 mice/group) and (C–D) BAT neurotransmitter levels (n = 6 mice/group) of wildtype and *db/db* mice intravenously injected with control or SF1-AMPKα1-DN loaded sEVs. Data are expressed as mean ± SEM. Statistical significance was determined by Student's *t*-test (one-sided in D). *P < 0.05 *vs.* control.

(National Institutes of Health; Bethesda, MD, USA).

2.12. Statistical analysis

Data are expressed as mean ± SEM; when data are relativized, they are given as percentage of the appropriate controls. Statistical significance was determined by two-sided (at least one-sided is specified) Student's *t*-test (when two groups were compared), and by hierarchical regression models for longitudinal measures, ANOVA (ITT analyses), or Mixed effect analysis (for time course treatments), followed of Holm-Sidak's multiple comparisons test; sphericity was not assumed and therefore Geisser-Greenhouse method applied. Fixed effects (type III) for i) time, ii) treatment and iii) time × treatment were calculated and specified in figure legends. For the association analyses, to consider both the temporal evolution of the variables and the presence of autocorrelation in the measurements of each mouse, a hierarchical random intercept and slope model have been estimated, which will allow the temporal variability in the measurements to be studied. P < 0.05 was considered significant. Data analysis was performed using Prism 8.0.2 Software (GraphPad; San Diego, CA, USA) and packages lme4 and emmeans from free software R [20,23–25].

3. Results

3.1. Systemic SF1-AMPKα1-DN sEVs induced weight loss in db/db mice

Intravenous injections of SF1-AMPKα1-DN sEVs promoted a significant feeding-independent weight loss in wildtype (Fig. 1A, C, E and F) and *db/db* mice (Fig. 1B, D, G and H), associated with decreased adiposity in *db/db* mice (but, somewhat expected, not in wildtype mice, likely due to the limit of sensitivity of the NMR analyses; Fig. 1I–J and L–M) and serum triglyceride levels, both in wildtype and *db/db* mice (Fig. 1K and N). Despite these positive effects on energy metabolism, SF1-AMPKα1-DN sEVs did not improve insulin sensitivity either in wildtype or *db/db* mice (Supplemental Fig. 1A–B).

3.2. Systemic SF1-AMPKα1-DN sEVs increased BAT thermogenesis in db/db mice

Inhibition of AMPKα1 in SF1 neurons of the VMH is a canonical mechanism increasing sympathetic tone on BAT, leading to thermogenesis, increased energy expenditure and weight loss [8,9,14]. It is known that leptin or LEPR deficiency leads to decreased sympathetic nervous system (SNS) tone on BAT [12,26]. In keeping with this, our data showed that activation of AMPK is increased in the VMH of *db/db* mice (Supplemental Fig. 2A). Therefore, our rationale was that inhibition of AMPK at that level, using SF1-AMPKα1-DN sEVs, would lead to increased SNS tone and subsequently BAT thermogenesis. Our data showed that intravenous injection of SF1-AMPKα1-DN sEVs decreased pACCα levels in the VMH of both mouse models (Fig. 2A–B), demonstrating the efficacy of our treatment in inhibiting AMPK activity in this hypothalamic nucleus. To control the specificity of our treatment, we assayed the effect of SF1-AMPKα1-DN sEVs in non-neuronal SF1-expressing tissues, such as pituitary, adrenal, and testis. As formerly demonstrated in a diet-induced obesity model (DIO) [14], our data showed no changes in pACCα content (and therefore in AMPK activity) in those organs (Supplemental Fig. 3A–B). Overall, those results demonstrated that the specificity of SF1-AMPKα1-DN was restricted to SF1 cells of the VMH.

To address whether increased BAT function was due to an activation of SNS tone, we analyzed BAT neurotransmitter content. Our data showed that both wildtype and *db/db* mice treated with SF1-AMPKα1-DN sEVs had higher levels of norepinephrine (NE) in the brown fat (Fig. 2C–D), which is indicative of higher adrenergic tone. No changes were found in other neurotransmitters analyzed, such as dopamine (DA) or serotonin (5-HT) (Fig. 2C–D). Next, we evaluated the effect of the sEV-mediated treatment on thermogenic mechanisms. We found that both wildtype and *db/db* mice intravenously treated with SF1-AMPKα1-

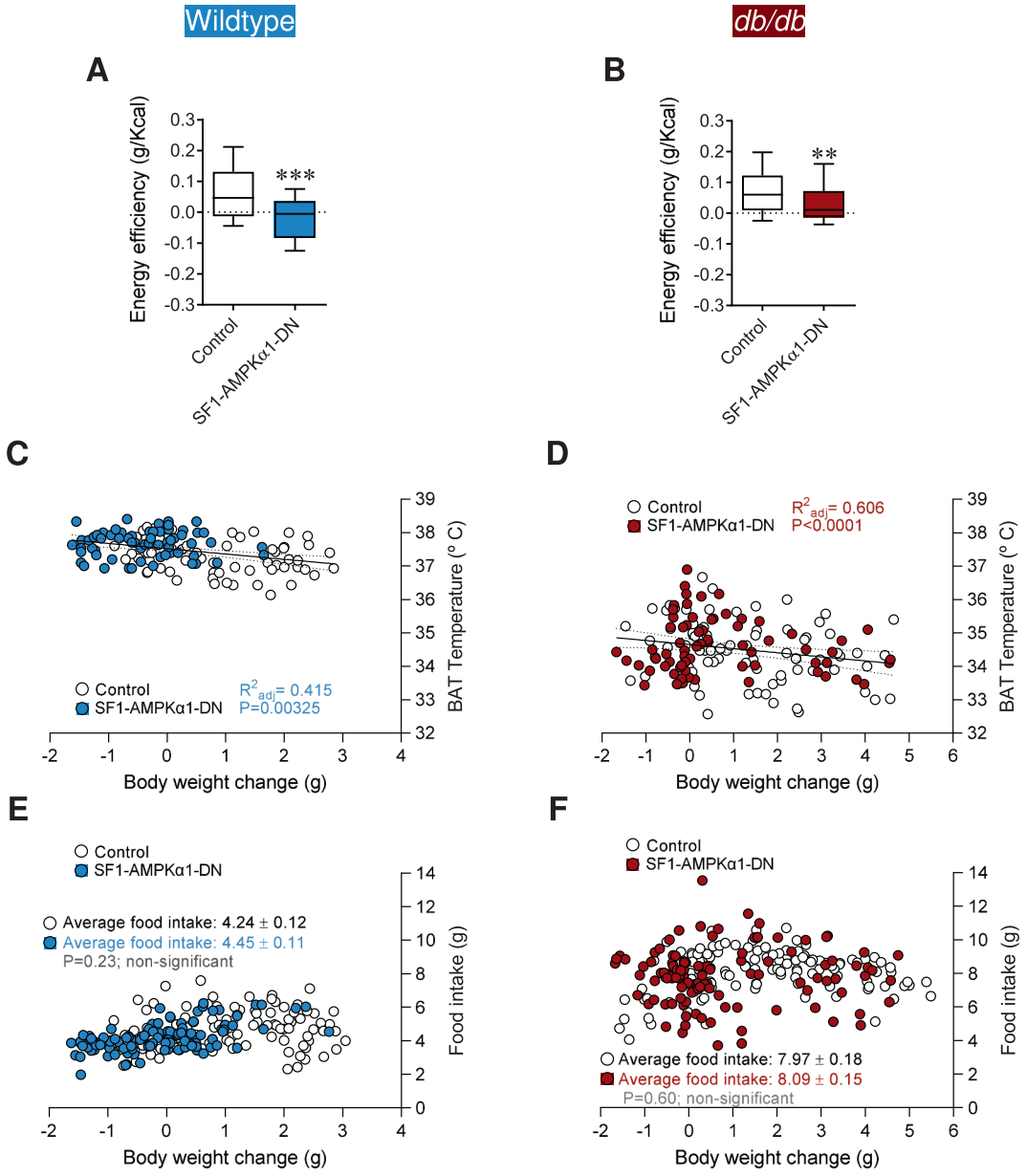

Fig. 4. Effect of systemic treatment with SF1-AMPKα1-DN sEVs on energy efficiency in *db/db* mice.
(A–B) Energy efficiency [n = 7–8 mice/group; n = 110–126 individual values/group; box plot indicate median (middle line), 25th, 75th percentile (box) and 10th–90th percentiles]; (C–D) association analyses between body weight changes in grams and BAT temperature (°C) (n = 131–147 pair values) and (E–F) food intake in grams (n = 238 pair values) of wildtype and *db/db* mice intravenously injected with control or SF1-AMPKα1-DN loaded sEVs. Data are expressed as mean ± SEM. Statistical significance was determined by Student's t-test (A–B) and statistical differences in means and association between continuous variables were assessed through hierarchical regression models (C–F). **P < 0.01, ***P < 0.001 *vs.* control. Note the total number of individual values is higher in the food intake analyses (E–F) because feeding was analyzed daily, while BAT temperature (C–D) was not, to avoid stress reactions in the mice.

DN sEVs exhibited increased BAT temperature (Fig. 2E–H), because of increased UCP1 protein levels and therefore immunoreactivity (Fig. 2I–L).

3.3. Systemic SF1-AMPKα1-DN sEVs increased browning in db/db mice

Current data have shown that browning of WAT is modulated by AMPK downstream signaling [18,27,28] and ameliorates obesity in LEPR conditions in a feeding independent manner [21]. Therefore, we planned to investigate whether SF1-AMPKα1-DN sEVs could induce browning in *db/db* mice. Our data showed that *db/db* mice (but not wildtypes) displayed higher UCP1 immunoreactivity in sWAT, being indicative of browning (Fig. 3A–B). In keeping with this, *db/db* (but not wildtype) mice treated with SF1-AMPKα1-DN sEVs tended (P = 0.07) to higher levels of NE in the WAT (Fig. 3C–D), which is indicative of higher adrenergic tone. No changes were found in other neurotransmitters analyzed, such as DA or 5-HT (Fig. 3C–D).

3.4. Systemic SF1-AMPKα1-DN sEVs promoted decreased energy efficiency in db/db mice

Finally, we aimed to investigate the effect of SF1-AMPKα1-DN sEVs on energy efficiency (the ratio of calories consumed by body weight gain over the whole treatment period). The energy efficiency was reduced in both wildtype and *db/db* mice (Fig. 4A–B). Overall, the presented evidence suggested that peripheral treatment with SF1-AMPKα1-DN sEVs

induced a BAT thermogenic-, but not feeding-associated decrease in body weight in wildtype and *db/db* mice. Therefore, we intended to address the possible correlations between these variables. Our results revealed a highly significant negative association between body weight change and BAT temperature ($P < 0.00325$ in WT and $P < 0.0001$ in *db/db*; Fig. 4C–D): the mice that received the SF1-AMPKα1-DN sEVs being the ones that lost most weight and had higher BAT temperature. Food intake was similar in both groups (inside each genotype) and no negative association was found (Fig. 4E–F). Altogether, this evidence reinforces the idea that increased BAT function, leading to increased energy expenditure (and subsequently decreased energy efficiency), accounted for the body weight reducing effects of this sEV strategy, either in wildtype or *db/db* animals.

4. Discussion

Many of the current treatments against genetic forms of obesity are mechanistically grounded on replacement therapies. Such approaches are based on the use of agonists that substitutes the lack of a ligand binding to a key receptor [1–3]. Leptin replacement therapy shows great effects in leptin deficient patients by substantially reducing their food intake, body weight and adiposity and normalizing their endocrine axes [1,29,30]. Similarly, patients with POMC or PCSK1 (proprotein convertase subtilisin/kexin type 1, a prohormone convertase required for processing POMC) deficiency are treated with setmelanotide (a selective MC4R agonist that has been recently approved by the FDA) reducing feeding and body mass [1,2,5,7]. Interestingly, this treatment has also been demonstrated to be successful, in individuals with severe obesity due to LEPR deficiency [4,7]. Of note, some differences in therapeutic response were observed when compared POMC and LEPR-deficiencies. Up to 80 % of patients with POMC-deficiency and just 46 % of patients with LEPR deficiency obesity met the primary endpoint, achieving a ≥10 % weight loss after 1 year of treatment with setmelanotide [4,7]. This evidence demonstrates that targeting a mechanism downstream LEPR is a suitable strategy for the management of obesity-induced by LEPR-deficiency [1,2,4,7], but also highlights the need to attain a greater degree of efficacy.

Hypothalamic AMPK acts downstream LEPR to modulate both feeding and BAT thermogenesis [10–12]. Moreover, we have recently shown that central targeting of AMPKα1 using a sEV-based strategy is a suitable approach against DIO in preclinical models, by specifically modulating BAT thermogenesis [14]. With this in mind, we evaluated the efficacy of SF1-AMPKα1-DN sEVs in *db/db* mice, a model of complete leptin resistance due to LEPR-deficiency. Our results demonstrated that intravenous injections of SF1-AMPKα1-DN sEVs inhibited AMPK activity in the VMH, leading to BAT thermogenesis and weight loss in wildtype and *db/db* mice, in which WAT browning was also increased. Notably, SF1-AMPKα1-DN sEVs-induced weight loss was totally feeding-independent; this suggests that the combination of our approach with other strategies modulating appetite, such as setmelanotide [4,7] or even with sEVs targeting AMPK in other hypothalamic cell populations regulating homeostatic and/or hedonic food intake [8] could allow to achieve a more significant ponderal losses. This would permit a more complete treatment against LEPR deficiency, and by extension any leptin-resistant state, by targeting both sides of the energy balance equation.

5. Conclusions

The search of new therapeutic strategies against obesity-induced LEPR-deficiency is still an unmet clinical need. Many of these patients failed to achieve the desire therapeutic response even when treated with the new MC4R agonists [4,5,7]. Our data reveal, for the first time, that sEV-mediated targeting of hypothalamic AMPK [14] could be a suitable approach against genetic LEPR deficiency-induced obesity, which might have a clear translational potential. The major strength of this approach is the innovative and very specific hypothalamic targeting by using a peripheral and secure way of administration, which has not been achieved before [8,14]. The main limitation is that only one side of the energy balance equation, namely thermogenesis, is affected. Further work will be necessary to address whether this method could be extensible to other monogenetic (and polygenetic) obesity forms and could be implemented for a more integrated strategy, *i.e.*, a simultaneous regulation of feeding and energy expenditure; something that becomes recently feasible with the emergence of incretin-based drugs as single or dual agonists [1,3].

CRediT authorship contribution statement

EM, ND, VR-L loaded the sEVs, performed the *in vivo* experiments, analytical methods and collected the data.
PG-G and JLL-G performed the HPLC analyses.
XV-G, MW, SR, MCM and RA provided the sEVs.
MPP and ML performed the statistical analysis.
EM, MPP, CD, RN, MCM, RA and ML interpreted and discussed the data.
EM and ML made the figs.
ML wrote the manuscript; all authors revised and edited the manuscript.
RA and ML supervised this work, secured funding, coordinated the project and serve as guarantors.
ML developed the hypothesis and is the lead contact of this study.

Conflict of interest

EM, MCM, RA and ML declare that the research described in this article is included in the PCT Application entitled 'Populations of small extracellular vesicles for use in the treatment of obesity' with application number PCT/EP2022/071463, filed on July 29, 2022, before European Paten Office (EPO). The other authors declare no competing interests.

Data availability

Data that support the findings of this study are available from the corresponding authors upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2022.155350.

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