



Srebf1a is a key regulator of transcriptional control for adipogenesis

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Adipogenesis is regulated by a complex cascade of transcriptional factors, but little is known about the early events that regulate the adipogenic program. Here, we report the role of the *srebf1a* gene in the differentiation of fibroblastic 3T3-F442A cells. We found that expression of *srebf1a* depended on GSK3 β activity and that GSK3 β activity was necessary for C/EBP β phosphorylation at Thr188. Knockdown of *srebf1a* inhibited the adipogenic program because it blocked the expression of genes encoding PPAR γ 2, C/EBP α , SREBP1c and even FABP4, demonstrating that SREBP1a activation is upstream of these three essential adipogenic transcription factors. Kinetic analysis during differentiation illustrated that the order of expression of adipogenic genes was the following: *cebpb*, *srebf1a*, *pparg2*, *cebpa*, *srebp1c* and *fabp4*. Our data suggest that *srebf1a* acts as an essential link between the GSK3 β -C/EBP β signaling axis and the beginning of the adipogenic transcriptional cascade.

White adipose tissue plays an important role in energy storage and organismal homeostasis through secretion of molecules with endocrine activities. The study of adipose differentiation has been highly facilitated by cell culture models, such as the fibroblastic 3T3-L1 and the 3T3-F442A cell lines. Both sister cell lines undergo adipose differentiation following stimulation with adipogenic serum proteins or growth hormones^{1–4}. 3T3-F442A cells have the ability to differentiate into fat pads *in vivo*, suggesting that they respond to physiological signals in the body⁵, whereas 3T3-L1 cells do not⁶. 3T3-F442A cells can also differentiate into an osteoblast phenotype⁷, demonstrating the plasticity of this cell line. Thus, the 3T3-F442A cell line is suitable for the study of adipose differentiation, adipogenesis-regulating molecules and the expression of very early genes involved in this pathway.

Adipogenesis has previously been studied in culture conditions using highly adipogenic serum that is supplemented without or with molecules such as methyl isobutyl xanthine (MIX) and dexamethasone (Dex) that enhance adipose conversion⁸. However, none of these molecules have been shown to induce commitment in the absence of adipogenic serum. The use of small molecules to assist in adipocyte 3T3-F442A cells conversion provides the opportunity to study some of the mechanisms involved in the early stages of differentiation. Such an approach also facilitates the examination of signaling and transcriptional cascades involved in adipogenesis and discriminates between other stimulated pathways that could be unnecessarily and simultaneously activated by serum or growth hormones.

Recently, we demonstrated the ability of staurosporine (St) to induce adipose differentiation in the absence of adipogenic proteins⁹ and the ability of dexamethasone (Dex) to enhance differentiation of 3T3-F442A cells induced with St¹⁰. This model offers the following two advantages: 1) St/Dex can promote a maximum differentiation in 4 h without adipogenic serum (incubation with the serum alone requires approximately 48 h for the cells to reach the same level of adipogenesis⁹); and 2) St induces two well-defined stages for differentiation before clonal expansion that then permit the identification and analysis of early gene regulation. The first stage consists of 4 h of induction, where St induces progenitor cells to differentiate, and the second stage consists of a subsequent 44 h of stabilization, where differentiation continues in the absence of the inducer but can still be reversed by anti-adipogenic substances or cytokines⁹. It is important to note, that this model of 3T3-F442A cells, allows an early response to adipogenesis, since only 4 hours of treatment with St is sufficient to trigger the adipose program, whereas adipogenic serum and MIX/Dex require, at least, 48 hours for induction in 3T3-L1 cells⁸. All the studies published with the 3T3-L1 cells are carried out in medium supplemented with adipogenic serum during growth phase of the culture, and MIX/Dex are added later at confluency to enhance adipose conversion. The 3T3-L1 cells



are able to differentiate with adipogenic serum without the addition of MIX/Dex, which later were found to enhance, but not initiate adipose conversion^{2,8}. These data, shows that the 3T3-F442A model we are using, is suitable to carry out kinetic analysis of gene expression, mainly during the early stages of induction.

Studies using cell lines have shown that a complex cascade of transcription factors is engaged in the generation of fat cells¹¹. The peroxisome proliferator activated receptor gamma (PPAR γ , *pparg*), particularly isoform 2, and CCAAT enhancer binding protein alpha (C/EBP α , *cebpa*) are considered the orchestrators of adipogenesis, and their expression patterns determine adipose differentiation^{12–14}. Also, it has been suggested that in 3T3-L1 cells, expression of PPAR γ to promote adipogenesis depends on glucocorticoids, cAMP and serum mitogens via C/EBP β ¹¹.

The principal transcription factor involved in the early steps of adipogenesis is C/EBP β (gene: *cebpb*). Its expression precedes the expression of *pparg2* and *cebpa* by approximately 30 h¹⁵, raising the question of whether other genes that are expressed such a long time after *cebpb* expression can regulate the expression of *pparg2* and *cebpa*.

The Sterol Responsive Element Binding Proteins (SREBPs) are transcription factors encoded by the *srebfl1* and *srebfl2* genes. Recently, the importance of the SREBPs has been highlighted; these proteins function as the central hubs in lipid metabolism¹⁶. Using genome-wide expression analysis, we identified *srebfl1* as an early gene induced during adipogenesis of 3T3-F442A cells. The *srebfl1* gene encodes two proteins, SREBP1a and SREBP1c, with isoform -1c being the predominant isoform in most tissues analyzed¹⁷. However, the roles of SREBP1a and -1c during the early events of adipogenesis remain unknown. In this study, we found that *srebfl1a* was expressed very early in induction and preceded the expression of all other adipogenic genes, including *pparg2*, *cebpa*, *srebfl1c* and *fabp4*. Loss-of-function experiments revealed that expression of *srebfl1a* was necessary for adipogenesis. We also showed that expression of *srebfl1a* depended on the activity of GSK3 β probably via the phosphorylation of C/EBP β and that GSK3 β activity played an essential regulatory role in the very early stages of induction of adipogenesis and maintenance of the adipose cell phenotype. Our results indicate the importance of the *srebfl1a* gene during early adipogenesis and demonstrate that *srebfl1a* acts as a link between the GSK3 β -dependent signaling pathway and the beginning of the transcriptional cascade that results in the expression of *pparg2*.

Results

Adipogenesis involves lineage commitment, clonal amplification of committed cells, and expression of the adipose cell phenotype. In 3T3-F442A cells, replication of DNA and clonal amplification precede the expression of the adipose phenotype when cells are induced to differentiate upon treatment with adipogenic serum¹⁸. Clonal amplification, which marks the beginning of adipose phenotype expression, has been shown to occur approximately 68 h after adipogenic serum supplementation in non-differentiating resting cultures, and DNA synthesis takes place about 12 h earlier¹⁸. Because adipogenesis of 3T3-F442A cells has been shown to occur more rapidly following induction with St/Dex compared to adipogenic serum⁹, we attempted to identify the time course of clonal amplification under these conditions. Two-day post-confluent cultures of 3T3-F442A cells were incubated in DMEM (Dulbecco's Modified Eagle Medium) containing non-adipogenic serum with St/Dex for 4 h to induce cells to differentiate. At various time points, cell numbers were assessed in each culture. We found that the total cell number increased approximately 56 h following treatment, which corresponded to clonal amplification (Figure 1A). Based on our previous data, these results suggested that DNA synthesis likely occurred about 44 h into induction. In combination with previously described studies⁹, our data demonstrate the following two early

stages for for differentiation: a 4-hour induction stage initiated by St/Dex, followed by a stabilization stage that lasts up to 44 h, which is followed by DNA synthesis and clonal amplification at 56 h post-treatment with St/Dex (Figure 1B). Diaz-Velasquez et al. (2008) found that at about 14 h after clonal amplification, the activity of the lipogenic enzyme, glycerol phosphate dehydrogenase, an early marker of phenotypic expression, began to increase in a time-dependent manner. These results are essential to more precisely identify the molecular events that regulate each adipogenesis stage.

To quantify differentiation into adipocytes, we counted the number of adipocyte clusters in cultures at the conclusion of the experiment. Cells induced to differentiate into adipocytes form adipose clusters⁹, and as each cluster arises from the selective multiplication of one parental cell¹⁹, the number of adipose clusters corresponds to the number of induced cells. When we cultured 3T3-F442A cells in non-adipogenic conditions and then induced them with St/Dex for 4 h, differentiation to adipocytes was 10-fold higher compared to non-induced cultures, and the number of adipocyte clusters was in agreement with the number of adipose clusters observed in cultures incubated with fetal bovine serum, which is highly adipogenic (Figure 1C).

The genes coding for the SREBPs are early and highly expressed during differentiation of 3T3-F442A cells. We performed a global screening using genome-wide expression microarrays to identify genes that may be involved in the various stages of adipogenesis. We compared 3T3-F442A cells under three distinct culture conditions: 1) cells in the induction stage with St/Dex (4 h); 2) cells in the stabilization stage (30 h from induction, prior to clonal amplification); and 3) cells in the phenotype expression stage (144 h), which consisted primarily of terminally differentiated mature adipocytes. We observed an early increase in the expression of the *srebfl1* gene. This gene differentially expresses two isoforms, -1a and -1c; however, microarray analyses did not reveal isoform-specific expression changes (data not shown).

Although the participation of the *srebfl1* gene in lipid metabolism has been described, the roles of the -1a and -1c isoforms have not been documented. Our results demonstrated that compared to non-induced cells, expression levels of both isoforms of the *srebfl1* gene were increased several fold during induction and stabilization stages, and these levels continued to increase (Figure 1D). The *Srebfl1a* isoform showed a rapid and sharp increase in expression after 4 h of induction, and by the stabilization stage (8 h), the expression of *srebfl1a* reached a maximum level at about 15-fold, which was maintained thereafter. During the first 3 h of the induction stage, *srebfl1a* expression remained at levels similar to non-induced cells (Figure 1E). *Srebfl1c* expression increased significantly at the end of the stabilization stage (48 h), reaching a maximum of 350-fold well into the phenotypic expression stage (72 h), and remained at those levels during terminal differentiation, forming mature adipocytes (Figure 1D). It is remarkable that during commitment, expression of *srebfl1a* was higher than *srebfl1c* and it took about 60 h longer for *srebfl1c* to reach its maximum level of expression compared with *srebfl1a* (Figure 1D). *Srebfl2*, the third isoform in this family, which regulates cholesterol metabolism, did not undergo any significant change in expression in either of the culture conditions (Figure 1D), suggesting that cholesterol metabolism was not significantly stimulated during adipose conversion. In agreement with this, other studies have shown that total cholesterol synthesis is not altered during 3T3-L1 fat cell differentiation¹.

The increase in *srebfl1a* expression preceded the changes in the expression of other genes that have been described as characteristic regulators of 3T3 cell adipogenesis. We found that expression of *pparg2*, the regarded master regulator of adipogenesis¹⁴, did not change during induction but began to increase after 8 h and was 50-fold higher by the end of stabilization (48 h); at the phenotypic

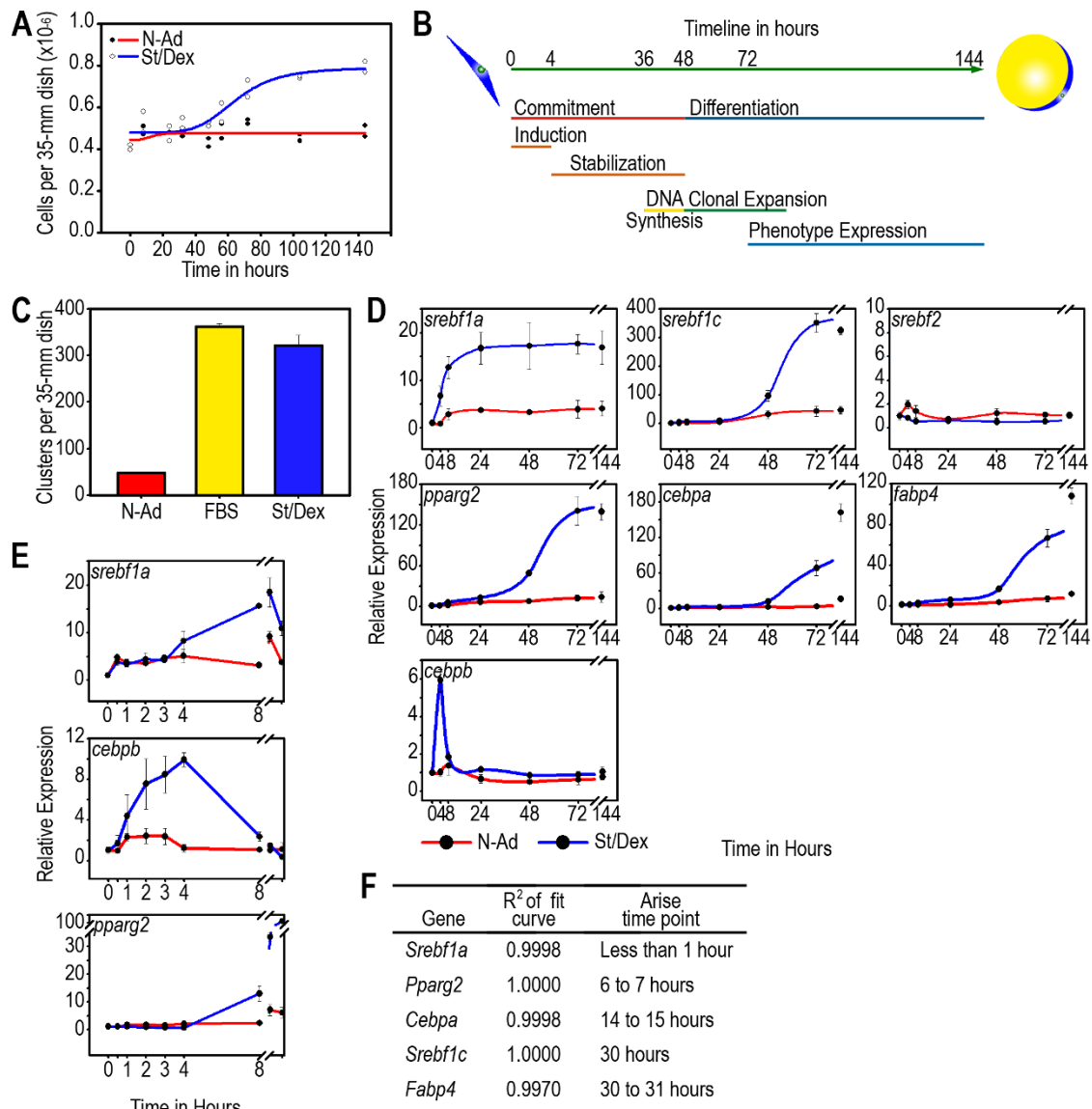


Figure 1 | Expression of *srebf1a* and adipogenic genes during adipose differentiation of 3T3-F442A cells. (A) Clonal expansion of 3T3-F442A cells induced to differentiate with staurosporine and dexamethasone (St/Dex). Post-confluent 3T3-F442A cells were induced to differentiate into adipose cells with St/Dex for 4 h followed by incubation in non-adipogenic conditions (N-Ad). Cells were harvested and counted at the indicated times. (B) Schematic representation of the time course of the adipogenic program induced by St/Dex. The main stages are shown at the indicated time frames. (C) Adipose conversion in post-confluent cultures induced with fetal bovine serum (FBS) for 48 h or St/Dex for 4 h in non-adipogenic medium. After induction, cultures were switched to non-adipogenic medium and grown for 144 h. Adipose clusters were assessed by staining with Oil Red O. (D) and (E) Expression of *srebf1a* and other adipogenic genes. Post-confluent cultures under non-adipogenic conditions (N-Ad) or induced with St/Dex were harvested at different time points, and gene expression was evaluated by qRT-PCR. (F) The calculated time for the increase in the expression of adipogenic genes based on the gene expression kinetics. Briefly, we calculated the fit curve at a maximal R² and determined the inflection points from the second derivative. Data are presented as the mean \pm s.d.

expression and terminal differentiation stage, *pparg2* expression was 140-fold higher (Figure 1D, E). The *cebpa* gene did not show any change in expression during induction, but its expression was increased (12-fold) at the end of the stabilization stage, reaching its maximum (160-fold) after clonal expansion and at the beginning of phenotype expression (72 h) (Figure 1D).

The *fabp4* gene, which encodes aP2, a lipid carrier expressed during adipose differentiation²⁰, had similar expression kinetics as *cebpa*; *fabp4* reached its maximum level of expression of 108-fold in terminally differentiated adipocytes (Figure 1D). The expression of *cebpb* transiently increased (6-fold) during the 4 h induction stage but returned quickly to the basal levels found in non-induced cells by 8 h (Figure 1D, E). The early and transient increase in expression was accompanied by changes in the phosphorylation state of the C/EBP β protein.

The expression kinetics for each gene was different. To more precisely identify the time at which expression of each gene began to increase, we determined the mathematical equation for each kinetic plot. We used the second derivative of each function and calculated the inflection point of the derivative that corresponded to the time point at which expression of each gene began to increase. We observed the following time course of gene expression (Figure 1F): *srebf1a* was the first expressed gene, with expression beginning at less than 1 h of the induction stage. The other genes began to be expressed well into the stabilization stage of commitment. Increasing expression levels of *pparg2* followed the expression of *srebf1a*, and then *cebpa*, *srebf1c* and *fabp4* expression followed. This analysis demonstrated that these genes began to be expressed during induction and stabilization and likely before clonal expansion. It also suggests that SREBP1a may be the transcription factor that orchestrates



the adipogenic program, regulating even *pparg2*, the well-known adipogenic master regulator.

With the available data on *cebpb* expression, we could not calculate the second derivative, but there was a significant increase in *cebpb* expression that clearly occurred in the first hour of induction (Figure 1E). Thus, *cebpb* and *srebfla* were the two genes that changed their expression in the very early stage of induction.

Loss of function of *srebfla* inhibits adipose differentiation of 3T3-F442A cells. To study the necessity of the expression of the *srebfla* gene, we knocked down its expression by shRNA targeting. 3T3-F442A cells were transfected with the pSingle-shSRE1a vector, which expresses an shRNA targeting *srebfla* upon treatment with doxycycline. After transfection, cells were seeded and induced to differentiate with St/Dex. Silencing of the *srebfla* gene by pSingle-shSRE1a led to about a 70% inhibition in *srebfla* mRNA expression compared to control cultures not treated with doxycycline (Figure 2A). As expected, parallel cultures transfected with a vector containing a non-related sequence did not show silencing of the gene (results not shown). The expression of the adipogenic genes that are characteristic of adipose differentiation, including *pparg2*, *cebpa* and *srebflc*, also decreased by an amount similar to *srebfla* (Figure 2A).

These results demonstrate that SREBP1a activity is upstream of *pparg2*, *cebpa* and *srebflc* and that SREBP1a is required for the regulation of these adipogenic genes.

GSK3 β regulates the expression of *srebfla* through C/EBP β phosphorylation. The C/EBP β protein is a transcription factor that targets distinct genes in several tissues. Phosphorylation of C/EBP β is required for DNA binding and determines the specificity of target genes transactivated by C/EBP β . For the adipogenic program, phosphorylation of C/EBP β at Thr188 is required. Phosphorylation of Thr188 could be mediated by ERK or GSK3 β , as Thr188 is part of the ERK/GSK3 phosphorylation consensus site²¹. An antibody against phospho-C/EBP β Thr188 showed that phosphorylation of the protein began to increase within 0.5 h of the induction stage, reaching a maximum at 2 h, and then it began to decrease to basal levels after 4 h, corresponding with the end of the induction stage (Figure 2B). Our results also showed that this phosphorylation event preceded the expression of various adipogenic genes, including *srebfla*, the earliest expressed gene in the adipogenic pathway (compare Figure 2B with Figure 1D and E). We have previously demonstrated that GSK3 β activity is essential for the induction of the adipogenic program⁹. The activity of GSK3 β is regulated by

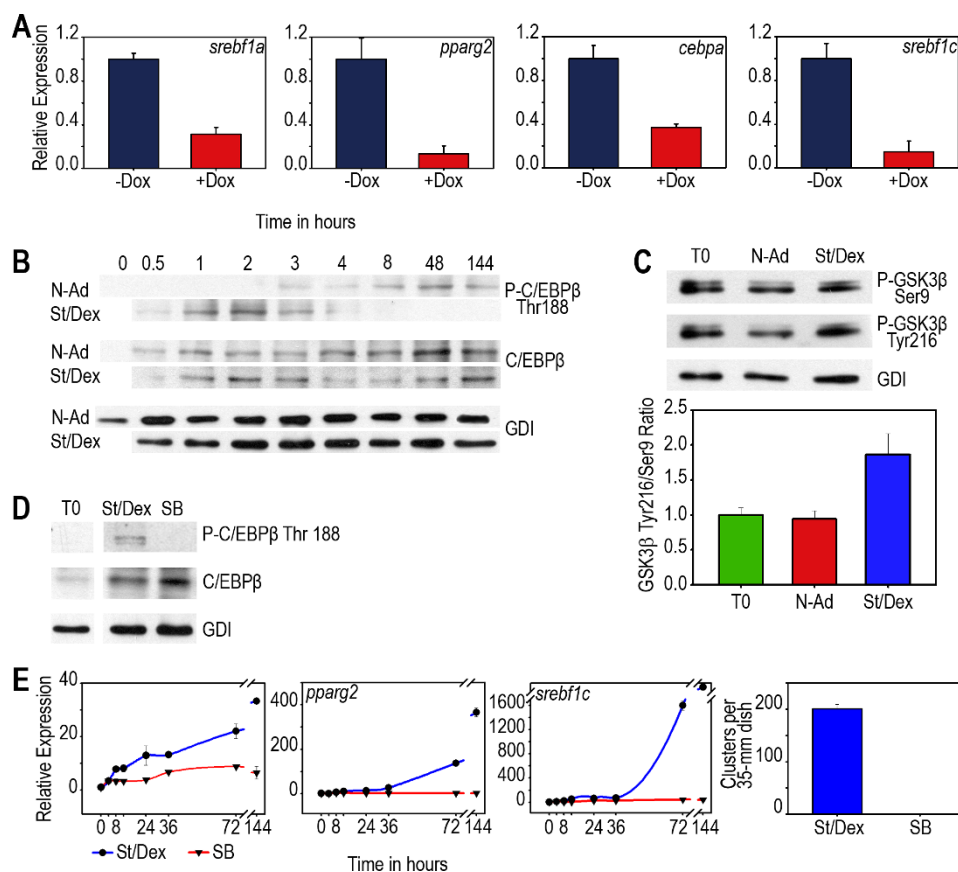


Figure 2 | *Srebfla* is necessary for adipogenesis of 3T3-F442A cells. (A) Knockdown of *srebfla* expression by specific shRNA blocks the adipogenic program. 3T3-F442A cells were transfected with pSingle-shSRE1a containing a specific shRNA against *srebfla*. Transfected cells were then induced to the adipogenic program with St/Dex for 4 h. Expression of shRNA was induced by doxycycline (Dox), and gene expression was evaluated by qRT-PCR at 48 h after induction. (B) St/Dex induced early phosphorylation of C/EBP β at Thr188 during adipocyte differentiation. Cultures of 3T3-F442A cells were induced with St/Dex, and total protein was extracted and immunoblotted at the indicated times. (C) St/Dex induction stimulates GSK3 β phosphorylation at Tyr216. Protein extracts from 3T3-F442A cells at time zero (T0) in non-adipogenic conditions at 4 h (N-Ad) or induced with St/Dex for 4 h were immunoblotted with specific antibodies. (D) Inhibition of GSK3 β activity inhibits phosphorylation of C/EBP β at Thr188. Cells were induced with St/Dex for 4 h, and cultures were then switched to fresh medium supplemented with 50 μ M SB415286 (SB), a specific inhibitor of GSK3 β activity, for 20 h. Protein extracts were obtained at time zero (T0) or in the presence (SB) or absence (St/Dex) of the inhibitor and analyzed by immunoblotting. (E) GSK3 β activity is necessary for the expression of the adipogenic genes and subsequent adipocyte differentiation. Cells were treated as in D. Cultures in the presence (SB) or absence (St/Dex) of SB415286 were followed for 144 h to evaluate adipose conversion. Gene expression was evaluated at the indicated times by qRT-PCR. Data are presented as the mean \pm s.d.



posttranslational modifications. Phosphorylation of GSK3 β at Tyr216 activates the kinase, but phosphorylation at Ser9 inactivates the kinase even in the presence of Tyr216 phosphorylation²². In cultures induced with St/Dex, we found that GSK3 β phosphorylated at Tyr216 was the more predominant form than GSK3 β phosphorylated at Ser9 (Figure 2C). By densitometric analysis of the immunoblots, GSK3 β -Tyr216 was approximately 80% more abundant than the inactive form of GSK3 β , GSK3 β -Ser9 (Figure 2C). We also inhibited the activity of GSK3 β using its selective inhibitor, SB415286. Phosphorylation of C/EBP β at Thr188 was completely abolished in cultures treated with SB415286 (Figure 2D). These results strongly suggest that the activity of GSK3 β regulates the phosphorylation of C/EBP β .

The GSK3 β inhibitor, SB415286, also blocked the expression of the adipogenic genes tested and thus prevented adipose differentiation (Figure 2E). We next analyzed the promoter sequence of the *srebf1* gene. Murine *srebf1* has two separate promoters that give rise to isoforms -1a and -1c; these forms mutually exclude each other¹⁷. Sequence analysis of the *srebf1* promoter using the TFSearch algorithm²³ revealed the existence of at least 5 putative binding sites for C/EBP β between positions -1075 to -3527. Additionally, there was a consensus site for C/EBP at position -841. These results show that GSK3 β is involved in regulating phosphorylation and activation of C/EBP β and that this transcription factor is required to transactivate *srebf1* expression, leading to the early steps of adipogenesis.

PPAR γ does not transactivate *srebf1* expression. We showed that *srebf1* is one of the earliest genes expressed during the adipogenic program, even prior to *pparg2* expression (Figure 1D, E). *Srebf1c* and *srebf1a* share a high level of sequence homology and differ in the first exon; here, SREBP1a is 24 amino acid residues longer. Sequence analysis of the *srebf1c* promoter revealed a putative binding site for PPAR γ that was about -6900 bp from the transcription start site. Similar analyses of the *srebf1a* promoter did not reveal any recognition site for PPAR γ . To date, there has been no experimental evidence that PPAR γ transactivates the expression of *srebf1a* or -1c. Nevertheless, due to the high homology between *srebf1a* and -1c and the putative binding site for PPAR γ in the *srebf1c* promoter, it is conceivable that basal levels of PPAR γ in pre-adipocytes could be sufficient to transactivate, either directly or indirectly, the expression of *srebf1a* or -1c.

To exert its transcriptional activity, PPAR γ requires an endogenous lipid ligand¹⁴, but rosiglitazone (Ros), a synthetic ligand, can also activate the transcriptional function of PPAR γ ²⁴. Therefore, we attempted to identify by functional experiments with Ros whether *srebf1a* or -1c expression relied on PPAR γ transcriptional activity. Early treatment with Ros during induction should activate the basal levels of PPAR γ and thus transactivate PPAR γ target genes. This process would be evident by an early shift in the expression curve of those genes. We induced post-confluent cultures of 3T3-F442A cells with St/Dex and then added Ros to the cultures during induction (0–4 h). Cultures were maintained for up to 144 h to assess adipose conversion. Gene expression was evaluated by qRT-PCR at various time points.

Ros accelerated the expression of *pparg2*, *cebpa*, *srebf1c*, and *fabp4*, suggesting that Ros activation of basal PPAR γ leads to increased self-activation and the transactivation of *cebpa*, *srebf1c*, and *fabp4*. More importantly, *srebf1a* expression did not increase upon addition of Ros (Figure 3A), demonstrating that the expression of this gene is not under the regulation of PPAR γ or other transcription factors that are responsive to this drug.

In parallel cultures, we determined the number of adipose clusters at the end of the experiment. We found that the number of adipose clusters was similar in all culture conditions either with or without Ros (Figure 3B). This indicated that the accelerated gene expression did not increase induction into differentiation of the adipose

precursors in the cultures. However, the cluster size was larger in the cells treated with Ros (Figure 3C), suggesting that the early activation of the genes might lead to higher adipose conversion through larger adipose clusters and/or higher lipid accumulation.

Discussion

Adipose differentiation of 3T3 cells is regulated by networks of transcriptional factors¹¹. *Cebpb* is one of the earliest transcription factors expressed during adipose differentiation, and it precedes *pparg2* and *cebpa* expression by 30 h¹⁵. Recently, SREBPs have been regarded as important central hubs in lipid metabolism¹⁶.

The ratio of the SREBP1c protein to the -1a protein varies widely in different tissues. The -1c isoform is more abundant in adipose tissue and liver than the -1a isoform¹⁷, although -1a shows higher transcriptional activity²⁵. Deletion of *srebf1a* is lethal to the embryo, suggesting an unknown important role of the protein during embryonic development²⁶. Overexpression of *srebf1a* in transgenic mice produced adipocyte hypertrophy and fatty liver²⁷. These and other data suggest that isoforms -1a and -1c may be controlled independently, and the two may respond differentially to organ-specific and metabolic factors¹⁷. Despite the key function of these transcription factors in lipid homeostasis, a role for SREBP1a in adipose differentiation has not been reported.

To study the adipogenesis program, we took advantage of the two-stage model in 3T3-F442A cells, which includes an induction stage of 4 h followed by stabilization for 44 h, a time in which the adipogenic inducer is no longer required⁹. We also used St as a specific small molecule to induce differentiation without any adipogenic proteins⁹, circumventing the ERK/MAPK signaling pathway that is activated by serum adipogenic proteins or growth hormone to promote adipogenesis²⁸. It has been described that St directly activates GSK3 β ²⁹, a molecular switch in the Wnt pathway. Here, we report that GSK3 β induces C/EBP β phosphorylation at Thr188, which then promotes the expression of the *srebf1a* transcription factor. These events precede the expression of *pparg2* and *cebpa*, and regulate the early stages of adipogenesis. As deletion of *srebf1a* leads to embryonic lethality²⁶, expression studies of this gene in 3T3-F442A cells offer a suitable experimental model to elucidate the role of this transcription factor during adipogenesis. The early expression of *srebf1a* and the loss-of-function experiments showed that the expression of *srebf1a* was essential for adipogenesis through regulation of *pparg2*, *cebpa* and *srebf1c*. *Srebf1a* expression increased at least 10-fold in the 4 h of the induction stage and increased prior to augmentation of *pparg2* expression, which occurred after the induction stage. After the inducer (St/Dex) was removed, cells were at the stage of stabilization well after *srebf1a* mRNA had reached its maximum level. Therefore, the well-known, adipogenic transcriptional cascade involving PPAR γ is activated after the cells have begun to express *srebf1a* and no longer require an inducing agent.

ADD1/SREBP1³¹, which was later identified to be the -1c isoform, does not bind to the *pparg2* promoter³², but it can activate PPAR γ through the production of a fatty acid derivative, which acts as an endogenous ligand³³. Analysis of the *pparg2* sequence with the TFSearch algorithm did not reveal any recognition site in its promoter region for binding of SREBP1a or -1c. However, the same analysis of the *srebf1c* promoter revealed a recognition site for PPAR γ . In combination with previous work, our data demonstrating the up-regulation of *srebf1c* after *pparg2* and the accelerated expression of *srebf1c* in the presence of Ros (an exogenous ligand for PPAR γ activation) support the idea that PPAR γ may directly regulate *srebf1c* expression. Additionally, in the phenotype expression stage, a mutual regulation of PPAR γ and SREBP1c may take place to regulate adipogenesis and lipid metabolism through a feedback loop, where PPAR γ transactivates *srebf1c* expression, and SREBP1c produces an internal ligand to activate PPAR γ (Figure 3D). Due to the lack of a recognition site for SREBP1a in the *pparg2* promoter

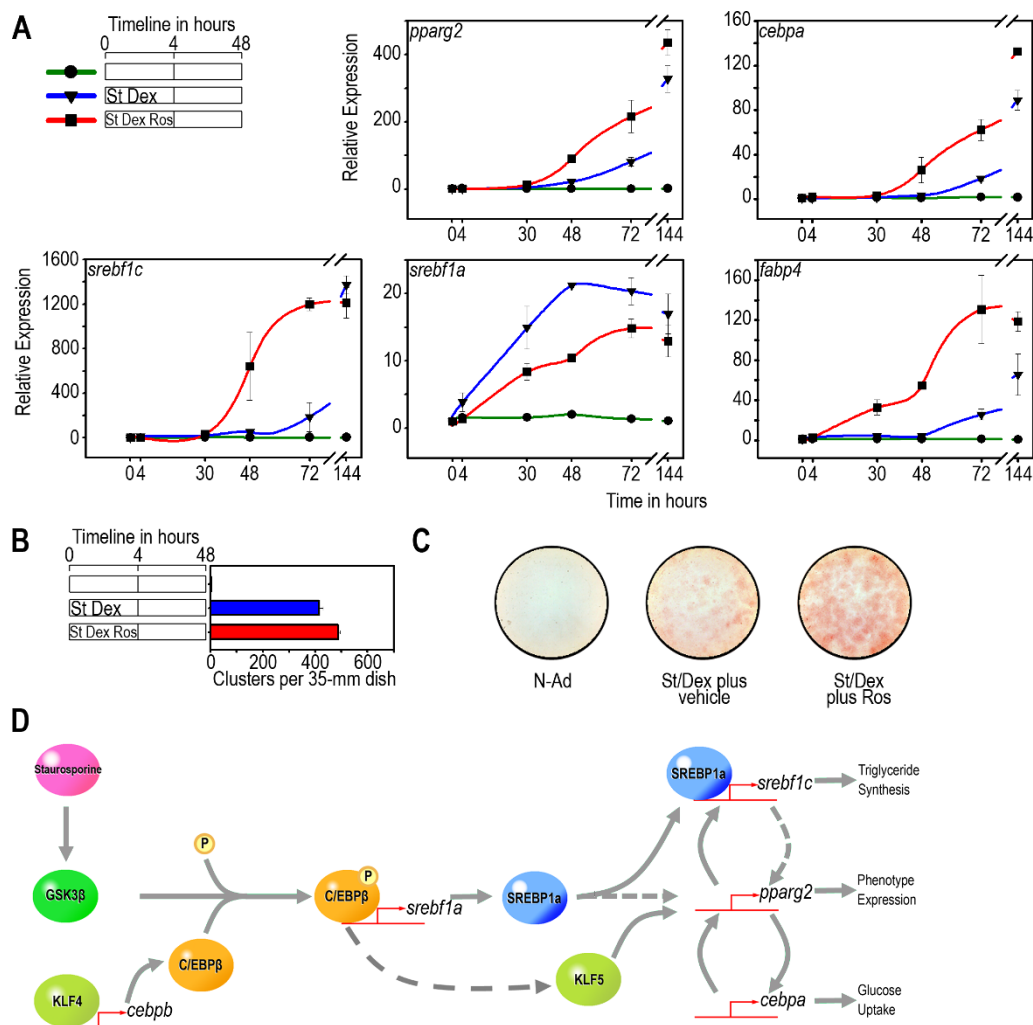


Figure 3 | *Srebf1a* expression does not depend on PPAR γ transcriptional activity. (A) Rosiglitazone (Ros), a potent PPAR γ activator, accelerates the expression of adipogenic genes, but not of *srebf1a*. Post-confluent cultures were induced with St/Dex supplemented with 1 μ M Ros during induction. Gene expression was determined by qRT-PCR. (B) Adipose differentiation induced with St/Dex with and without Ros. Data are presented as the mean \pm s.d. (C) Representative cultures differentiated in the presence or absence of Ros; note that the adipose clusters treated with Ros are larger in size and more intensely stained with Oil Red O. N-Ad, non-adipogenic condition. (D) Schematic representation of the proposed signaling pathway and transcriptional cascade during adipogenesis. The data show that the early expression of *srebf1a* depends on GSK3 β activity, and the expression of *srebf1a* is necessary for the expression of the adipogenic genes, *pparg*, *cebpa* and *sreb1c*.

sequence, we suggest that SREBP1a activity, similar to SREBP1c, may produce an intermediary that transactivates *pparg2* and upregulates its expression, warranting further investigation into a possible pathway of *pparg2* regulation by SREBP1a (Figure 3D).

SREBP1a appears to be the strongest activator of Sterol Responsive Element (SRE), SRE-like and E-box sequences²⁵. Previous work has shown that SREBP1a and -2, but not -1c, transactivate sites found in cholesterologenic genes²⁵. SREBP1a and -1c transactivate targets with E-box sequences in their promoters to activate the lipogenic pathway²⁵. Interestingly, SREBP1c has an SRE binding site in its promoter³⁴, suggesting that SREBP1a could transactivate it. Analysis of the promoter sequence of *srebf1a* did not show any SRE or E-box binding sites. *Srebf1a* appears to have a constitutively low expression pattern in liver, but its overexpression in transgenic mice leads to liver steatosis³⁵, which is consistent with a report indicating that the activation of the lipogenic program could also occur through SREBP1a²⁵. Based on our data, we propose that an SRE binding site in *srebf1c* could be occupied by SREBP1a to trigger *srebf1c* expression, which then contributes to the activation of the lipogenic program. It is conceivable that SREBP1a activates expression of *srebf1c*, and because it is required for expression of *pparg2*

either directly or indirectly, it would contribute to a feedback loop among these three factors and trigger the rest of the transcriptional adipogenic cascade (Figure 3D). The feedback loops between PPAR γ and CEBP α ³⁶ and between SREBP1a, SREBP1c and PPAR γ described here raise the possibility that they not only regulate induction to adipogenesis but also have a regulatory function in lipid metabolism, as their genes remain highly expressed in mature adipocytes.

One of the early steps for adipose conversion is the inhibition of the Wnt pathway³⁷. The Wnt pathway maintains pre-adipocytes in an undifferentiated state³⁷, and direct activation of GSK3 β inhibits intracellular Wnt signaling³⁸. Staurosporine activates GSK3 β by maintaining its Tyr216 phosphorylation, which is the active state of the enzyme²⁹. Our results show that staurosporine induces adipose conversion by preserving GSK3 β phosphorylation at Tyr216, which is subsequently required to induce C/EBP β phosphorylation. This is followed by the expression of *srebf1a* during the first 4 h of induction. After this point, the cells enter an intrinsic and autonomous pathway of commitment without the involvement of St or other adipogenic factors. The persistent GSK3 β activity has been found to be required not only for induction but also for its subsequent stabilization and clonal amplification of adipogenic cells⁹. Some of



the possible mechanisms of progression into intrinsic signaling during differentiation include persistent protein kinase activation, transcriptional autoactivation, and late stabilization of gene expression³⁹. Because GSK3 β remains active for progression into intrinsic signaling during commitment, terminal differentiation of adipose cells and lipid metabolism, this kinase should have a crucial role during adipogenesis. The persistent activation of GSK3 β is due to its autophosphorylation on Tyr216 that is initially mediated by chaperone proteins, but after maturation of the kinase, the chaperone proteins are no longer required⁴⁰.

More recently, it has been shown that two additional transcription factor genes, *klf4* and *klf5*, are expressed early during 3T3-L1 cell adipogenesis. KLF4 binds to the *cebpb* promoter⁴¹. *Klf5* expression is induced by C/EBP β and C/EBP δ , and it has been suggested that KLF5 directly affects the expression of *pparg2* and *cebpa*, probably by forming an enhanceosome with C/EBP β/δ on the *pparg2* promoter⁴². Despite the possible differences between the principal adipogenesis models, the 3T3-L1 and 3T3-F442A cells, it is clear that the expression of *klf4*, *klf5* and *srebfla* occurs very early. Based on data from this and other studies, we propose that induction of adipocyte commitment involves the following (see Figure 3D): 1) inhibition of the Wnt pathway through GSK3 β activation; 2) KLF4 binding to the *cebpb* promoter, activation of CEBP β through induction of phosphorylation by GSK3 β , and expression of *srebfla* via phosphorylated CEBP β ; and 3) following activation of *cebpb* by KLF4, one pathway could involve *klf5* and another *srebfla*, with both pathways converging on the expression of *pparg2*. It is equally likely that the expression of *srebfla* and *klf5* are coordinated and not independently regulated. This could be explored by determining the expression of *klf5* in cells in which *srebfla* is knocked down. Finally, after the expression of *klf4*, *klf5* and *srebfla* during the early stage of adipogenesis and without the requirement of the inducer, cells continue through the stabilization stage with the expression of *pparg2*, *cebpa* and *srebflc*. The progression into these stages during points to a regulatory transcriptional cascade for adipogenesis that is more complex than previously thought. The necessity of *srebfla* expression and the presence of feedback loops between SREBP1a/1c and PPAR γ and between CEBP α and PPAR γ (Figure 3D) suggest the existence of finely tuned regulatory pathways involving the SREBP1 transcription factors in adipose biology. These possible alternate pathways for adipogenesis warrants further study into these new lines of investigation. Several crucial points of adipogenesis regulation emerge from our data and offer new insights on obesity and its related pathologies. Our studies point to the activity of GSK3 β and SREBP1a/1c as new control points for adipogenesis and, thus, new targets for therapeutic intervention in obesity and diabetes. It is important to note from our studies and others that adipogenesis induction or stimulation by various substances (St/Dex, adipogenic proteins, growth hormones or MIX/Dex) all require GSK3 β activation. Therefore, this kinase appears to have a crucial function as the molecular switch to initiate a differentiation program. Our data clearly demonstrate the requirement for persistent activation of GSK3 β during all stages of adipose differentiation and phenotype expression and its close relationship with SREBP1a, indicating the importance of these molecules as regulatory factors for lipid homeostasis.

Methods

Materials. Eagle's medium modified by Dulbecco and Vögt (DMEM) and TRIzol® solution were purchased from Invitrogen-Life Technologies (Carlsbad, CA). Adult bovine serum was from M.A. Bioproducts (Walkersville, MD). We obtained adult cat serum by bleeding domestic adult cats in accordance with the NIH guidelines for the welfare of research animals. Epidermal growth factor was from IMCERA Bioproducts Inc. (Terre Haute, IN). Insulin, d-biotin, human transferrin, triiodothyronine, staurosporine, dexamethasone, β -mercaptoethanol, Oil Red O, dimethyl sulfoxide, 3-[[3-Chloro-4-hydroxyphenyl]amino]-4-(2-nitrophenyl)-1H-pyrrrol-2,5-dione (SB415286), and rosiglitazone were all from Sigma-Aldrich Corp. (Saint Louis, MO). All other reagents were of analytical grade.

Cell culture and induction of adipose differentiation. The 3T3-F442A cells were seeded in culture dishes at 1.25×10^3 cell/cm² in non-adipogenic growing medium consisting of DMEM supplemented with 4% adult cat serum, 5 μ g/ml insulin and 1 μ M d-biotin^{3,10}. For experiments, one- to two-day post-confluent cultures were grown in non-adipogenic medium containing DMEM supplemented with 2% adult cat serum, 0.2% adult bovine serum, 5 μ g/ml insulin, 5 μ g/ml transferrin, 1 μ M d-biotin, 2 nM triiodothyronine, 40 μ M β -mercaptoethanol and 0.01 ng/ml epidermal growth factor⁴. Adipogenesis was induced in post-confluent cultures with staurosporine (St) and dexamethasone (Dex) for 4 h in the non-adipogenic medium as previously described^{3,10}. Cultures were incubated for up to 144 h, and adipose conversion was evaluated by counting adipose clusters stained with Oil Red O⁴³. All cultures were incubated at 37°C in a 10% CO₂ atmosphere.

Short hairpin RNA cloning and cell transfection. A short hairpin RNA (shRNA; Supplementary Table S1) against *srebfla* mRNA was cloned into the pSingle-tS-shRNA plasmid (Clontech Laboratories; Mountain View, CA) at the *XhoI* and *HindIII* cloning sites. The resulting construct was termed pSingle-shSRE1a. Expression of shRNA was driven by a tetracycline-responsive Pol III hybrid promoter. For experiments, purified endotoxin-free plasmid extracts were transfected into 3T3-F44 cells using Amaxa's Nucleofector™ (Lonza; Basel, Switzerland) with a Cell Line Nucleofector Kit V (Amaxa; Cologne, Germany). The 3T3-F442A cells were then harvested and resuspended in Nucleofection™ solution at 3.0×10^6 cells/100 μ l. After the addition of 2.5 μ g shRNA expression vectors, the cells were transfected using Nucleofector and seeded onto 35-mm plates at a near-confluent density of 4×10^5 in the absence or presence of 1 μ g/ml doxycycline. Transfected cells were induced to differentiate with St/Dex 2 days after transfection. Differentiation experiments were carried out using the same media and culture conditions as described earlier, and the knockdown efficiency was verified by relative quantitative RT-PCR assays.

Relative-quantitative RT-PCR. Cellular RNA was isolated with TRIzol® from cultured cells. Two micrograms of total RNA was transcribed into cDNA using reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Relative-quantitative PCR (qRT-PCR) was carried out using the FastStart Universal Sybr Green Master (Rox) kit (Roche Applied Science; Mannheim, Germany), and amplification was performed on a 7500 real-time thermal cycler (Applied Biosystems; Carlsbad, CA). Specific primers for each gene are listed in Supplementary Table S2. Expression values were determined by the $2^{-\Delta\Delta CT}$ formula using the expression of the ribosomal phosphoprotein large P0 (*rplp0*) gene as the normalizing factor.

Immunoblotting. At the times indicated, cells were lysed with ProteoJet Mammalian Cell Lysis Reagent (Fermentas Inc., Glen Burnie, MA) supplemented with Complete® protease inhibitor (Roche Applied Science) and phosphatase inhibitors. Proteins were separated by SDS/PAGE and immunoblotted with antibodies against human phospho-C/EBP β Thr235 (the equivalent of murine Thr188) from Cell Signaling Technology (Berkeley, CA), phospho-GSK3 β Ser9 (Abcam; Cambridge, UK), C/EBP β and phospho-GSK3 β Tyr216 from Santa Cruz Biotechnology (Santa Cruz, CA), and with Rab Guanine Nucleotide Dissociation Inhibitor (GDI, Invitrogen).

Data management and statistical analysis. Data are presented as the mean plus/minus standard deviation of the mean. Data were analyzed by the two-tailed Student's *t*-test for means analysis when comparing two data groups, or analysis of variance (ANOVA) when comparing three or more data groups. Statistical significance was identified if the *P* value was lower than 0.05.

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Author contributions

JTAS, CVV, JCS and WKH contributed for the experimental design and writing manuscript. JTAS carried out cell culture, molecular cloning, Western blots, qRT-PCR assays, and figure artwork. CVV and MMM carried out cell culture and cell transfection assays. ABL cultured the cells and performed inhibitors and agonists-related experiments. All authors reviewed manuscript.

Additional information

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Competing financial interests: The authors declare no competing financial interests.

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