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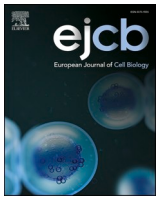

GIPR expression is induced by thiazolidinediones in a PPARγ-independent manner and repressed by obesogenic *stimuli*

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ABSTRACT

Adipose tissue (AT) dysfunctions are associated with the onset of insulin resistance (IR) and type 2 diabetes mellitus (T2DM). Targeting glucose-dependent insulinotropic peptide receptor (GIPR) is a valid option to increase the efficacy of glucagon-like peptide 1 (GLP-1) receptor agonists in T2DM treatment. Nevertheless, the therapeutic potential of targeting the GIP/GIPR axis and its effect on the AT are controversial. In this work, we explored the expression and regulation of *GIPR* in precursor cells and mature adipocytes, investigating if and how obesogenic *stimuli* and thiazolidinediones perturb *GIPR* expression. Using publicly available gene expression datasets, we assessed that, among white adipose tissue (WAT) cells, adipocytes express lower levels of *GIPR* compared to cells of mesothelial origin, pericytes, dendritic and NK/T cells. However, we report that *GIPR* levels markedly increase during the *in vitro* differentiation of both murine and human adipocytes, from 3T3-L1 and human mesenchymal precursor cells (MSCs), respectively. Notably, we demonstrated that thiazolidinediones – ie. synthetic PPARγ agonists widely used as anti-diabetic drugs and contained in the adipogenic mix – markedly induce *GIPR* expression. Moreover, using multiple *in vitro* systems, we assessed that thiazolidinediones induce *GIPR* in a PPARγ-independent manner. Our results support the hypothesis that PPARγ synthetic agonists may be used to increase *GIPR* levels in AT, potentially affecting in turn the targeting of GIP system in patients with metabolic dysfunctions. Furthermore, we demonstrate *in vitro* and *in vivo* that proinflammatory *stimuli, and especially the TNFα,* represses *GIPR* both in human and murine adipocytes, even though discordant results were obtained between human and murine cellular systems for other cytokines. Finally, we demonstrated that *GIPR* is negatively affected also by the excessive lipid engulfment. Overall, we report that obesogenic *stimuli* - ie. proinflammatory cytokines and the increased lipid accumulation – and PPARγ synthetic ligands oppositely modulate *GIPR* expression, possibly influencing the effectiveness of GIP agonists.

1. Introduction

The impaired response to insulin and the overall functional deficit of insulin signaling in key metabolic organs (*e.g.*, liver, skeletal muscle and adipose tissue) is one of the hallmarks of obesity and a driving force for type 2 diabetes mellitus (T2DM) onset (Blüher, 2020; Bray et al., 2017). Adipose tissue (AT) dysfunctions - both in terms of variation in the cell number (and type) as well as of soluble factors released in AT microenvironment - play causal roles in the establishment and progression of obesity-associated local and peripheral insulin resistance (IR) (Wang et al., 2013; Arner et al., 2010). Indeed, altered adipokine/cytokines secretion, oxidative stress, and a chronic low-grade inflammatory *milieu* impair both adipocytes' and precursor cells' functions (*e.g.*, reduced lipid storage, insulin resistance and adipogenic capacity), favoring ectopic fat accumulation (Gustafson et al., 2009; Blüher, 2016; Smith and Kahn, 2016; Lim et al., 2021).

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Different therapeutic approaches have been so far developed to improve insulin sensitivity in obese and diabetic patients. To date, targeting the primary incretin hormones, ie. glucose-dependent insulinotropic peptide (GIP) and the glucagon-like peptide 1 (GLP-1), represent two among the most valuable options (Min and Bain, 2021; Frias et al., 2020, 2018; Samms et al., 2020; Killion et al., 2018; Christensen et al., 2014; Finan et al., 2013). GLP-1 based therapy is a well-established treatment in diabetic patients and its co-infusion with GIP - targeting the AT - has been proposed about 20-years ago as a strategy to enhance its therapeutic efficacy (Miyawaki et al., 2002a). The insulin-sensitizer activity of the GIP incretin in adipocytes is mediated by its receptor GIPR that, in normoxic conditions, interacts with GRK2 decreasing its binding to IRS1. Such physiological effects are markedly reduced in hypoxic adipocytes of obese patients (Ceperuelo-Mallafré et al., 2014). Moreover, reduced *GIPR* expression has been reported in subcutaneous and visceral adipose tissue (SAT and VAT, respectively) of non-diabetic obese individuals (Ceperuelo-Mallafré et al., 2014; Rudovich et al., 2007), and fasting hyperinsulinemia has been suggested as possible negative regulator of *GIPR* levels in the SAT (Rudovich et al., 2007). Hence, normalizing GIP/GIPR signaling could be a potential combinatorial strategy in the treatment of IR and obesity (Ceperuelo-Mallafré et al., 2014). However, the direct effects of GIPR agonism and antagonism in adipocytes are a matter of debate and data are confusing and controversial (Killion et al., 2020; Kim et al., 2010, 2007; Song et al., 2007; Getty-Kaushik et al., 2006a; Mohammad et al., 2011; Getty-Kaushik et al., 2006b; Starich et al., 1985), indicating that further studies are needed to address the specific mechanisms of GIPR signaling in adipose cells. Indeed, although it has been demonstrated that GIP promotes insulin sensitivity in human adipocytes (Song et al., 2007; Mohammad et al., 2011; Starich et al., 1985), GIPR agonists fail to improve insulin responsiveness of adipose cells isolated from obese patients (Ceperuelo-Mallafré et al., 2014). Moreover, the reduced levels of *GIPR* in SAT and VAT of obese patients (Ceperuelo-Mallafré et al., 2014; Rudovich et al., 2007) could represent one of the factors influencing the therapeutic efficacy of GIP agonists.

Here, browsing public RNA-Seq data we investigated – both in human and mouse - *GIPR* expression pattern in distinct tissues and AT subpopulations. Moreover, using *in vivo* and *in vitro* models (Cataldi et al., 2021; Aprile et al., 2020, 2018) we assessed the modulation of *GIPR* levels during adipocyte differentiation, the effects induced by the obesity-associated factors (ie. lipid overload and pro-inflammatory mediators) as well as by the insulin-sensitizer factor PPARγ and its synthetic ligands.

2. Materials and methods

2.1. In silico data analysis

The expression data regarding the human *GIPR* gene (ENSG00000010310.8) used for the analyses described in this manuscript were obtained from the GTEx Portal (accessed on 01/05/22), GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2). From this database, bulk tissue RNA-Seq (reported as Transcript Per Million, TPM) and single cell snRNA-seq pilot data were used to investigate *GIPR* expression across all available post-mortem tissues.

Additionally, the evaluation of *GIPR* expression in the human and murine WAT at single-cell level was carried out through the Single Cell portal (https://singlecell.broadinstitute.org/single_cell; acc. n. SCP1376; "*A single cell atlas of human and mouse white adipose tissue*"), in which snRNA-Seq and scRNA-Seq data generated from Emont and colleagues (Emont et al., 2022) are deposited. In particular, this atlas includes single-cell expression data for 363870 total cells, 166149 of which from human WAT and 197721 from mouse WAT. The visual inspection was carried out on the web portal using the "Explore" tab and the following options: "Clustering" - Human (or Mouse) WAT; "Annotation" - cluster; "Subsampling" - All cells.

2.2. Animals and tissues

C57BL/6J mice were intraperitoneally injected with LPS (2 μg/g of body weight, InvivoGen, San Diego, USA) or with vehicle (NaCl 0.9%) for 5 h. Epididymal WAT (eWAT) samples were isolated, as previously described in (Cataldi et al., 2021; Pastor et al., 2017) and according to the Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985) and EU guidelines on animals' laboratory care. Procedures were approved by Animal Care Committee of the Faculty of Medicine of Nice-Sophia Antipolis University, Nice, France, and the French ministry of national education (#05116.02 and #201505&9143792_v2).

2.3. Cell lines and culture conditions

hTERT immortalized Adipose Derived Mesenchymal Stem Cells (hMSCs) were purchased from American Type Culture Collection (Cat#SCRC-4000, ATCC, Manassas, Virginia, United States), cultured in DMEM-F12 (1:1) supplemented with 10% South American fetal bovine serum (FBS) 2 mM glutamine, 30 units/ml penicillin, 30 μg/ml streptomycin.

Human Embryonic Kidney 293 cells T (HEK239T, Cat#CRL-1573, ATCC, Manassas, Virginia, United States, available in the lab from a previous work (Aprile et al., 2018) were cultured in DMEM supplemented with 10% South American FBS, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin.

3T3-L1 mouse fibroblasts (Cat#CL-173TM, ATCC, Manassas, Virginia, United States) were cultured in DMEM supplemented with 10% of newborn calf serum, 2 mmol/L glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin.

The J774A.1 and RAW264.7 mouse macrophage cell lines (Cat#85011428 and Cat#91062702, respectively) were obtained from the European Collection of Authenticated Cell Culture (ECACC, Porton Down, Salisbury, UK), and cultured in DMEM supplemented with 5% South American FBS, 2 mmol/L glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin.

Human *in vitro* monocytes, THP-1 (Cat#TIB-202, ATCC, Manassas, Virginia, United States) were cultured in suspension by supplementation of RPMI culture medium with 10% South American FBS, 2 mmol/L glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin. HMSCs, HEK293T, J774A.1, RAW264.7 and THP-1 were cultured at 37 °C, in a humidified atmosphere with 5% CO2, whereas 3T3-L1 cells required 7% CO2. Media, sera and antibiotics were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

2.4. Activation of immune cells and conditioned media

THP-1 monocytes were differentiated in macrophages by treatment with 50 ng/ml of Phorbol 12-Myristate 13-Acetate (PMA, Cat#P8139, Sigma-Aldrich Inc, St. Louis, MO, United States) in a complete culture medium. Fresh medium supplemented with PMA was replaced every two days until the complete macrophage differentiation was reached (ie. 6 days upon induction). Conditioned medium was obtained by treatment of THP-1 derived-macrophages with 20 ng/ml of LPS (Cat# L2630, Sigma-Aldrich Inc, St. Louis, MO, United States) in RPMI (0.5% BSA) for 24 h. Afterward, the medium was collected, centrifuged at 1000 rpm for 10 min and cell free supernatant was used for treating hMSCs, as described below. Conditioned media were similarly obtained from J774A.1 and RAW264.7 macrophages by stimulation with LPS (0.5 ng/ml and 100 ng/ml, respectively) in DMEM (5% FBS). The medium was collected after 24 h, centrifuged at 1300g for 5 min and cell free supernatant used for 3T3-L1 treatment.

2.5. Adipocyte differentiation

Adipocyte differentiation of hMSCs and 3T3-L1 was performed as we

previously reported (Cataldi et al., 2021; Aprile et al., 2020, 2018). Particularly, hMCSs - plated at a density of 2–5 × 10^3/cm^2 and grown to 100% confluence – were treated alternating two different mixes every three days, obtained supplementing the complete cell culture medium with 850 nM insulin (Cat#AIC025707011/M, Humulin, Lilly, Indianapolis, IN, USA), 10 μM dexamethasone (Cat#D4902, Sigma-Aldrich Inc., St. Louis, MO, USA), 0.5 mM 3-isobutyl-1-methylXanthine (Cat#I7018, Sigma-Aldrich Inc., St. Louis, MO, USA), 33 μM biotin (Cat#B4639, Sigma-Aldrich Inc., St. Louis, MO, USA), 17 μM pantothenate (Cat#P5155, Sigma-Aldrich Inc., St. Louis, MO, USA) and 1 μM rosiglitazone (Cat#R2408, Sigma-Aldrich Inc., St. Louis, MO, USA; Adipocyte differentiation Induction Mix, AIM) or with 850 nM insulin and 1 μM rosiglitazone (Adipocyte differentiation Maintaining Mix, AMM).

Similarly, 3T3-L1 were cultured at confluence and induced to differentiate alternating two mixes every two days. Thus, the complete culture medium was supplemented with isobutyl methylxanthine 0.5 μM, dexamethasone 0.25 μM, rosiglitazone 10 μM and insulin 5 μg/ml or, alternatively, with insulin (5 μg/ml) and rosiglitazone (10 μM).

Adipocyte differentiation of hMSCs and 3T3-L1 was reached in 19–21 days and 8–10 days, respectively.

Moreover, hMSCs-derived mature adipocytes were pulsed toward hypertrophic-like stages. Afterward, hypertrophic-like cells were obtained by treating differentiated cells for an additional 12 days with AMM mix supplemented with palmitic or oleic acid (350 μM) or their combination (175 μM of each fatty acid), as we previously described (Aprile et al., 2020).

The high rate of differentiation was assessed by microscopic visualization and quantification of lipid accumulation by Oil Red O Staining (Cat#O1391, Sigma-Aldrich Inc., St. Louis, MO, USA), as previously described (Cataldi et al., 2021; Aprile et al., 2020, 2018).

2.6. Cell transfection

Expression vectors (pcDNA3.1/V5-His-TOPO, Cat# K480001, Invitrogen, Carlsbad, CA, USA) containing coding regions of canonical PPARG or PPARGΔ5 isoforms, were obtained as we reported in (Aprile et al., 2018). For the silencing of canonical transcripts of *PPARG,* a human DsiRNA duplex (HSC.RNAI.N005037.12.3) was specifically designed against *PPARG* exon 5 (IDT,Coralville, Iowa, USA) (Aprile et al., 2018). According to manufacturer's instructions, Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) was used in culture medium without antibiotics and serum for the transfection of HEK293T and hMSCs with *PPARG* DsiRNA (30 nM) and of HEK293T with expression vectors (1 μg/ml; pcDNA3.1/V5-His-TOPO).

hMSCs - at early stage of differentiation (ie., 9 days after adipogenic induction) - were transfected with expression vectors by electroporation, using Amaxa Human MSC Nucleofector Kit (Cat#VPE-1001, Amaxa Inc, MD, USA) and the U23 nucleofection program of Nucleofector 2b Device, according to manufacturer's instructions.

The transfection efficiency of expression vectors (estimated between 80% and 96%) was estimated using a GFP-containing vector (Cat#-pEGFN3, Clontech, Mountain View, CA, USA) and by fluorescence microscopy visualization and FACS analysis. The *PPARG* silencing efficiency (estimated between 90% and 98%) was specifically estimated by analyzing *PPARG* expression by qPCR.

2.7. Cell treatments

For all treatments, cells were starved for 16–18 h with culture medium without serum. Serum starved hMSCs and HEK293T cells were treated for 48 h with rosiglitazone (10 μM,) or troglitazone (10 μM, T2573, Sigma Aldrich St Louis, MO, USA), respectively, and pre-treated or not with GW9662 (15 μM, Cat#M6191, Sigma Aldrich St Louis, MO, USA) for 6 h. Twenty-four hours after transfection, hMSCs knockdown for canonical *PPARG* transcripts were similarly treated with rosiglitazone.

Moreover, murine mature adipocytes differentiated from 3T3-L1 were treated with the CM of J774A.1 (*vs* control cells exposed to LPS 0.5 ng/ml) and the RAW264.7 (*vs* control cells exposed to LPS 100 ng/ml). Similarly, human mature adipocytes differentiated from hMSCs were exposed to the CM of human THP-1-derived macrophages (*vs* control cells exposed to LPS 20 ng/ml).

Both murine and human mature adipocytes were treated for 24 h with recombinant cytokines (10 ng/ml), ie. murine IL-1β (Cat#211–11B, Preprotech, Rocky Hill, NJ, USA), murine TNF-α (Cat#315–01 A, Preprotech, Rocky Hill, NJ, USA) or murine IL-6 (Cat#216–16, Preprotech, Rocky Hill, NJ, USA) and human IL-1β (Cat#200–01B, Preprotech, Rocky Hill, NJ, USA), human TNF-α protein (Cat#10602-HNAE-50, SinoBiological, Beijing, China) or human IL-8 (Cat#200–08, Preprotech, Rocky Hill, NJ, USA).

2.8. RNA extraction and qPCR assays

Murine eWAT samples were homogenized by TissueLyser LT (QIAGEN, Hilden, Germany). RNA was isolated by tissues and different cellular samples by TRIzol Reagent (Cat#15596018, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and quantified by NanoDrop spectrophotometer (Life Technologies, Carlsbad, CA, USA).

High Capacity cDNA Reverse Transcription kit (Cat#4368814, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for reverse transcription of RNA samples, according to the manufacturer's protocols. Oligo 4.0. software was used for designing specific primers to be used in qPCR assays for the following human and mouse genes: *GIPR* 5'-CAATGTGAGAACCCAGAGAAG-3' (Fw), 5'-GACAGGGAGTAGCCGACAG-3' (Rv); *PPIA*: 5'-TACGGGTCCTGGCATCTTGT-3' (Fw), 5'-GGTGATCTTCTTGCTGGTCT-3' (Rv); *HPRT*: 5'-TGGCGTCGTGATTAGTGATG-3' (Fw), 5'-CCCATCTCCTTCATCACATC-3' (Rv); *Gipr*: 5'-CGGAGGAACAGGTTGAAGG-3' (Fw), 5'-TTTGTTGATGAAGCAGTAGAG-3' (Rv); *36b4*: 5'-TCCAGGCTTTGGGCATCA-3' (Fw), 5'-CTTTATCAGCTGCACATCACTCAGA-3' (Rv); *Adipoq*: 5'-AGAGAAGGGAGAGAAAGGAG-3' (Fw), 5'-GCCAGTGCTGCCGTCATAAT-3' (Rv); *Adgre1*: 5'-TGAGAACAAAAGTGCCCCAG-3' (Fw), 5'-AGAGGGTATCAGAAGAGCAG-3' (Rv); *Jam2*: 5'-CCACCGTCAAGAAGTCACAG-3' (Fw), 5'-TTGGAGAGCCTGTTGGTAGT-3' (Rv); *Msln*: 5'-AAGGGGCTGGCTATGGCTG-3' (Fw), 5'-GAAGAGCAGCAGGTCCAGT-3' (Rv); *Pdgfra*: 5'-AGATCGAAGGCAGGCACATT-3' (Fw), 5'-CTTGAACGTCCTCCCTTTGA-3'. CFX Connect Detection System (Bio-Rad, Hercules, CA, USA) and iTaq Universal Sybr Green Supermix (Cat#172–5124, Bio-Rad, Hercules, CA, USA) were used for qPCR assays, according to manufacturer's instructions. Relative quantification of gene expression was measured using the $2^{-\Delta\Delta Ct}$ method and *PPIA* was used as a housekeeping gene. All reactions were performed at least in triplicate.

2.9. Elisa

Levels of murine IL-6 inflammatory cytokine was determined by ELISA (R&D Systems, MN, USA) in cell-free supernatants according to the manufacturer's instructions. Absorbance of assay wavelength was measured at 450 nm using a Cytation 3 imaging reader (BioTek, Winooski, VT, USA).

2.10. Statistical analysis

Normal data distribution of expression values obtained by qPCR was assessed by the Shapiro–Wilk test ("shapiro.test function" implemented in R language). Differences between testing and control samples were analyzed by two-tailed (one or two sample) Student's t test (GraphPad Software Inc., La Jolla, CA, USA) and defined as significant as p value$\leq$ 0.05.

3. Results and discussion

3.1. Thiazolidinediones induce GIPR overexpression in a PPARγ-independent manner

The capacity of GIP to increase the glucose-dependent secretion of insulin in β-cells is well established (Kashima et al., 2001; Trümper et al., 2001; Ehses et al., 2002; McIntosh et al., 2012), whereas conflicting results about GIPR signaling have been described for the AT (Killion et al., 2020; Kim et al., 2010, 2007; Song et al., 2007; Getty-Kaushik et al., 2006a; Mohammad et al., 2011; Getty-Kaushik et al., 2006b; Starich et al., 1985). Indeed, both agonism and antagonism of GIPR have been reported to impair AT mass and weight gain in presence of nutrient overload (Miyawaki et al., 2002b; Nasteska et al., 2014; Mroz et al., 2019; Svendsen et al., 2020; Bailey, 2020). Moreover, the regulation of *GIPR* expression within WAT has been poorly investigated.

To this aim, first we *in silico* explored the overall expression across human tissues and then the localization - in terms of cell types/subtypes - of *GIPR* in the WAT. Using public gene expression data (bulk RNA-Seq available in GTeX, https://www.gtexportal.org/home/), we observed that *GIPR* is expressed in multiple human tissues (Fig. S1A). However, looking at single-cell expression patterns - available in GTeX as single *nuclei* RNA-Seq (snRNA-Seq) data - adipocytes do not display the highest *GIPR* expression, especially if compared to other cell types that generally populate the AT, such as macrophages, pericytes and smooth muscle cells (Fig. S1B). However, snRNA-Seq data of adipocytes available in the GTeX database derive from cells isolated from breast, esophagus and skin, whereas data from AT depots are not available.

Therefore, we used single-cell (scRNA-Seq) and snRNA-Seq data - recently generated by Emont et al., 2022 (Emont et al., 2022) and released in the single cell portal (https://singlecell.broadinstitute.org/; acc. n. SCP1376) - to evaluate *GIPR* expression in multiple cell populations composing the human and murine WAT. Of a total of about 166,000 single cells obtained from micro-dissected human WAT and undergoing RNA-Seq, only a very small fraction of the 25,000 cells - marked as *bona fide* adipocytes - expresses *GIPR* (Fig. 1A). Indeed, most *GIPR*-expressing cells in the human (Fig. 1B) and mouse (Figs. S1C and S1D) WAT are of mesothelial origin or pericytes, dendritic (DCs) and NK/T cells. Notably, these unexpected data are in line with a very recent paper - published during the writing of this manuscript - of Campbell and colleagues (Campbell et al., 2022), reporting the predominant non-adipocyte expression of *GIPR* in mouse WAT.

However, as several studies focused on the study of *GIPR* in adipose tissue and related cells (Ceperuelo-Mallafré et al., 2014; Killion et al., 2020; Starich et al., 1985; Kim et al., 2011; Mohammad et al., 2014), we aimed to clarify the expression levels of *GIPR* at different stages of adipogenesis, ie. in AT-derived mesenchymal precursors, in differentiating cells and in mature adipocytes. To this aim, we used human (hMSCs) and murine (3T3-L1) commercially available adipocyte precursors' cell lines, inducing *in vitro* the adipogenesis and obtaining well-differentiated mature adipocytes. However, both murine and human mature adipocytes display increased levels of *GIPR* compared to precursor and mesenchymal cells, respectively (Fig. 1C and 1D).

However, it is plausible that culturing conditions may affect its expression. Hence, to evaluate it, as PPARγ is the master adipogenic transcription factor and its activity is potently induced during *in vitro* adipocyte differentiation by the thiazolidinediones - contained in the adipogenic mix - we investigated whether PPARγ might modulate *GIPR* expression, as also reported in pancreatic β-cells (Gupta et al., 2010). Although *PPARG* overexpression in differentiating hMSCs – which are grown in presence of PPARγ agonist - modestly induces *GIPR* (Fig. 1E), the overexpression of *PPARGΔ5* - ie. a dominant negative isoform demonstrated to impair PPARγ activity (Aprile et al., 2018) - has no effect on *GIPR* levels (Fig. 1F), possibly suggesting that *GIPR* transcription is not directly affected by PPARγ *trans*-activation capacity. Notably, the treatment of undifferentiated hMSCs - displaying almost undetectable *GIPR* levels (Fig. 1D) - with the PPARγ agonist rosiglitazone induces a significant increase of *GIPR* levels. However, the rosiglitazone-mediated increase of *GIPR* expression is not affected either when PPARγ is chemically inhibited by the irreversible PPARγ antagonist (ie, GW9662, which prevents the PPARγ-ligand induced activation) nor after *PPARG* silencing with siRNAs targeting canonical *PPARG* isoforms (Fig. 1G). To further confirm these data, the HEK293T cell line - expressing *PPARG* at barely undetectable levels (Aprile et al., 2018; Costa et al., 2016) - was transfected to over-express functional *PPARG* full-length. These cells did not display any *GIPR* induction, whereas the treatment with the PPARγ agonist (troglitazone) - alone or in combination with PPARG over-expression - was able to induce it (Fig. 1H). Overall, these data suggest that PPARγ agonists, contained in the adipogenic mix, may - at least partially - contribute to *GIPR* induction in a PPARγ-independent manner. In the last two decades, independent studies have reported that TZDs can exert multiple PPARγ-independent actions, mostly *via* Ca^{2+} mobilization (Gras et al., 2009), including the functional activation of G-protein coupled receptors (Gras et al., 2009; Kotarsky et al., 2003) or AMPK (LeBrasseur et al., 2006) and the recruitment of transcriptional coactivators (Matthews et al., 2009). In this regard, despite experimental evidence of Sp1 and Pparγ binding in *GIPR* promoter being reported in murine and human adipose and pancreatic cells (Kim et al., 2011; Gupta et al., 2010; Baldacchino et al., 2005; Tharp et al., 2020), the complete landscape of *GIPR* transcriptional regulation has not been fully explored. Indeed, as schematized in Fig. S1E, public human ENCODE ChIP-Seq data available for various transcription factors (TFs) indicate that the putative *GIPR* promoter contains many ChIP-Seq peaks (indicative of TF binding) for different TFs. Notably, some of them - such as CREB and NFAT family members - are Ca^{2+} -sensitive, suggesting that the TZD-induced Ca^{2+} mobilization may be one of the potential mechanisms accounting for PPARγ-independent *GIPR* regulation. However, we cannot exclude that other molecules included in the adipogenic mix might also contribute to *GIPR* upregulation.

3.2. GIPR expression is impaired by obesogenic stimuli in vitro and in vivo

The mechanisms that regulate GIPR signaling, the incretin/s system and consequently the efficacy of targeting GIP/GIPR axis - especially in obese patients in which GIPR signaling is impaired in the SAT and VAT (Ceperuelo-Mallafré et al., 2014; Rudovich et al., 2007) - are still debated.

The analysis of eWAT isolated from mice treated with LPS (Pastor et al., 2017) revealed a very marked repression of *Gipr* (Fig. 2A), suggesting a negative role of obesity-related *stimuli* (*e.g.*, inflammatory molecules) on GIPR signaling. The expression analysis of multiple cell-specific markers in eWAT biopsies confirmed the marked enrichment of adipose cells in the analyzed tissue (Figs. S2A and S2B), allowing us to exclude a relevant contribution by other sub-populations expressing *Gipr* (Figs. S1C and S1D). Hence, we evaluated if the thiazolidinediones-mediated induction of *GIPR*, observed in our *in vitro* murine and human systems, is negatively affected by obesity-related *stimuli,* such as inflammation and high lipid content. To this aim, we exposed mature adipocytes - differentiated from 3T3-L1 cells in presence of rosiglitazone - to the conditioned media (CM) of two distinct LPS-activated murine macrophage cell lines, ie., the J774A.1 and the RAW264.7. Interestingly, despite differentiated in presence of rosiglitazone, cells exposed to the CM of J774A.1 macrophages display a strong repression of *Gipr* (Fig. 2B), whereas the CM of RAW264.7 cells induces it (Fig. 2C). These opposite results might be - at least partially - explained by a different repertoire of proinflammatory molecules secreted by the two different cell lines at the concentration of LPS used to stimulate the macrophages.

To clarify this point, we tested the direct effect of proinflammatory molecules secreted by the two macrophage cell lines. Particularly, we treated differentiated 3T3-L1 cells with murine recombinant TNF-α or

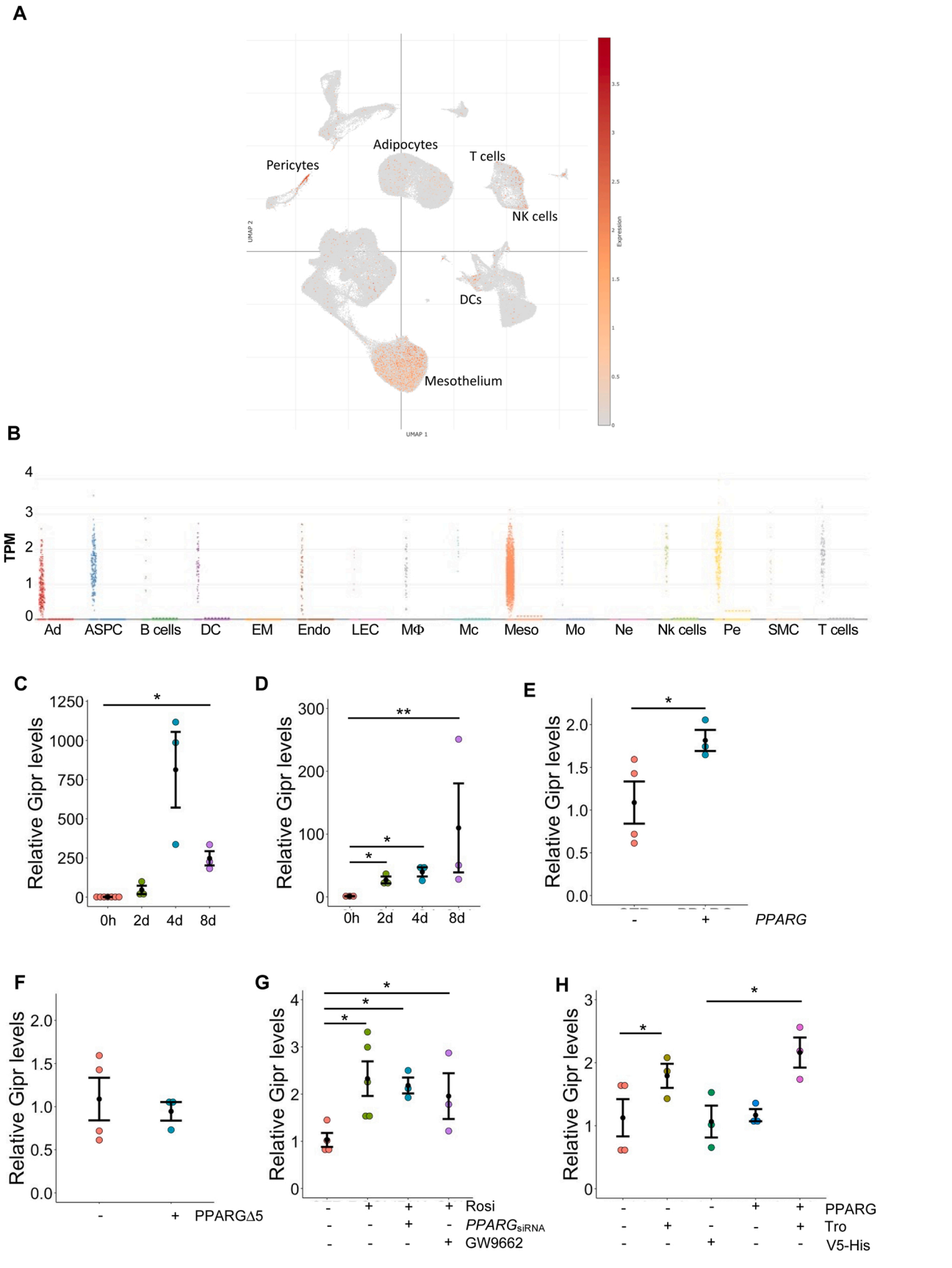

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Fig. 1. **(A)** Uniform manifold approximation and projection (UMAP) of all 166,129 sequenced human cells split by cell type clusters in the human WAT and reporting *GIPR* expression where each colored dot (color heat map on the right) corresponds to a single cell (downloaded and modified from the Single Cell portal, study n. SCP1376). **(B)** Boxplot showing the expression of human *GIPR* gene for each cell type in the human WAT; each colored dot corresponds to a single cell (downloaded from the Single Cell portal, study n. SCP1376). Ad: adipocytes; ASPC: (adipose stem and progenitor cells). DC: dendritic cells; EM: endometrium; Endo: endothelium; LEC: lymphatic endothelial cells; MΦ: macrophages; Mc: mast cells; Meso: mesothelium; Mo: monocytes; Ne: neutrophils; Pe: pericytes; SMC: smooth muscle cells. **(C)** Relative mRNA quantification (qPCR) of *Gipr* levels at different time points of mouse adipocyte differentiation (ie. 2, 4 and 8 days upon 3T3-L1 differentiation induction). 3T3-L1 cells at undifferentiated stage (ie. 0 h) were used as reference samples and *36b4* as reference gene. Data are reported as mean ± SEM of three independent experiments. **(D)** Relative mRNA quantification (qPCR) of *GIPR* levels at different time points of human mesenchymal stem cells (hMSCs) adipocyte differentiation (ie. 9, 12 and 21 days upon differentiation induction). hMSCs at undifferentiated stage (ie. 0 h) were used as reference samples and *PPIA* as reference gene. Data are reported as mean ± SEM of three independent experiments. **(E-F)** Relative quantification (qPCR) of *GIPR* mRNA levels in hMSCs derived-mature adipocytes transfected with PPARG **(E)** or PPARGΔ5 **(F)** expression vectors at early stage (ie., 9 days after adipogenic induction). Differentiating hMSCs - grown in presence of rosiglitazone-containing adipogenic mix - transfected with empty vectors were used as control samples. *PPIA* was used as a reference gene. Data are reported as mean ± SEM of three independent experiments. **(G)** *GIPR* relative mRNA quantification (qPCR) in undifferentiated hMSCs (ie. 0 h) treated for 48 h with rosiglitazone (10 μM) or knockdown for canonical *PPARG* (estimated silencing efficiency=75%, $p = 0.0024$) and treated for 48 h with rosiglitazone (10 μM) or pre-treated for 6 h with GW9662 (15 μM) and treated for 48 h with rosiglitazone (10 μM). hMSCs at 0 h similarly treated with vehicle (ie. DMSO) or transfected with scrambled were used as control samples. *PPIA* was used as a reference gene. Data are reported as mean ± SEM of at least three independent experiments. **(H)** Relative mRNA quantification (qPCR) of *GIPR* levels in HEK293T cells transfected with PPARG expression vector and/or treated for 48 h with troglitazone (10 μM). HEK293T cells transfected with empty vector (V5-His) or treated for 48 h with vehicle (ie. DMSO) were used as control samples respectively. *HPRT* was used as reference gene. Data are reported as mean ± SEM of at least three independent experiments. Statistical significance (p value ≤0.05) has been assessed by using two-tailed (one sample or two samples) Student's t test. *$p \leq 0.05$; **$p \leq 0.01$.

IL-1β or IL-6 proteins. Accordingly, cells treated with TNF-α exhibit a marked *Gipr* repression, whereas the treatment with IL-6 induces the expression of the *Gipr* and IL-1β do not produce any significant change (Fig. 2D). As IL-6 treatment unexpectedly induced *Gipr* expression, we measured its levels in the CM of both unstimulated (basal) and LPS-stimulated RAW264.7 and J774.1 cell lines. Both untreated and LPS-treated RAW264.7 cells secrete significantly higher amounts of IL-6 compared to LPS-stimulated J744.1 cells that - at basal growing conditions (ie. not stimulated by LPS) - display almost undetectable IL-6 levels (Fig. S2C). This finding suggests that IL-6, differently from the other pro-inflammatory cytokines, at least in mice, may differentially affect *Gipr* expression. Then, to verify the effect of pro-inflammatory *stimuli* on *GIPR* expression in human adipocytes, we treated mature cells - differentiated (in presence of rosiglitazone) from hMSCs - with the CM of the THP-1-derived macrophages. As shown in Fig. 2E, mature adipocytes exposed to macrophage CM display a marked repression of *GIPR,* even though they have been differentiated in presence of the PPARγ agonist. Moreover, differently from what is observed in murine cells, independent treatments with TNF-α, IL-1β or IL-8 induce strong repression of *GIPR* in human mature adipocytes (Fig. 2 F). In this context, these conflicting results could be - at least in part - explained by the inter-species differences between mouse and human in the inflammatory response (Mestas and Hughes, 2004; Seok et al., 2013; Zschaler et al., 2014).

Finally, to evaluate if the thiazolidinediones-induced over-expression of *GIPR* is sensitive to increased lipid accumulation, we used a highly reproducible *in vitro* model of hypertrophic-like adipocytes (HAs) (Aprile et al., 2020). Differentiated human adipocytes treated with rosiglitazone were cultured in a medium containing saturated (ie. palmitic acid) and/or monounsaturated fatty acids (ie. oleic acid) highly abundant in western diets. As previously assessed (Aprile et al., 2020), treated cells supplemented with FFAs become HAs containing large LDs (Fig. 2G).

Interestingly, despite the presence of the PPARγ agonist, terminally differentiated adipose cells, supplemented either with single - or a combination of - FFAs, display a marked *GIPR* repression (Fig. 2H). We excluded any confounding effect due to the presence of inflammatory factors in cell culture media, as we could not detect IL-1β, TNF-α or other proinflammatory cytokines (*e.g.*, IL-1R2 and IL-10) in the media collected from adipocytes supplemented or not with FFA mix (data not shown).

Overall, our findings indicate that obesity-related pro-inflammatory cytokines and increased lipid accumulation negatively affect *GIPR* expression in adipocytes.

4. Conclusions

Obesity is a driving force for the onset of metabolic disorders, including IR, T2DM and dysfunctions of the adipose tissue associate with disrupted metabolic homeostasis (Blüher, 2020; Arner et al., 2010). Hence, pharmacological interventions focused on preserving AT health have been proposed for IR and T2DM treatment (Gustafson et al., 2009; Blüher, 2016; Smith and Kahn, 2016). Notably, the downregulation and desensitization of GIPR have been proposed to impair the response to GIP agonism (Mohammad et al., 2014; Shu et al., 2009) and contrasting results on the targeting GIP/GIPR axis in AT - partially due to inter-species differences between rodents and humans - challenged the therapeutic potential of GIP agonists (Killion et al., 2020; Kim et al., 2010, 2007; Song et al., 2007; Getty-Kaushik et al., 2006a; Mohammad et al., 2011; Getty-Kaushik et al., 2006b; Starich et al., 1985). Our work, by investigating *in silico*, *in vitro* and *in vivo GIPR* expression and regulation, reveals that thiazolidinediones - routinely used for IR treatment - induce *GIPR* expression in a PPARγ-independent manner and that multiple proinflammatory *stimuli* and excessive lipid engulfment promote *GIPR* downregulation, opening new interesting perspectives. Indeed, despite most of our results being obtained in murine and human cell lines, overall these findings suggest that, in obese individuals, the inflammatory *milieu* of AT and the hypertrophic state of adipose cells may modify the efficacy of GIP agonists, such as the co-administration of thiazolidinediones.

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CRediT authorship contribution statement

S. Cataldi: conceptualization, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review & editing. **M. Aprile:** conceptualization, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review & editing. **C. Perfetto**: investigation, validation, visualization. **B. Angot:** formal analysis, investigation, validation, visualization. **M. Cormont**: formal analysis, investigation, validation, visualization. **A. Ciccodicola:** resources, supervision, writing – review & editing. **J. F.**

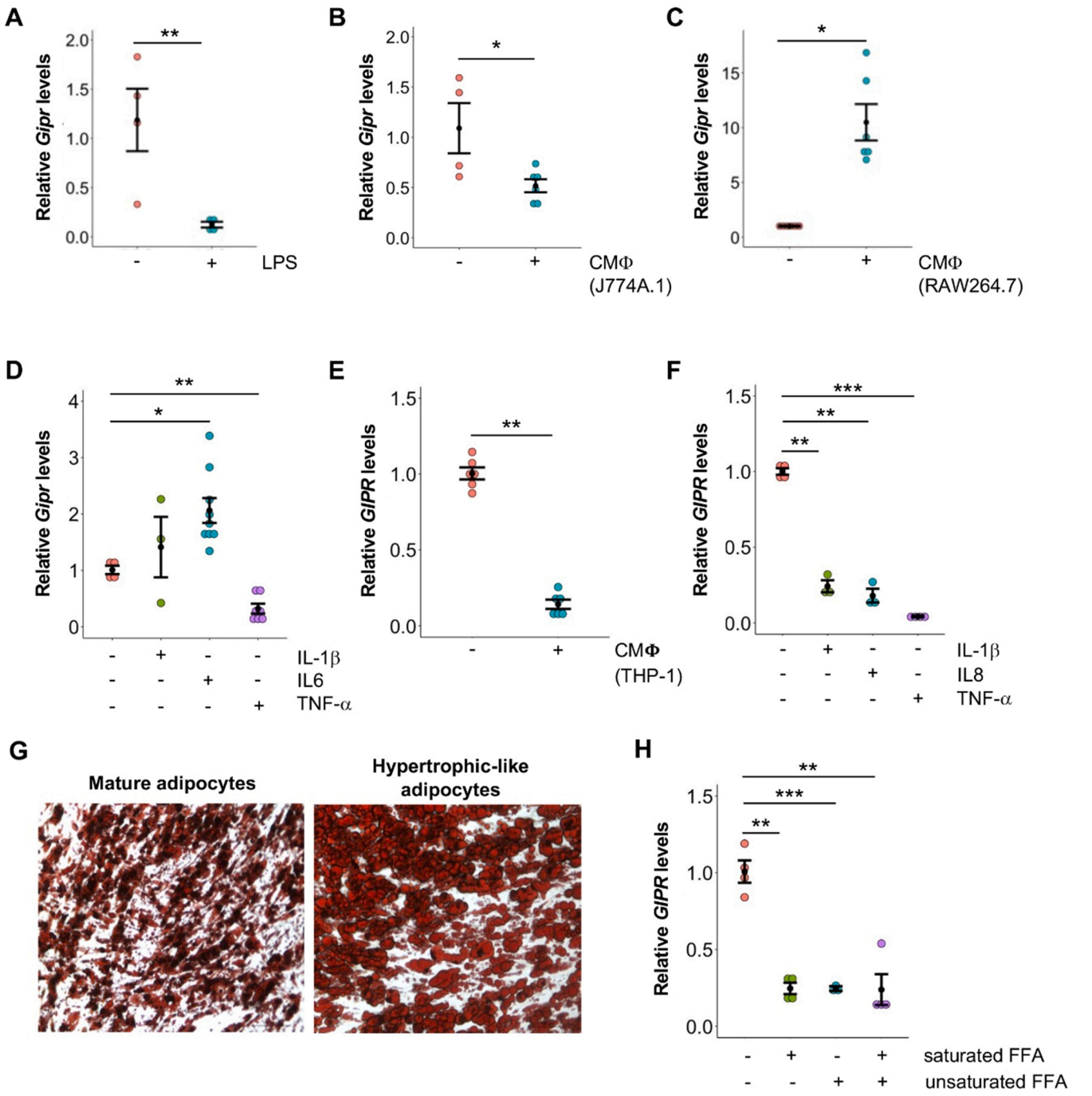

Fig. 2. **(A)** Relative mRNA quantification (qPCR) of *Gipr* in epididymal adipose tissue of C57BL/6JB-LPS injected mice (n = 6). Epididymal adipose tissue from control mice (n = 7) was used as reference samples and *36b4* as reference gene. Data are reported as mean ± SEM from independent experiments. **(B-D)** Relative mRNA quantification (qPCR) of *Gipr* in mature adipocytes differentiated from 3T3-L1 cells (ie. 8 days upon adipocyte differentiation induction) treated for 24 h with conditioned medium of LPS activated J774.A1 (B) or RAW264.7 macrophages or with mouse recombinant cytokines (ie. TNF-α, IL-1β or IL-6; D). Mature adipocytes treated with control medium plus LPS (B, C) or treated with vehicle (ie. PBS; D) were used as control samples. *36b4* was used as reference gene. Data are reported as mean ± SEM from at least three independent experiments. **(E-F)** Relative mRNA quantification (qPCR) of *GIPR* in mature adipocytes differentiated from hMSCs (ie. 21 days upon adipocyte differentiation induction) treated for 24 h with conditioned medium of LPS activated THP-1 macrophages (E) or with human recombinant proteins (ie. TNF-α, IL-1β or IL-8; F). Mature adipocytes treated with control medium plus LPS (E) or treated with vehicle (ie. PBS; F) were used as control samples. *PPIA* was used as reference gene. Data are reported as mean ± SEM from at least three independent experiments. **(G)** Representative images of hMSCs derived-mature adipocytes (ie. 21 days upon differentiation induction) and hypertrophic-like adipocytes (ie. 32 days upon differentiation induction using palmitate fatty acid) stained with Oil red O. **(H)** Relative mRNA quantification (qPCR) of *GIPR* in hypertrophic-like adipocytes generated by the treatment of hMSCs derived-mature adipocytes with saturated/unsaturated fatty acids (ie. palmitate/oleate). hMSCs derived-mature adipocytes were used as control samples and *PPIA* as reference gene. Data are reported as mean ± SEM from at least three independent experiments. Statistical significance (p value ≤0.05) has been assessed by using two-tailed (one sample or two samples) Student's t test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Tanti: conceptualization, data curation, funding acquisition, project administration, supervision, writing – review & editing. **V. Costa:** conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, visualization, writing – original draft, writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

For in silico analyses we have used public data and all the links have been reported in the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2023.151320.

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