Adaptive changes of the Insig1/SREBP1/SCD1 set point help adipose tissue to cope with increased storage demands of obesity.

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ABSTRACT
The epidemic of obesity imposes unprecedented challenges on human adipose tissue (WAT) storage capacity that may benefit from adaptive mechanisms to maintain adipocyte functionality. Here, we demonstrate that changes in the regulatory feedback set point control of Insig1/SREBP1 represents an adaptive response that preserves WAT lipid homeostasis in obese and insulin resistant states.

In our experiments we show that Insig1 mRNA expression decreases in WAT from mice with obesity-associated insulin resistance, from morbidly obese humans and in in vitro models of adipocyte insulin resistance. Insig1 down-regulation is part of an adaptive response that promotes the maintenance of SREBP1 maturation, facilitates lipogenesis and availability of appropriate levels of fatty acid unsaturation partially compensating the antilipogenic effect associated with insulin resistance. We describe for the first time the existence of this adaptive mechanism in WAT, which involves Insig1/SREBP1 and preserves the degree of lipid unsaturation under conditions of obesity-induced insulin resistance. These adaptive mechanisms contribute to maintain lipid desaturation through preferential SCD1 regulation and facilitate fat storage in WAT, despite on-going metabolic stress.
INTRODUCTION

The epidemic of obesity is testing the capacity of white adipose tissue (WAT) to cope with an unprecedented nutritional pressure and demand to expand. We and others have proposed that the expansion and dysfunction of WAT may be an important pathogenic contributor to obesity-associated metabolic complications (1). However, a recurrent observation from clinical studies is that obese patients maintain a remarkable capacity to store fat before adverse metabolic effects occur. Moreover, we have recently demonstrated using a monozygotic twin cohort discordant for obesity that while expanding, the WAT adapts its biochemical features to maintain its biophysical characteristics and preserves its functionality by increasing the degree of fatty acid (FA) unsaturation and chain length in adipocyte membrane phospholipids. This implies the existence of adaptive homeostatic mechanisms that preserve WAT functionality (2).

Intrigued by these and other observations showing that improving WAT expandability and storage capacity ameliorates insulin sensitivity (3), we hypothesised the existence of adaptive responses that optimise fat storage and preserve WAT homeostasis. These adaptations may help WAT to cope with the added storage demands imposed by positive energy balance through changes in transcriptional and biochemical cycles. The interest in identifying these adaptive mechanisms is fuelled by their potential as biomarkers of metabolic stress (4) with diagnostic, prognostic and therapeutic value in human obesity.

SREBPs are transcription factors that control genes involved in FA and triglyceride (TG) synthesis via SREBP1, or cholesterol biosynthesis through SREBP2. Due to alternative splicing, two isoforms of SREBP1 exist. The highly active Srebp1a isoform is predominantly expressed \textit{in vitro}, whereas Srebp1c, a less active form, is predominantly expressed \textit{in vivo}. Demonstrated SREBP1 targets
include genes involved in rate-limiting de novo lipogenesis steps e.g acetyl-CoA carboxylase (Acc) and fatty acid synthase (Fas) (5-7), FA desaturation (e.g. Stearoyl-CoA desaturase 1, Scd1) (6, 8) and elongation (e.g elongase 6, Elovl 6) (9, 10), FA re-esterification (e.g mitochondrial glycerol-3-phosphate acyltransferase, Gpat1) (11) and phospholipid synthesis (e.g CTP:phosphocholine cytidylyltransferase α, Ccαtα) (12, 13). Given these pleotropic effects of SREBP1 on lipid metabolism we hypothesised that SREBP1 may play a leading role in adaptive responses in WAT. Moreover, since a key event in this response is the post-translational maturation and activation of SREBP1, and this process is highly dependent on the activity of Insulin-induced genes 1 and 2 (Insig1 and 2), we hypothesised that the set point linking SREBP1 and Insig1 may be important for the process of WAT adaptation to excess lipid storage.

It is well established that under conditions characterised by high sterol levels or elevated polyunsaturated/unsaturated FAs, SREBP1 is physiologically inactivated. Under these conditions, SREBP1 remains anchored in the ER as part of a tripartite complex with SREBP cleavage activating protein (SCAP) and Insig1 or Insig2 (14, 15) which exert a permissive retaining effect on SREBP1 preventing its maturation and activation. This regulatory system is highly sensitive and rapidly activated in response to conditions requiring SREBP1 activation (e.g. decreased polyunsaturated FA, anabolic effects of insulin, or excess saturated FA). In these situations the ER-anchoring activity of Insigs is diminished, allowing SCAP to escort SREBP1 from the ER to the Golgi to be cleaved producing a soluble and transcriptionally active SREBP1 mature protein.

The importance and dependence on Insig1 for the regulation of SREBP1 activity and lipogenic capacity has been demonstrated in vitro and in vivo. Overexpressing Insig1 in 3T3-L1 adipocytes inhibits both adipogenesis and lipid
accumulation (16). Similarly, PPARγ agonists can induce Insig1 expression in vitro and in vivo in WAT (17, 18) while promoting adipocyte differentiation, suggesting that Insig1 acts as a control set point to limit the amount of fat that can be stored under anabolic conditions. Coordinating this regulation, insulin itself can activate SREBP1 and promote lipogenesis whilst, simultaneously, Insig1 expression is induced and restricts further SREBP1 activation. This has been demonstrated both in vitro (17, 19, 20) and in vivo (21). These findings indicate a physiological negative feedback regulatory loop consisting in the regulation of Insig1 expression by SREBP1 in order to finely control its own cleavage. Akin to that this type of auto-regulatory feedback loop has already been suggested for the physiological control of cholesterol synthesis in liver via Insig1/SREBP (21, 22). However pathophysiological dysregulation of this loop in WAT in the context of obesity has never been considered.

In addition to genes involved in de novo synthesis of FAs and TG storage, SREBP1-target genes are also involved in the supply of specialised FAs used for critical cellular roles including maintenance of membrane lipid homeostasis, lipid modification of proteins and effectors of intracellular signalling (23). Based on previous knowledge, we hypothesised that in insulin resistance (IR) state, the down-regulation of Insig1 will lead to a resetting of the physiological negative Insig1/SREBP1 regulatory feedback loop to optimise active SREBP1 levels in an attempt to maintain adipose lipid homeostasis and preserve the synthesis of specific lipid species essential for cell functionality. Similar concept has been described in a recent report that showed that in C. elegans and mammalian cells, genetic limitation in the biosynthesis of phosphathydylcholine (PC) phospholipids lead to changes in membrane composition and functionality, and promoted increased levels of nuclear mature SREBP1, and elevated lipogenesis. This study proposes a conserved feedback
loop responding to PC levels and ensuring adequate levels of PC production (24). In a different experimental model, perturbation of phosphatydylethanolamine (PE) synthesis in the Drosophila heart leads to alteration in PE distribution in cardiac sarcolemmal membranes. Attempts to compensate this specific lipid perturbation involved adaptive elevation in SREBP1 mature protein levels and inappropriately increased lipogenesis (25) and associated pathology.

Here, we investigate whether coordinated regulation of Insig1/SREBP1 could work as an adaptive mechanism facilitating lipid biosynthesis to maintain WAT lipid homeostasis defying the recognised antilipogenic effect determined by IR. In agreement with this model, our results indicate that decreased Insig1 expression in WAT is a common feature of murine models of diet- and genetic-induced obesity, exposed to excessive nutrient pressure and is operative in human obesity and IR. In support to this concept we have in vitro and in vivo evidence that decreased Insig1 as observed in obese individuals, facilitates adipocyte differentiation and fat deposition. Finally, we discuss whether this adaptive response may contribute to preserve lipid generation and storage under adverse metabolic conditions.
RESEARCH DESIGN AND METHODS

Human samples. All subjects from the five cohorts studied gave their consent after being informed of the nature, purpose and possible risks of the different studies. All Ethical Committees gave their approval.

Animal care. Animals were housed in a temperature-controlled room with a 12-h light/dark cycle. Food and water were available ad libitum. Mice were fed with standard chow diet or a 45% high fat diet (HFD) (Research diet D12451) according to the experiment. All animal protocols were approved by the UK Home Office and the University of Cambridge, and mice were cared for according to the Guiding Principles for Research Involving Animals and Human Beings. Ob/ob and lean control (mix 129Sv-C57BL6) were generated as described in Medina-Gomez et al. (26).

Insig1 KO mouse. This mouse was generated by AstraZeneca Transgenics and comparative Genomics (ATCG), AstraZeneca R&D Molndal (Sweden) (Figure S11). For this study, tissue samples were collected from 11-12 weeks old male mice. Dual-energy X-ray absorptiometry (DEXA, Lunar Corporation) was used to measure body composition.

Cell culture and in vitro insulin resistance studies. 3T3-L1 preadipocytes were cultured as previously reported in (27). Insulin resistance was induced at day 9 of differentiation by incubating the cells in DMEM (25mM glucose) containing 100nM of insulin for 16 hours.
Stable Insig1 knockdown 3T3-L1 cell line. Insig1 stable knockdown and control cell line were generated as previously described in (28). Cells were cultured, differentiated into adipocytes, and stained for Oil-Red-O as described previously (29). TG content was determined as reported in (30).

RNA preparation and qRT-PCR. RNA extraction qRT-PCR were performed as previously reported (26, 28). The input value of the gene of interest was standardized to 18S.

Western blot analysis. Protein isolation from cells and murine and human adipose tissue was carried out as reported in (28, 31). Proteins were electrotransfered on PVDF membranes (Millipore) and probed with pAKT (Thr308), total AKT, α-tubulin (Cell Signalling), SREBP1 (Santa Cruz Biotechnology), INSIG1 and β-actin (Abcam) antibodies.

Total FA profiling-GC-MS experiments. Lipid profiling of adipose tissue (human and murine) was assessed by GC-MS as described in (32). Subfractionation of adipose tissue was performed according to (33). Shotgun lipidomics were performed as described in (26, 34).

De novo FA synthesis assay. Insig1 KD and control cells were differentiated in 24 well plates. After overnight serum starvation both cell lines were treated with or without 200nM of C75 (a Fas inhibitor) and with or without insulin, for 30 min previous to add $^{14}$C-sodium acetate into the medium. Incorporation of $^{14}$C-sodium acetate into lipids was assessed during 60 min. Cells were then lysed with 1 vol. of 1N KOH and the supernatant transferred to a screw top vial. Subsequently 1 vol. of
5M KOH in 15% methanol was added and the lysate heated for 45 min at 65°C to saponify. Then 1 vol. of cold 5M HCL was added to acidify the reaction in fume cupboard, followed by the addition of 1 vol. cold absolute ethanol. Saponified lipids were extracted twice by adding 7.5 vol. of n-hexane. Hexane containing the radioactive lipid mixture was mixed with the compatible organic-solvent scintillate and radioactivity was measured using liquid scintillation counter.

**Statistical analysis.** The data presented here were analysed SPSS version 19 (IBM) using ANOVA, General-Linear Model for ANCOVA and the Student t-test (unpaired). Significance levels was set at p<0.05. Principle Component Analysis was carried out using Simca 12 (Umetrics).
RESULTS

Down-regulation of *Insig1* and *Srebp1* mRNA and maintenance of SREBP1 mature protein in WAT of murine models of diet- and genetic-induced obesity.

We examined whether *Srebp1* and *Insig1* mRNA levels were altered in hypertrophic white adipocytes from mice undergoing nutritional excess, obesity and IR. Our data show that 6 months on HFD caused obesity and IR, as indicated by decreased levels of p-AKT in WAT (Figure S1A). At transcriptional level *Srebp1c* and *Insig1* mRNA expression was decreased (Figure 1A). These changes were not observed after 15 days on HFD (Figure 1B), when carbohydrate metabolism was still unaffected and the animals were healthy and actively expanding their WAT (Figure S1B). Interestingly, despite a reduction in *Srebp1c* mRNA expression, 6 months on HFD was associated with increased levels of transcriptionally active mature SREBP1 protein in WAT when compared to chow fed animals (Figure 1C and S1C). Maintenance of SREBP1 maturation may result from reduced *Insig1* mRNA expression. Of note, the level of SREBP1 protein was similar in WAT from 15 days HFD and chow fed animals (Figure 1D and S1D).

We also examined *Srebp1c* and *Insig1* in WAT from genetically obese and IR *ob/ob* mice. At 4 months of age, when *ob/ob* mice are severely obese, hyperglycaemic and IR (26), expression of both, *Srebp1c* and *Insig1* mRNA in WAT, is decreased compared to lean, insulin sensitive control mice (Figure 1E). Of note, this is not a leptin deficiency dependent effect since levels of *Srebp1c* and *Insig1* mRNA are increased in young 1 month old *ob/ob* mice (Figure 1F), when they are still insulin sensitive and glucose tolerant as indicated by increased markers of insulin sensitivity e.g Glut4 (Figure 1F) and *Adiponectin* (26). At one month of age, *ob/ob* mice show active expansion of their WAT and as observed previously in the DIO model, the WAT of *ob/ob* mice of both, 1 and 4 months of age, showed similar levels of
SREBP1 active protein with respect to WT littermates (Figure 1G, H, S1E and S1F) irrespectively of their gene expression pattern. Further support for discordant regulation of SREBP1 in lean and obese mice came from analyses of adipocyte sizes in 10 week old ob/ob mice. In line with the concept that larger adipocytes are associated with insulin resistance, we found a negative correlation between adipocyte size and SREBP1 mature protein levels (Figure S10E) in both the ob/ob and wild-type mice. However, the levels of SREBP1 for a given size of adipocyte were 5 times higher in the ob/ob.

*Insig1 and Srebp1 mRNA are specifically down-regulated in adipocytes of morbidly obese humans.*

We next determined whether the same Insig1/SREBP1 adaptive changes were observed in human WAT. We analysed *Srebp1* and *Insig1* in WAT from lean (BMI 22.2±0.8), obese (BMI 30.2±1.1) and morbidly obese (MO) (BMI 48.9±1.8) human subjects (Table S1, cohort 1). Expression of *Insig1* mRNA was severely decreased in both omental (Om) and subcutaneous (SC) isolated adipocytes from obese and MO when compared to lean individuals (Figure 2A). In the same isolated adipocyte samples, *Srebp1c* and *Srebp1a* mRNAs were also specifically reduced in MO compared to lean and obese individuals (Figure 2B). In an independent set of human-samples (Table S1, cohort 2), we confirmed that INSIG1 protein levels were reduced in SC WAT from MO (BMI 50.5±5.0) compared to lean subjects (BMI 25.1 ± 0.9) (Figure 2C).

*Insulin resistance leads to Insig1 down-regulation in subcutaneous adipose tissue resulting in maintenance of mature SREBP1 protein.*
We then compared SC WAT from MO patients showing different degrees of IR (MO Non-IR, BMI 39.0 ± 4.27, HOMA 2.31 ± 0.44 and MO IR, BMI 41.9 ± 1.84, HOMA 4.40 ± 0.51 respectively) vs. slightly overweight controls (BMI 26.1 ± 1.19, HOMA 1.57±0.19) (Table S1, cohort 3) to assess whether different degrees in obesity (BMI) and IR status affected Insig1/SREBP1. *Srebp1* mRNA level was preserved in adipocytes from MO Non-IR vs. controls, yet it was significantly decreased in MO IR subjects adipocytes (Figure 2D). In addition, *Insig1* expression was significantly decreased in MO Non-IR and further decreased in MO IR (Figure 2D) vs. overweight individuals and this was associated with maintenance of similar levels of mature SREBP1 protein among the three experimental groups (Figure 2E). Assessment of INSIG1 and SREBP1 protein expression in SC WAT of an independent cohort (Table S1, cohort 4) of MO subjects with more extreme BMI and different degree of IR, MO Non-IR (BMI 56.72 ±1.71, HOMA 3.60 ± 0.32) and IR (BMI 56.30 ± 1.65, HOMA 12.76 ± 1.25) showed significantly lower INSIG1 protein levels in the more IR patients (Figure S2A), whereas the mature form of SREBP1 was present in similar amount between the two groups (Figure S2B). Similar type of analysis performed in omental adipose tissue of the same cohort (Figure S3A and B) revealed comparable level of expression of mature SREBP1 and INSIG1 proteins among lean, MO Non-IR and MO IR. Globally considered, these results indicate that the changes in Insig1 may be predominantly driven by IR, and that changes in Insig1 protein levels are preferentially affected in the human SC AT, suggesting that the SC depot may be more affected by metabolic stress than the Om WAT depot (35, 36).

Using an *in vitro* model of induced IR in 3T3-L1 adipocytes, showing decreased levels of p-AKT (Figure S4A), *Glut4* and *Adiponectin* mRNA (Figure S4B), we recapitulated the changes in *Srebp1* and *Insig1* observed *in vivo* (Figure
S4C,D), further supporting a primary mechanistic role for IR in Insig1 and SREBP1 changes.

**Maintained mature SREBP1 levels in MO-derived adipocytes maintain expression of genes regulating unsaturation and triglycerides re-esterification.**

Expression analysis of SREBP1-target genes in isolated Om- and SC-derived human adipocytes from lean, obese and MO individuals (Figure 3A and B, respectively) (Table S1, cohort 1) showed that the *de novo* FA synthesis genes- *Acc1* and *Fas* -were significantly reduced in Om and SC adipocytes from MO (BMI 48.9±1.8) humans when compared to either lean (BMI 22.2±0.8) or obese (BMI 30.2±1.1) adipocytes. Of relevance, the expression of *Scd1* was relatively conserved in obese, only showing a moderate decrease when comparing lean and MO SC adipocytes. Similarly, *Cctα* expression was conserved in obese and only significantly reduced in the MO derived Om adipocytes compared to lean. Expression of genes involved in FA re-esterification, e.g *Gpat1* and *Dgat1* (diacylglycerol O-acyltransferase 1), was maintained in the obese population in both adipose depots independently of their IR status. As previously shown (37, 38), *Glut4* mRNA was markedly decreased in adipocytes from IR obese and MO humans when compared to lean.

The same pattern of SREBP1 target genes expression was observed in our IR 3T3-L1 *in vitro* model (Figure S4E) and our DIO and *ob/ob* murine models (Figure S5A,C) when they developed IR. When still insulin sensitive, WAT from mice fed 15 days a HFD and 1 month old *ob/ob* mice exhibited similar lipogenic gene expression pattern than their control littermates (Figure S5B, D).

Altogether, these data suggest that the combination of reduced Insig1 expression and optimised maturation of SREBP1 in WAT of the MO population was
associated with maintained FA desaturation and re-esterification SREBP1-dependent pathways.

**Decreased expression of *Insig1* facilitates triglyceride accumulation and accelerates differentiation of 3T3-L1 cells.**

It has previously been shown that *Insig1* mRNA levels increase during differentiation of 3T3-L1, and its overexpression decreases lipid accumulation during adipogenesis (16). We examined whether decreasing Insig1 as observed in SC WAT of obese IR individuals facilitated lipid accumulation in adipocytes. To address this question, stable Insig1 KD and control cell lines were generated. Gene expression analysis performed in these cells cultured at both low (5mM) and high glucose concentration (25mM), showed a reduction of *Insig1* mRNA of 60-80% (Figure 4A and S6A). Notably, *Insig2* mRNA levels were unchanged (Figure 4A and S6A). In the absence of Insig1, *Srebp1c* was up-regulated, regardless of glucose concentration (Figure 4A and S6A), whereas *Srebp1a* mRNA levels were significantly increased only at 5mM glucose (Figure 4A). *Srebp1* up-regulation was associated with a significant increase in mature SREBP1 in I1KD cells vs. controls (Figure 4B and S6B).

I1KD cells showed increased lipid accumulation compared to controls, regardless of glucose concentration, as shown by Oil-Red-O staining (Figure 4C and S6C) and measurement of TG content (Figure 4D and S6D). Increased TG levels in I1KD cells corresponded with increased lipogenic genes expression (Figure 4E and S6E), particularly *Scd1*, at both low and high glucose concentrations. The primary effects of decreased Insig1 on fat deposition and *Scd1* expression were confirmed by de novo lipogenesis assay and lipidomic analysis performed on I1KD cells vs. controls. The functional relevance of Insig1 knockdown was confirmed as de novo
lipogenesis functional assay showed that I1KD cells incorporated more $^{14}$C-sodium acetate than control cells after 1 hour incubation, effect that was further enhanced by insulin (Figure 4F). In support of a preferential effect on desaturation, global FFA profile performed on Insig1 KD cells showed that Insig1 depletion lead to a significant increase in the desaturation indexes (C18:1n9/18:0; C16:1n7/16:0 and C14:1n5/14:0) associated with SCD1 activity (Figure 4G).

**Insig1 KO WAT and adipocyte primary culture show increased Srebp1, SREBP1 lipogenic target genes expression and augmented lipid accumulation.**

The results of the Insig1 KD *in vitro* model were validated *in vivo* using an Insig1 KO mouse (generation strategy described in Figure S11). Although this murine model has a complex phenotype characterised by being lighter (Figure S7A-C) and shorter (Figure S7B), it is useful to study the effects of Insig1 genetic ablation without the confounding effect of IR on lipogenesis. In support of a facilitated fat deposition phenotype, Insig1 KOs exhibit increased % of fat mass (Figure 5A), and increased gonadal and subcutaneous depot mass (% of body weight) (Figure S7D). Concerning the main metabolic parameter such as food consumption (Figure S7E), glucose tolerance (Figure S8A), insulin sensitivity (Figure S8B), RER (Figure S8C) and energy expenditure (Figure S8D), this mouse model does not present any difference with respect to their wt littermates. Concerning blood biochemistry the Insig1 KO mice show decreased TG when compared to controls (Table S3). Moreover, Insig1 KO mice show increase number of smaller adipocyte in gonadal WAT depot (Figure S9). We also performed transcriptional analysis of gonadal WAT from Insig1 KO model that revealed increased *Srebp1c* and a trend for increased *Srebp2* mRNA levels (Figure 5B). Protein analysis confirmed that SREBP1 mature protein form was also increased (Figure 5C). As seen in I1KD 3T3-L1 adipocytes, no compensatory
alteration in *Insig2* was observed and in the context of preserved insulin sensitivity, expression levels of *Fas*, *Acc1*, and *Scd1* were increased in parallel with *Srebp1c* (Figure 5D).

*In vitro* differentiation of primary cultures of Insig1 KO WAT preadipocytes, showed increased lipid accumulation (Figure 5E) and augmented expression of lipid accumulation markers e.g adipose differentiation-related protein (*Adrp*) and *Perilipin* at day 4 of differentiation (Figure 5F), compared to WT counterparts. As shown for gonadal WAT gene profiling performed on Insig1 KO WAT primary culture confirmed an increase in the expression of SREBP1 target genes, including *Scd1*, *Fas* and *Acc1* (Figure 5F).

**Increased SCD1 activity in the white adipose tissue of Insig1 KO mice associated with increased Scd1 expression.**

Next, we assessed whether the depletion in *Insig1* expression and associated up-regulation of *Scd1* affected the FA composition of WAT *in vivo*. FA profiling of TGs revealed a significant increase in the levels of oleate and palmitoleate as well as an increase in the SCD1 ratio of the essential FFAs of the Insig1 KO mouse compared to wild-type mice (Table 1). Of note, we did not observe changes in products of SCD1 or a change in the SCD1 ratio of the phospholipids of the Insig1 WAT, consistent with defence of the PL compartment. Of note, these analyses also identified an increased amount of non-essential fatty acids in the TGs, consistent with higher rates of de novo fatty acid synthesis in this model. A similar phenotype of preserved PL SCD1 ratio with elevated TG SCD1 ratio was also observed in the WAT of ob/ob mice via two independent analyses (S10 A-D). Similarly, we checked if the observed change in the Insig1/SREBP1 set point in WAT of MO patients vs. lean controls was associated with maintenance of the pool of unsaturated FAs. Lipid composition of SC
WAT from lean (BMI 23.2±1.2), and MO (BMI 54.0±4.9, HOMA 4.0± 1.4) (Table S1, cohort 5) revealed that the absolute amount of unsaturated FAs was conserved between the two groups. Moreover, we observed significant reduction of specific saturated FAs (20:0, 22:0) in WAT of MO vs. lean controls (Table S2). This result agrees with our recent analysis of WAT of weight-discordant monozygotic co-twins, showing that obese twins have a reduction in specific saturated FAs (e.g 12:0, 18:0, and 20:0) and increased proportion of specific unsaturated FAs such as palmitoleic (16:1n-7) and arachidonic acid (20:4 n-6) (2).
DISCUSSION

Here, we present strong supporting evidence for an adaptive mechanism designed to maintain cellular FA unsaturation when WAT is under adverse metabolic conditions imposed by obesity and IR. This adaptive response involves facilitated lipid biosynthesis mediated by down-regulation of Insig1 and optimised activation of SREBP1 and SCD1. This change in the set point of the Insig1/SREBP1 regulatory feedback loop helps to maintain adipocytes lipid desaturation in situation of metabolic stress.

Using genetically modified *in vitro* and *in vivo* models, we have shown that down-regulation of Insig1 *per se* facilitates TG accumulation in parallel with favoured biosynthesis of unsaturated lipids through preferential induction of SCD1. We observed this down-regulation of Insig1 in WAT of dietary and genetic mouse models of obesity-induced IR, in obese and MO IR humans and in cellular models of IR.

Our data indicate that it is the IR associated with obesity, more than the amount of fat stored, the main factor responsible for the decreased Srebp1c WAT mRNA expression observed in MO individuals. Insig1 mRNA expression is decreased in the SC depot of MO individuals compared with obese patients and is further reduced in the presence of IR. Based on the role of Insig1 controlling SREBP1 activation, the decrease in Insig1 is expected to help to maintain the levels of SREBP1 active form. From the clinical perspective, this change in set point may be important to reconcile the perceived paradox between the excessive amounts of lipids that MO individuals are able to store in their WAT despite their IR and decreased mRNA expression of fundamental prolipogenic genes such as Srebp1 (39-41).

We found that reducing Insig1 mRNA to a similar degree observed in MO human WAT accelerates adipocyte differentiation enhances lipogenesis and
consequently lipid accumulation. Notably, the effect of Insig1 KD enhancing lipid accumulation in 3T3-L1 adipocytes could be reproduced in conditions that mimicked both physiological and pathophysiological glucose levels. In our opinion, this data indicates that the regulation of Insig1 in WAT may be more important under conditions of IR and metabolic stress than for the AT expansion observed in anabolic states.

Maintenance of SREBP1 mature protein levels in morbid obesity or under IR conditions is not associated with conserved global expression or restoration of all known SREBP1 target genes. Indeed, genes involved in the early stages of de novo lipogenesis from glucose such as Acc1 and Fas were down-regulated in WAT of MO IR patients, in our DIO and ob/ob IR mouse models and in our IR in vitro model despite maintained levels of mature SREBP1. This suggests that other transcription factors regulated by glucose such as ChREBP may be required for their full expression. Moreover it has been shown, that in livers of C57BL6 mice fed with HFD there is upregulation of SREBP1 and preferentially SCD1 as its target gene when compared to unaltered FAS expression (42). Conversely, pathways involved in modification of FA chain or FA storage such as Scd1, Cctα, Gpat1 and Dgat1 were maintained to a greater extent in obese subjects, being closer to those observed in lean individuals.

One of the most revealing findings, both in vivo and in vitro, is the identification of SCD1 as a preferentially regulated target for Insig1/SREBP1. From an adaptive perspective, maintenance of a proper degree of unsaturation could be the main homeostatic function of this regulatory loop. This view is supported by the fact that in our in vitro studies, inactivation of Insig1 under both physiological and high glucose conditions preferentially induced Scd1 gene expression and consequently
increase in SCD1-dependent lipid unsaturation indexes such as C18:1n9/18:0, C16:1n7/16:0 (43, 44) and C14:1n5/14:0 (45).

To consolidate the concept that down-regulation of Insig1 promotes a prolipogenic effect in WAT, we generated an Insig1 KO mouse. This murine model allowed us to study the effect of Insig1 depletion without the confounding effects of obesity and/or insulin resistance. Mice lacking intact Insig1 are characterised by increased % fat mass with respect to WT littermates. Also primary cultures of Insig1 KO preadipocytes showed increased and accelerated lipid accumulation after induction of differentiation as previously observed in the Insig1 KD 3T3-L1. Based on these previous results we hypothesised that the adaptive changes in Insig1/SREBP1 set point will maintain the pool of unsaturated FAs in WAT. Lipid composition analysis performed on WAT of MO vs. lean controls, supports this concept as the absolute amount of unsaturated FA was maintained at similar levels in both groups. Most interestingly, profiling of specific lipid species in WAT of our models of genetic induced obesity and insulin resistance as well as our Insig1 KO mouse indicated that the level of unsaturation and SCD1 activity is specifically maintained in the membrane phospholipid fraction.

This result strengthens our previous observation that in weight-discordant monozygotic twin pairs (2), the obese co-twins displayed reduced specific saturated FA and augmented proportions of specific unsaturated FAs such as palmitoleate in membrane phospholipid, alongside increases in indexes reflecting augmented activity of desaturation enzymes.

In summary, our results indicate the existence of an adaptive response in human WAT involving changes in the set point of the Insig1/SREBP1/SCD1 axis in order to optimise lipogenic gene expression programmes that ensure availability of structural and biologically essential unsaturated lipids, under disadvantageous
metabolic conditions such as obesity or IR. Thus, defects in insulin-regulated lipid metabolism associated with IR may at least be partially ameliorated by a reduction of Insig1 and optimised maturation of the active form of SREBP1 as a consequence of a resetting of Insig1/SREBP1 negative regulatory feedback loop (Figure S12).

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No potential conflicts of interest relevant to this article were reported.

The author(s) have made the following declarations about their contributions. R.M.H., S.C., C.J.L., M.S., M. L performed the experiments and analyzed the data. T.W. M.B-Y and M.B. contributed to the generation of the Insig1 KO mouse. J.L.G., H.J.A., C-Y.T, M.O and S.V. developed analytical platforms, performed and analyzed lipidomic experiments. N.B. F.J.T, A.T., A.S., E.L., M. Laville, R.V.C., H.V., D.L., J-M.F.R. characterized human cohorts and provided human omental and SC adipose tissue samples. R.M.H., S.C., C.J.L. and M.S., M. López. and A.V.-P. designed the experiments. R.M.H., S.C., C.J.L., M.S., G.M.-G., M. López, J.K.S and A.V.-P. discussed the manuscript. R.M.H., S.C., C.J.L. J.K.S, A.V.-P. coordinated and directed the project. R.M.H., S.C., C.J.L and A.V.-P. developed the hypothesis and wrote the manuscript. A.V.-P is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES


**ABBREVIATIONS:** ACC1, acetyl-CoA carboxylase 1; AKT, protein kinase B; aP2, adipocyte fatty acid binding protein; AT, adipose tissue; BMI, body mass index; CCTα, CTP:phosphocholine cytidylyltransferase alpha; DGAT1, diacylglycerol O-acyltransferase 1; ER, endoplasmic reticulum; FA, Fatty acid; FAS, Fatty acid synthase; GLUT4, Insulin-sensitive glucose transporter 4; HOMA, Homeostatic Model Assessment; I1KD, Insig1 knockdown; INSIG, INSulin Induced Gene; IR, Insulin Resistance; GPAT1, mitochondrial glycerol-3-phosphate acyltransferase 1; KO, knockout; MO, morbidly obese; Om, omental; PPARγ, peroxisome proliferator-activated receptor γ; SC, subcutaneous; SCAP, SREBP cleavage activating protein; SCD1, Stearoyl-CoA desaturase 1; SEM, standard error of the mean; SREBP, Sterol receptor element-binding protein; TG, triglyceride; vol., volume; WAT, white adipose tissue; WT, wild type.

**FIGURES LEGENDS**

**FIGURE 1**

Analysis of *Insigs* and *Srebp1* mRNA expression and of SREBP1 mature protein levels in murine models of diet- and genetic-induced obesity. RT-PCR analysis was performed on RNA obtained from gonadal WAT of males WT C57BL6 mice fed a HFD (white bars) for 6 months (A, n=7 HFD; n=9 chow) or 15 days (B, n=8 each
group) vs. their respective controls fed a standard chow diet (black bars); from gonadal WAT of males ob/ob (white bars) and WT littermates (black bars) when mice were either 4 months (E, n=9 WT n= 8 ob/ob) or 1 month old (F, n=8 for WT, n=6 ob/ob). Western blot analysis of SREBP1 mature protein levels was performed on gonadal WAT of the same groups of mice e.g. WT C57BL6 mice fed a HFD (white bars) for 6 month (C, n=7 HFD; n=7 chow) or 15 days (D, n=7 each group) vs. their respective controls fed a standard chow diet (black bars); from gonadal WAT of males ob/ob (white bars) and WT littermates (black bars) when mice were either 4 months (G, n=4 for both WT and ob/ob) or 1 month old (H, n=4 for both WT and ob/ob). SREBP1 mature protein was normalized to α-tubulin as loading control. Blots were analyzed by densitometry. WT C57BL6 6 months on HFD vs. chow diet; *: p<0.05; **: p<0.01. ***: p<0.001; WT vs. ob/ob; *:p<0.05; **: p<0.01. ***: p<0.001. Data are presented as Mean ± SEM.

FIGURE 2

*Insig1 and Srebp1 mRNA and protein levels in white adipocytes of Non-IR and IR MO subjects.* RT-PCR analysis was performed on RNA obtained from mature adipocytes isolated from either SC or Om depots from lean (BMI 22.2±0.8, white bars), obese (BMI 30.2±1.1, dark grey bars) and MO (BMI 48.9±1.8, black bars) human subjects (Table S1, cohort 1), examining Insig1/2 (A), Srebp1c/a (B) mRNA levels, standardized to 18S mRNA within each group, (n=10 lean, n=10 obese, n=12 morbid obese). MO vs. lean;*: p<0.05; **: p<0.01; ***: p<0.005; ****: p<0.0005; MO vs. Obese; &: p<0.05. ANOVA: $: p<0.05; ^$: p<0.005. (C) Western blot analysis of INSIG1 protein in SC white adipocytes from lean (BMI 25.1±0.9, white bars) and MO (BMI 50.5±5.0, black bars) (Table S1, cohort 2). The blots from SC samples were analyzed by densitometry and INSIG1 protein levels were normalized to β-actin.
as loading control (n=6 lean, n=6 MO) MO vs. lean;*: p<0.05 **: p<0.01. (D) RT-PCR analysis was performed on RNA obtained from SC WAT from overweight (BMI 26.1±1.19, HOMA 1.57±1.19, grey bars), MO Non-IR (BMI 39.0±4.27, HOMA 2.31±0.44, black bars) and MO IR (BMI 41.9±1.84, HOMA 4.40±0.51, striped bars) human subjects (Table S1, cohort 1) examining Srebp1/Insig1 mRNA expression. (n=6 Overweight, n=4 MO Non-IR, n=4 MO IR). MO Non-IR and IR vs, Overweight * p<0.05; ** p<0.005; MO Non-IR vs IR; k p<0.05; ANOVA: $ p<0.005. (E) Mature SREBP1 protein levels were determined in these groups by western blot analysis and normalized to β-actin. Blots were analyzed by densitometry (n=4 Overweight, n=3 MO Non-IR, n=3 MO-IR). Data are presented as Mean ± SEM.

FIGURE 3

SREBP1 target genes expression in omental and subcutaneous white adipocytes of lean, obese and MO. RT-PCR analysis was performed on RNA obtained from mature adipocytes isolated from lean (BMI 22.2±0.8, white bars), obese (BMI 30.2±1.1, dark grey bars) and MO (BMI 48.9±1.8, black bars) (Table S1, cohort 1). Adipocytes derived from Om (A) and SC depots (B) were examined and standardized to the average 18S mRNA value within each group. (n=10 lean, n=10 obese, n=12 MO). Obese and MO vs. lean; *: p<0.05; **: p<0.01; ***: p<0.005; ****: p<0.001. MO vs. obese #: p<0.05 vs. obese; ##: p<0.01; ###: p<0.005. ANOVA: $: p<0.05; $$: p<0.005; $$$: p<0.001. Data are presented as Mean ± SEM.

FIGURE 4

Insig1 knockdown results in increased triglycerides accumulation, accelerated differentiation and augmented Srebp1 and lipogenic gene expression. (A) I1KD and Control 3T3-L1 cells were differentiated at 5mM glucose concentration for 8 days when RNA was isolated and Srebp1c, Srebp1a, Insig1 and Insig2 gene expression
analysed using qRT-PCR (n=6). I1KD vs. Controls *: p<0.01; **: p<0.001. (B) Western blot analysis was performed in I1KD and Control cells to assess mature SREBP1 levels. Mature SREBP1 was normalised to α-tubulin. The blot was quantified by densitometry (n=6). I1KD vs. Controls *: p<0.05. (C) Microscope pictures of Oil-Red-O stained I1KD and Control 3T3-L1 cells, 8 days after induction of differentiation at 5mM glucose. (D) Triglyceride levels were measured 10 days after induction of differentiation at 5mM glucose of I1KD and Control cells (n=3). I1KD vs. 5mM Controls **: p<0.001. (E) Real time PCR analysis was performed on RNA and gene expression levels of lipid related SREBP1 target genes analysed in cells differentiated in 5mM glucose (n=6). I1KD vs. Controls *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001. (F) De novo FA synthesis radioactive assay was carried out in Insig1 KD and Control cells at day 8 of differentiation after overnight serum starvation and at the condition indicated (n=8-9). I1KD vs. Control: *: p<0.005; I1KD treated with C75 vs. Control treated with C75: p<0.001. Control vs. Control treated with C75 and I1KD vs. I1KD treated with C75: p<0.01; Control vs Control treated with Insulin and I1KD vs I1KD treated with Insulin: ^ p<0.005; Control treated with Insulin vs I1KD treated with Insulin: $ p<0.005. (G) Lipidomic analysis and subsequent evaluation of SCD1 activity were performed in I1KD and Control cells. Cells were harvested at day 8 of differentiation and processed as described in Research design and Method section (n=12). I1KD vs. Controls *: p<0.05; **: p<0.01. Data are presented as Mean ± SEM.

FIGURE 5

Insig1 depletion in vivo and in vitro shows increased fat accumulation, SREBP1 levels and SREBP1 target gene expression. (A) % fat mass respect to body weight was established for Insig1 KO males vs. WT littermates (WT n=8, Insig1 KO n=6). (B, D) Real time PCR analysis was performed on gonadal WAT from 11 week-old
males Insig1 KO and WT littermates. (WT n=4, Insig1 KO n=5). (C) mature SREBP1 levels in gWAT of Insig1 KO and wt littermates (WT n=4, Insig1 KO n=5) (E) Oil-Red-O staining of WT and Insig1 KO primary adipocytes at day 4 and 8 of differentiation. Cells were induced to differentiate with standard MDI induction cocktail using 25mM Glucose DMEM. (F) Gene expression analysis of Insig1 KO and WT WAT primary culture after 4 days of differentiation (n=3-4).Insig1 KO vs. WT *: p<0.05; **: p<0.01; ***: p<0.0001. Data are presented as Mean ± SEM.

TABLES
Table 1 - Lipid composition of Phospholipids and Triglycerides as well as major elongases and desaturase activity ratios.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>PL</th>
<th>KO</th>
<th>TG</th>
<th>KO</th>
<th>P values:</th>
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<td>WT</td>
<td>S.E.M.</td>
<td>WT</td>
<td>S.E.M.</td>
<td></td>
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<td>C12:0</td>
<td>0.697±0.298</td>
<td>0.386±0.215</td>
<td>0.087±0.005</td>
<td>0.091±0.004</td>
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<td>C14:0</td>
<td>1.006±0.06</td>
<td>0.744±0.104</td>
<td>1.318±0.047</td>
<td>1.436±0.088</td>
<td>0.046±0.232</td>
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<td>C16:0</td>
<td>27.515±0.523</td>
<td>26.004±1.081</td>
<td>25.036±0.725</td>
<td>21.079±0.073</td>
<td>0.198±0.002</td>
</tr>
<tr>
<td>C16:1n7</td>
<td>4.760±0.479</td>
<td>4.160±0.494</td>
<td>7.819±0.363</td>
<td>9.832±0.845</td>
<td>0.426±0.037</td>
</tr>
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<td>C18:0</td>
<td>13.310±1.883</td>
<td>13.578±1.081</td>
<td>25.036±0.725</td>
<td>21.079±0.073</td>
<td>0.198±0.002</td>
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<td>C18:1n7</td>
<td>2.003±0.308</td>
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<td>3.637±0.155</td>
<td>0.767±0.002</td>
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<td>C18:2n6</td>
<td>25.733±1.712</td>
<td>26.004±1.081</td>
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<td>0.198±0.002</td>
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<td>C18:3n3</td>
<td>0.993±0.129</td>
<td>0.576±0.136</td>
<td>4.160±0.494</td>
<td>7.819±0.363</td>
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<td>C20:0</td>
<td>0.202±0.049</td>
<td>0.160±0.112</td>
<td>2.048±0.155</td>
<td>1.631±0.141</td>
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<td>C20:1n9</td>
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<td>0.369±0.039</td>
<td>0.523±0.023</td>
<td>0.550±0.039</td>
<td>0.949±0.525</td>
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<td>C20:2n6</td>
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<td>26.004±1.081</td>
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<td>C20:3n3</td>
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<td>C20:4n6</td>
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<td>5.592±1.175</td>
<td>3.666±0.059</td>
<td>3.13±0.074</td>
<td>0.244±0.594</td>
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<td>C22:0</td>
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<td>C22:2n6</td>
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<td>0.102±0.866</td>
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<td>C22:6n3</td>
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<td>0.052±0.145</td>
<td>0.199±0.013</td>
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<tr>
<td>C23:0</td>
<td>0.044±0.018</td>
<td>0.061±0.013</td>
<td>0.021±0.003</td>
<td>0.017±0.002</td>
<td>0.523±0.365</td>
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<tr>
<td>C24:0</td>
<td>0.034±0.008</td>
<td>0.056±0.023</td>
<td>0.009±0.003</td>
<td>0.008±0.002</td>
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<td>C22:6n3</td>
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<td>0.002±0.001</td>
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<td>C22:6n6</td>
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<td>1.020±0.252</td>
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<td>68.471±1.153</td>
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<td>70.914±0.742</td>
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<tr>
<td>Essential</td>
<td>32.329±1.384</td>
<td>31.529±1.153</td>
<td>33.009±0.483</td>
<td>29.086±0.742</td>
<td>0.693±0.002</td>
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<tr>
<td>SCD ratio</td>
<td>0.618±0.101</td>
<td>0.696±0.142</td>
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<td>2.017±0.014</td>
<td>0.655±0.000</td>
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<tr>
<td>Elovl6 Ratio</td>
<td>1.014±0.025</td>
<td>1.200±0.102</td>
<td>0.978±0.049</td>
<td>1.223±0.075</td>
<td>0.063±0.021</td>
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Lipid composition analysis was carried out in gonadal adipose tissue of Insig1 KO and WT littermates following solid phase extraction to separate neutral lipids (TG) and phospholipids (PL). Data is presented as Molar-percentages (Mol%) SCD1 ratio is calculated as: (18:1n9+18:1n7+16:1n7)/(18:0+16:0), Elovl6 ratio calculated as (18:1n9+18:1n7+18:0)/(16:1n7+16:0) and reflecting the activity of the two major desaturase and elongase classes acting on de novo synthesised lipids. (Insig1 KO n=5, WT n=6). Insig1 KO vs WT : *: p≤ 0.05.Data are presented as Mean± SEM.
Figure 1

A 6 months HFD mRNA expression

mRNA expression

WT chow

WT HFD

B 15 days HFD mRNA expression

mRNA expression

WT chow

WT HFD

C 6 months HFD SREBP1 protein level

SREBP1 protein level

WT chow

WT HFD

D 15 days HFD SREBP1 protein level

SREBP1 protein level

WT chow

WT HFD

E 4 months old mRNA expression

mRNA expression

WT

ob/ob

F 1 month old mRNA expression

mRNA expression

WT

ob/ob

G 4 months old SREBP1 protein level

SREBP1 protein level

WT

ob/ob

H 1 month old SREBP1 protein level

SREBP1 protein level

WT

ob/ob
Figure 2

**Cohort 1**

**A** mRNA expression

- **Insig1**
  - Lean: OM, SC
  - Obese: OM, SC
  - MO: OM, SC
  - Fold change (vs Lean): $\$, **$, ****$

- **Insig2**
  - Lean: OM, SC
  - Obese: OM, SC
  - MO: OM, SC
  - Fold change (vs Lean): $\$, **$, ****$

**B** mRNA expression

- **Srebp1c**
  - Lean: OM, SC
  - Obese: OM, SC
  - MO: OM, SC
  - Fold change (vs Lean): $\$, **$, ****$

- **Srebp1a**
  - Lean: OM, SC
  - Obese: OM, SC
  - MO: OM, SC
  - Fold change (vs Lean): $\$, **$, ****$

**Cohort 2**

**C** Subcutaneous WAT

**INSIG1 protein level**

- Lean
- MO

**Cohort 3**

**D** Subcutaneous WAT

**INSIG1 protein level**

**Cohort 3**

**E** Subcutaneous WAT

**SREBP1 protein level**

- Overweight
- MO Non-IR
- MO IR

**Mature SREBP1**

- Overweight
- MO Non-IR
- MO IR
Cohort 1

A  OMENTAL WAT

B  SUBCUTANEOUS WAT
Figure 4

A) 5mM glucose
mRNA expression

B) mature SREBP1
fold change (vs Control)

C) 5mM glucose

D) 5mM glucose

E) 5mM glucose
mRNA expression

F) De novo FA synthesis assay

G) Desaturation indexes
Figure 5

A

Fat percentage (%)

WT

Insig1 KO

Gonadal adipose tissue

mRNA expression

WT

Insig1 KO

p=0.10

Insig1

Insig2

Srebp1c

Srebp1a

Srebp2

fold changes (vs wt)

Gonadal adipose tissue

fold change (vs wt)

Fas

Acc1

Scd1

Dgat1

Pparγ2

mRNA expression

WT 4 days

KO 4 days

mRNA expression

Insig1

Insig2

Srebp1c

Srebp1a

Srebp2

Acc1

Scd1

Fas

Adip

Ppilipin

Glut4

Pparγ2

ap2

fold change (vs wt 4 days)

WT primary culture day 4 of differentiation

KO

WT

KO

WT

Day 4

Day 8
Figure S1. Decreased AKT phosphorylation and mature SREBP1 expression in mouse models of diet- and genetic-induced obesity. To determine AKT phosphorylation levels (p-AKT), western blot analysis was performed on protein lysate obtained from gonadal white adipose tissue of males WT C57BL6 mice fed a HFD (white bars) for 6 months (A, n=7 HFD; n=6 chow) or 15 days (B, n=7 HFD; n=6 chow) vs. their respective controls fed a standard chow diet (black bars). Blots were analysed by densitometry. Immunoblots were also carried out on the gonadal WAT of the same 6 months (C) and 15 days HFD (D) fed mice as well as 1 month (E) and 4 months (F) old ob/ob mice vs. controls in order to analyse mature SREBP1 protein expression. Data are presented as Mean ± SEM.
Figure S2. INSIG1 and SREBP1 protein levels in subcutaneous adipose tissue of MO Non-IR and IR subjects. Representative blots and quantification of INSIG1 (A) and mature SREBP1 (B) protein levels measured in subcutaneous WAT of MO Non-IR and IR individuals (Table SI, cohort 4). &: p<0.05 vs MO Non-IR. Data are presented as Mean ± SEM.
Figure S3. Insig-1 and SREBP1 protein levels in omental adipose tissue of MO subjects. Western analysis of INSIG1 protein (A) and SREBP1 protein (B) in Om white adipocytes from lean (gray bars), MO Non-IR (black bars) and MO IR (striped bars) (Table SI, cohort 4). Representative blots from Om samples were scanned and analyzed by densitometry (total n=6 lean, total n=14 MO Non-IR and n=14 MO IR) ;*: P<0.01 **: P<0.001 vs. lean; &: P<0.05 v.s MO Non- IR. ANOVA : a P<0.005. Data is presented as Mean ± SEM.
Figure S4. Induction of IR in 3T3L1 cells results in decreased Srebp1, Insig1 mRNA and increased SREBP1 mature protein levels. 3T3-L1 adipocytes were either incubated in high glucose media (Control) alone or in combination with 100nM insulin for 16 hours to induce insulin resistance (IR). Western analysis showing phosphorylated AKT (p-AKT) levels and total AKT levels in control and insulin resistant 3T3-L1 adipocytes subsequently stimulated with/without 1nM insulin for 10mins (A). Real time PCR analysis was performed on RNA and gene expression levels analysed from control and insulin resistant 3T3-L1 adipocytes (n=4) (B,C,E). Immature and mature SREBP1 protein levels were assessed using western blot analysis. Band intensity was determined by densitometry (n=6 for each condition). IR vs. Control cells *: p<0.05; **: p<0.01. Data are presented as Mean ± SEM.
Figure S5. SREBP1 target genes expression in gonadal adipose tissue of mouse models of diet- and genetic-induced obesity. RT-PCR analysis was performed on RNA obtained from gonadal adipose tissue of males WT C57BL6 mice fed a HFD (white bars) for 6 months (A, n=7 HFD, n=9 chow) or 15 days (B, n=8 each group ) vs. their respective controls fed a standard chow diet (black bars); from gonadal WAT of males ob/ob (white bars) and WT littermates (black bars) when mice were either 4 months (C, n=9 WT, n=8 ob/ob) or 1 month old (D, n=8 WT, n=6 ob/ob). WT C57BL6 on HFD vs. chow diet: *: p<0.05; **: p<0.01; ***: p<0.001; WT v.s ob/ob: *: p<0.05; **: p<0.01. ***: p<0.001. Data are presented as Mean±SEM.
Figure S6. Insig1 knockdown results in increased triglycerides accumulation, accelerated differentiation and augmented Srebp1 and lipogenic gene expression. (A) I1KD and Control 3T3-L1 cells were differentiated at 25mM glucose concentration for 8 days when RNA was isolated and Srebp1c, Srebp1a, Insig1 and Insig2 gene expression analysed using qRT-PCR (n=6) (B) Representative blot of SREBP1 mature protein analysed in I1KD vs. Control cells. (C) Microscope pictures of Oil-Red-O stained I1KD and Control 3T3-L1 cells, 8 days after induction of differentiation at 25mM glucose. (D) Triglyceride levels were measured 10 days after induction of differentiation at 25mM glucose of I1KD and Control cells (n=3); vs. 25mM Control #: p<0.01. (E) Real time PCR analysis was performed on RNA and gene expression levels of lipid related SREBP1 target genes analysed in cells differentiated in 25mM glucose (n=6). I1KD vs. Controls *: p<0.05; **: p<0.01; ***: p<0.001. Data are presented as Mean ± SEM.
Figure S7. Insig1 KO growth curve, fat depots weight, food intake and activity. All the analysis described in this figure have been performed in 12 weeks old Insig1 KO and wt littermates. Insig1KO n=6; WT n=8. A. Growth curve B. Body length during development C. Body weight at culling D. Fat depots weight E. Food intake F. Activity. For all other analysis showed in this figure:*p≤ 0.05, **p≤ 0.001 Data are presented as Mean± SEM.
Figure S8. Insig1 KO mice carbohydrate metabolism, RER and EE. GTT (A) and ITT (B) were performed in Insig1 KO mice vs. controls (n=9 KO; n=15 WT). Respiratory exchange ratio (C) and Energy Expenditure (D), assessed using ANCOVA, were also analysed for Insig1 KO and WT mice (WT n=4 Insig1 KO n=5). Data are presented as Mean ± SEM.
Figure S9. Adipose tissue morphology and adipocyte size. The histological analysis and the adipocyte size evaluation was performed in 12 weeks old Insig1 KO and WT littermates. For the histology 3 representative picture of each genotype are shown. For the adipocyte size evaluation, Insig1 KO n=6; WT n=8.
Figure S10. Maintenance of SREBP1 mature levels promotes preservation of membrane phospholipid unsaturation and adipocyte homeostasis in obese IR state. (A) SCD1 ratio defined as the sum of the concentration of monounsaturated PC or TG lipid species divided by the sum of the concentrations of the fully unsaturated PC or TG lipid species. (B) Scatter plot of SCD1 ratios of individual mice for PC against TG. N= 4 WT and 5 ob/ob mice, 4 month old males, mixed genetic background. (C) Principal component analysis plot showing the separation of the lipid profiles of ob/ob and WT adipocytes. (D) Average loading coefficients for the lipid species separating ob/ob and WT adipocytes. N= 3 WT and N=6 ob/ob. Each WT sample comprises of a pool of 4 mice. Males, C57BL/6, 16 weeks old. Analysis of mature SREBP1 protein levels and adipocyte area from 10 week old ob/ob males showing; (E) The relationship between adipocyte area and mature SREBP1 protein levels (significant effects of genotype, adipocyte area and a significant genotype* adipocyte area interaction with mSREBP1 protein levels as a dependent variable and adipocyte area as a covariate); (F) mature SREBP1 protein levels; (G) average adipocyte area; (H) mature SREBP1 protein levels adjusted for an adipocyte area of 3570 uM using ANCOVA analysis.
**Figure S11. Insig1 KO gene targeting strategy.** (A) partial genomic map of wild-type Insig1 locus from exon 1 (1) to 5 (5). SA; short arm; DF, deleted region; LA, long arm. The lower part of the panel shows the targeted locus after excision of the neomycin-resistance selection cassette (PGKneo, light blue rectangle) that is flanked by two loxP sites (red arrow); and replacement of exon 2, 3 and 4 (orange rectangles) that are flanked by two loxP sites (red arrowhead). The mice carrying the PGKneo cassette were crossed with Rosa26-Cre mice resulting in total Insig1 knockout animals (Insig1 KO). (B) Tissues collected from these animals were analysed by PCR to detect the KO and WT allele in different organs.
Figure S12. Hypothetical model of Insig1/SREBP1/SCD1 adaptive response during the evolution from insulin sensitive states to obesity-induced insulin resistance and final metabolic failure. A) Normal insulin sensitivity states (lean or healthy obese): under this condition glucose uptake and insulin action is maintained, levels of Srebp1 mRNA are normal due to the expression promoting effect of insulin, SREBP1 is cleaved and released to support lipid metabolism requirements. Insig1 expression and protein are also maintained providing feedback control to SREBP1 excessive activation. B) During obesity and insulin resistance with adaptive compensation. Circulating glucose and insulin may be increased, but due to tissue insulin resistance, Srebp1 mRNA levels are decreased. Under these conditions, maintenance of adequate SREBP1 cleavage requires progressive optimisation through a decrease of the Insig1 set point, illustrated by decreased gene and protein expression. Under these conditions, SCD1 is the most efficiently maintained gene target and results in an enrichment of unsaturated lipid species in adipose tissue. C) During insulin resistance with combined adaptive response failure. This stage occurs when excessive fat deposition, insulin resistance leading to diabetes, results in failure of the adaptive compensation of WAT. Here, the decrease in Insig1 is insufficient to maintain a minimum level of activity in SREBP1 and SCD1 expression able to maintain lipid homeostasis. The size of the fonts used in the figure represents the relative levels of the different parameters described.
**TABLE S1** - Baseline biological characteristics of the cohorts of individuals considered in this study

<table>
<thead>
<tr>
<th>cohort category</th>
<th>gender</th>
<th>Age (years)</th>
<th>BMI (Kg/m²)</th>
<th>Fasting Glucose (mg/dl)</th>
<th>Fasting Insulin (µU/ml)</th>
<th>HbA₁c (%)</th>
<th>HOMA</th>
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<tbody>
<tr>
<td>Lean 1</td>
<td>5F/5M</td>
<td>60.3 ± 5.0</td>
<td>22.2 ± 0.8</td>
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<td>-</td>
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<tr>
<td>Obese 1</td>
<td>7F/3M</td>
<td>49.3 ± 3.6</td>
<td>30.2 ± 1.1</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MO 1</td>
<td>12F/2M</td>
<td>40.2 ± 3.1</td>
<td>48.9 ± 1.8</td>
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<tr>
<td>Lean 2</td>
<td>6F</td>
<td>35.5 ± 4.7</td>
<td>25.1 ± 0.9</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>MO 2</td>
<td>6F</td>
<td>42.8 ± 3.6</td>
<td>50.5 ± 5.0</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Overweight 3</td>
<td>7F/1M</td>
<td>38.5± 3.15</td>
<td>26.1± 1.19</td>
<td>86.5± 2.9</td>
<td>7.3± 0.72</td>
<td>4.46±0.15</td>
<td>1.57± 0.19</td>
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<tr>
<td>MO-non IR 3</td>
<td>6F</td>
<td>52.2± 3.95</td>
<td>39.0± 4.27</td>
<td>85.5± 5.9</td>
<td>11.1±0.74</td>
<td>5.00± 0.18</td>
<td>2.31± 0.44</td>
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<td>MO-IR 3</td>
<td>4F/1M</td>
<td>45.5± 3.77</td>
<td>41.9± 1.84</td>
<td>113.5±4.6</td>
<td>21.0± 6.08</td>
<td>5.00± 0.04</td>
<td>4.40± 0.51</td>
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<tr>
<td>Lean 4</td>
<td>3F/3M</td>
<td>52.17± 5.1</td>
<td>25.0± 0.55</td>
<td>108.6±5.7</td>
<td>11.2±1.58</td>
<td>3.51± 0.48</td>
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<tr>
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<td>44.14± 3.43</td>
<td>56.72±1.71</td>
<td>95.8±4.1</td>
<td>15.4±1.44</td>
<td>3.60± 0.32</td>
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<tr>
<td>MO IR 4</td>
<td>7F/7M</td>
<td>39.00± 2.50</td>
<td>56.30±1.65</td>
<td>110.9±5.2</td>
<td>44.7±3.17</td>
<td>12.76± 1.25</td>
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<tr>
<td>Lean 5</td>
<td>6F</td>
<td>50.7 ± 5.9</td>
<td>23.2 ± 1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MO 5</td>
<td>5F</td>
<td>40.0± 2.8</td>
<td>54.0± 4.9</td>
<td>96.2±2.2</td>
<td>17.7±6.7</td>
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<td>4.0± 1.4</td>
</tr>
</tbody>
</table>

MO: morbidly obese; HOMA (fasting plasma glucose (mmol/l) * fasting plasma insulin (mU/l))/ 22.5.
Table S2-Total FA profile of subcutaneous adipose tissue of lean (BMI 23.2±1.2) and MO (BMI 54.0±4.9)

<table>
<thead>
<tr>
<th>FA</th>
<th>Lean (% total FA)</th>
<th>MO (% total FA)</th>
</tr>
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<tbody>
<tr>
<td>C10:0</td>
<td>0.035 ±0.008</td>
<td>0.025 ±0.004</td>
</tr>
<tr>
<td>C12:0</td>
<td>0.673 ±0.072</td>
<td>0.575 ±0.097</td>
</tr>
<tr>
<td>C13:0</td>
<td>0.034 ±0.002</td>
<td>0.030 ±0.003</td>
</tr>
<tr>
<td>C14:0</td>
<td>3.008 ±0.299</td>
<td>2.760 ±0.230</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.387 ±0.032</td>
<td>0.368 ±0.034</td>
</tr>
<tr>
<td>C16:0</td>
<td>21.805 ±1.122</td>
<td>21.845 ±0.532</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.435 ±0.049</td>
<td>0.366 ±0.043</td>
</tr>
<tr>
<td>C18:0</td>
<td>5.697 ±0.745</td>
<td>3.999 ±0.561</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.174 ±0.025</td>
<td>0.107 ±0.016*</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.041 ±0.005</td>
<td>0.025 ±0.004*</td>
</tr>
<tr>
<td>C14:1</td>
<td>0.340 ±0.038</td>
<td>0.456 ±0.073</td>
</tr>
<tr>
<td>C16:1</td>
<td>4.016 ±0.525</td>
<td>5.178 ±0.713</td>
</tr>
<tr>
<td>C18:1</td>
<td>38.429 ±1.421</td>
<td>39.789 ±1.730</td>
</tr>
<tr>
<td>C18:2</td>
<td>19.643 ±1.701</td>
<td>18.984 ±1.346</td>
</tr>
<tr>
<td>C18:3</td>
<td>1.213 ±0.105</td>
<td>0.982 ±0.105</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.670 ±0.053</td>
<td>0.627 ±0.059</td>
</tr>
<tr>
<td>C20:2</td>
<td>0.272 ±0.017</td>
<td>0.294 ±0.030</td>
</tr>
<tr>
<td>C20:3</td>
<td>0.368 ±0.040</td>
<td>0.428 ±0.059</td>
</tr>
<tr>
<td>C20:4</td>
<td>0.581 ±0.054</td>
<td>0.762 ±0.099</td>
</tr>
<tr>
<td>C20:5</td>
<td>0.261 ±0.040</td>
<td>0.305 ±0.043</td>
</tr>
<tr>
<td>C22:1</td>
<td>0.051 ±0.006</td>
<td>0.059 ±0.008</td>
</tr>
<tr>
<td>Others</td>
<td>1.817 ±0.097</td>
<td>1.978 ±0.087</td>
</tr>
<tr>
<td>SFA</td>
<td>32.287 ±2.081</td>
<td>30.099 ±1.141</td>
</tr>
<tr>
<td>USFA</td>
<td>65.896 ±2.028</td>
<td>67.923 ±1.090</td>
</tr>
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</table>

**Total FA profile of subcutaneous adipose tissue of lean and MO individuals.** Total FA profiling was performed on subcutaneous adipose tissue of lean (BMI 23.2 ± 1.2) and MO (BMI 54.0 ± 4.9), (Table S1, cohort 5) (Lean n=6, MO n=5). MO vs. lean: *: p ≤ 0.05. Data are presented as Mean± SEM.
Table S3 – Blood biochemistry of Insig1 ko and wt males 12 weeks old

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Glucose (mmol/l)</th>
<th>Insulin (µg/l)</th>
<th>TG (mmol/l)</th>
<th>Adiponectin (ng/ml)</th>
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<tr>
<td>WT</td>
<td>13.6±0.83</td>
<td>0.11±0.01</td>
<td>0.9±0.1</td>
<td>27875±1765</td>
</tr>
<tr>
<td>KO</td>
<td>12.8±0.92</td>
<td>0.10±0.01</td>
<td>0.6±0.1*</td>
<td>30761±1781</td>
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</tbody>
</table>

**Insig1 KO serum biochemistry.** All the analysis described in this figure have been performed in 12 weeks old Insig1 KO and wt littermates: *p≤ 0.05, Data are presented as Mean± SEM.
Acquisition of human samples

The present study includes white adipose tissue samples from subjects belonging to five different cohorts.

For analysis of Srebp1 and SREBP1 target genes mRNA levels omental (Om) and subcutaneous (SC) adipose tissue biopsies were obtained from lean, obese and morbidly obese subjects undergoing elective open-abdominal surgery at Addenbrooke’s Hospital (AH), Cambridge, UK (Table S1 cohort 1). The subjects were included in the study after informed consent and the Institutional Review Board at IU and the Cambridge Local Research Ethics Committee (at AH) approved protocols involving adipose biopsies.

For analysis of INSIG1 protein levels, SC adipose tissue samples were obtained from Dr R.V. Considine, Division of Endocrinology and Metabolism, Indiana University School of Medicine, Indianapolis (Table S1, cohort 2). All participants gave their consent after being informed of the nature, purpose and possible risks of the study and the protocol to obtain the samples was approved by the Ethical Committee of Indiana University School of Medicine.

For analysis of Srebp1 and Insig1 mRNA and SREBP1 protein levels in overweight, Non-IR and IR morbidly obese subjects, Om and SC adipose tissue samples were obtained from human subcutaneous fat depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia and gastric by-pass surgery), washed, fragmented and immediately flash-frozen in liquid nitrogen before being stored at -80°C. These fat samples were provided by Dr J.M Fernandez-Real from a group of subjects who were invited to participate at the Endocrinology Service of the Hospital Universitari de Girona (Girona, Spain) (Table S1 cohort 3). All subjects gave their informed consent to the use of the samples and the protocol of the study was approved by the Ethical Committee of the Hospital Universitari de Girona.
For the analysis of SREBP1 and INSIG1 protein in lean, morbidly obese non insulin resistant and insulin resistant subjects, SC adipose tissue samples were obtained from 28 morbidly obese (MO) subjects (BMI 56.51 ± 1.52 kg/m$^2$) (14 women and 14 men) who underwent bariatric surgery with mixed techniques, combining gastric reduction with an intestinal bypass, and 6 non-obese subjects (BMI 25.04 ± 0.55 kg/m$^2$) (3 women and 3 men) with no alterations in lipid or glucose metabolism, as controls. The morbidly obese patients were selected in base to similar clinical profile but different insulin resistance degree. Any patients received oral antidiabetic agents or insulin therapy. The weight of all individuals had been stable for at least one month and none had associated renal pathology. The morbidly obese patients were classified according to their insulin sensitivity. Specifically patients having HOMA-IR score <4.7 were considered non-insulin resistance (MO Non-IR) group. This cut point was established from the mean +/- 2SD of a healthy control population. The morbidly obese with a HOMA-IR score> 8, was considered as the insulin resistant (MO IR) group. Om and abdominal SC adipose tissue biopsies were obtained from MO patients undergoing bariatric surgery procedures or laparoscopic surgery procedures (hiatus hernia repair or cholecystectomies) in lean subjects. Tissue samples were washed in physiological saline, immediately frozen in liquid nitrogen and stored at -80°C. Clinical details of patients included in the study are indicated in (Table S1, cohort 4). All subjects were recruited in the Virgen de la Victoria Hospital, Malaga, Spain and were included after informed consent and the Ethical committee of the Hospital Virgen de la Victoria gave the approval for the protocols involving the adipose tissue.

Total FFA profile was performed on SC white adipose tissue of lean and morbidly obese individuals. These samples were provided from Dr R.V. Considine, Division of Endocrinology and Metabolism, Indiana University School of Medicine, Indianapolis (Table S1, cohort 5). All participants gave their written consent after being informed of the nature,
purpose and possible risks of the study and the protocol to obtain the samples was approved by the Ethical Committee of Indiana University School of Medicine.

**Anthropometric measurements.** BMI was calculated as weight (in kilograms) divided by height (in meters) squared. The subjects’ waist was measured with a soft tape midway between the lowest rib and the iliac crest.

**Analytical determinations.** The serum glucose levels were measured in duplicate by the glucose oxidase method with a Beckman Glucose Analyzer 2 (Brea, CA). The coefficient of variation (CV) was 1.9%. Serum insulin levels were measured in duplicate by monoclonal immunoradiometric assay (IRMA, Medgenix Diagnostics, Fleunes, Belgium).

### SUPPLEMENTAL TABLES

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
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<tr>
<td><em>TaqMan-Act1</em></td>
<td>ACTACCGGAACCTTCTTGCATAAAATG</td>
<td>CAACTTTATCCCTAAAAAGCCACAT</td>
<td>CATGGCTTTCATGGGTCCTCAAG</td>
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<td><em>TaqMan-CCTq</em></td>
<td>CCGAGAATCATTTGGAAGTTTTCT</td>
<td>GCCCCTCCCTTCCTTTAC</td>
<td>TTTGGTCGGAAGGAAGACTGAAA</td>
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<tr>
<td><em>TaqMan-Fas</em></td>
<td>GCTGTGAAACCCATTGCTT</td>
<td>AGGTCTAGAGGCCCTATCTGTG</td>
<td>AGCACCCTTTGATGACATGCTECA</td>
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<td><em>TaqMan-Glut4</em></td>
<td>TGCAGAAGAGCTGCTGAAGCG</td>
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<td><em>TaqMan-Scd1</em></td>
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<td><em>TaqManSrebp1a</em></td>
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<tr>
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TABLE S5 – Primers sequences for Quantitative PCR in mouse samples and in 3T3-L1 cells

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<td>CCCGCTCTCTCAACTTGCT</td>
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<td>TaqMan – Ceto</td>
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<td>GAATCGGCCCCACAATCCA</td>
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<td>TaqMan-Fas</td>
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<td>CTGACTCGGGCAACTTCCC</td>
<td>GAGGAGGATGGTGATAGCGGTAAGTC</td>
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<td>TaqMan-Glut4</td>
<td>ACTCATCTTTGGACGGTTCTTC</td>
<td>CACCCGAAGATGAGTGGG</td>
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