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SYNOPSIS.

The transcription factor SREBP1c is highly expressed in adipose tissue and plays a central role in several aspects of adipocyte development including the induction of PPARγ, the generation of an endogenous PPARγ ligand and the expression of several genes critical for lipid biosynthesis. Despite its significance, the regulation of SREBP1c expression during adipogenesis is not well characterised. We noted that in several models of adipogenesis SREBP1c expression closely mimics that of known C/EBPβ targets. Inhibition of C/EBP activity during adipogenesis by expressing either the dominant negative C/EBPβ LIP isoform, the co-repressor ETO, or using siRNAs targetting either C/EBPβ or C/EBPδ significantly impaired early SREBP1c induction. Furthermore, chromatin immunoprecipitation (ChIP) assays identified specific sequences in the SREBP1c promoter to which C/EBPβ and C/EBPδ bind in intact cells, demonstrating that these factors may directly regulate SREBP1c expression. Using cells in which C/EBPα expression is inhibited using shRNA and ChIP assays we show that C/EBPα replaces C/EBPβ and C/EBPδ as regulator of SREBP1c expression in maturing adipocytes. These data provide novel insight into the induction of SREBP1c expression during adipogenesis. Moreover, these findings identify an important additional mechanism via which the C/EBP transcription factors may control a network of gene expression regulating adipogenesis, lipogenesis and insulin sensitivity.

Abbreviations Used: serum response element binding protein 1, SREBP1; C/AAT enhancer binding protein, C/EBP; peroxisome proliferator activator gamma, PPARγ; chromatin immunoprecipitation, ChIP; short hairpin RNA, shRNA; small interfering RNA, siRNA; eight-twenty one/MTG8, ETO; phosphoenolpyruvate carboxykinase, PEPCK; diacylglycerol acyltransferase 2, DGAT2; liver inhibitory protein, LIP; liver activating protein, LAP;

INTRODUCTION.

Adipose tissue is a complex, highly active metabolic and endocrine organ [1]. Whilst the adverse metabolic consequences of excessive adiposity are well known, pathologically decreased lipid accumulation or impaired adipogenesis in lipodystrophic subjects also has similar deleterious metabolic consequences including insulin resistance, dyslipidemia, and the associated risk of cardiovascular disease [2, 3]. Thus optimal metabolic health likely requires restraint of adipose tissue mass whilst still maintaining the capacity to respond accurately to substrate availability to increase adipose mass when required. A fuller understanding of the pathways regulating the formation and maintenance of adipocytes is therefore likely to inform therapeutic strategies for the treatment of syndromes involving either decreased or increased adiposity.

The development of new adipocytes from pluripotent precursors involves a complex and tightly orchestrated program of gene expression [1]. Key early regulators of this process are the C/AAT enhancer binding proteins C/EBPβ and C/EBPδ. Acting alongside multiple co-activators, co-repressors and other transcription factors they play a central role in the subsequent induction of PPARγ and C/EBPα, transcription factors that have been dubbed the "master regulators of adipogenesis" due to their critical role in this process. The targets of these transcription factors include the promoters of many genes of the mature lipogenic and insulin-sensitive adipocyte such as aP2, PEPCK, lipoprotein lipase, adiponectin and Glut-4 [4-6]. Thus the C/EBP family of transcription factors has a critical role in adipocyte development and lipid accumulation. Studies investigating the importance of C/EBPβ and C/EBPδ have demonstrated that loss of one or both of these factors can lead to decreased adipose mass in mice and decreased adipogenesis in cellular models [7, 8]. In addition C/EBP factors may directly influence lipogenesis by controlling the early induction of the key lipogenic enzyme diacylglycerol acyltransferase 2 (DGAT2) during adipogenesis [9].

Sterol responsive element binding protein 1c (SREBP1c) is another important pro-adipogenic transcription factor that can directly regulate the expression of several key genes of lipid metabolism [10]. Moreover, in adipocyte differentiation SREBP1c appears to contribute both to the expression of PPARγ and the production of an endogenous PPARγ ligand [11, 12]. SREBP1c expression and activity, via cleavage and nuclear translocation, are acutely responsive to insulin [10, 13]. In addition to controlling genes involved in lipid metabolism, the regulation of the expression of the adipokines leptin and adiponectin by insulin is also mediated by SREBP1c [14, 15]. Thus in both developing and mature adipocytes SREBP1c can potentially integrate information of nutritional and metabolic status to control new adipocyte formation, lipid metabolism, insulin sensitivity and, via adipokines, whole body energy homeostasis and appetite. Despite the importance of SREBP1c and its established role in adipocyte development relatively little is known about the factors controlling its expression during adipogenesis although LXRα has been shown to be important for SREBP1c expression in these cells [16]. Here we demonstrate that the C/EBP family of transcription factors also play a critical role in both the early induction of SREBP1c and the maintenance of its expression in maturing adipocytes.

EXPERIMENTAL.

Preadipocyte Isolation and Cell Culture. Human preadipocytes were grown from the stromovascular fraction of collagenase digested abdominal subcutaneous adipose tissue as previously described [17]. At various times following induction of differentiation, cells were

harvested, and RNA extracted. 3T3-L1 preadipocytes were maintained and differentiated as described in [18]. 3T3-L1 preadipocyte cells stably expressing the LIP (liver inhibitory protein) isoform of C/EBPβ or ETO (eight-twenty one/MTG8) were as previously described [9]. Differentiating 3T3-L1 cells were assessed for lipid content by staining with oil-red O as in [18]. Murine E14 embryonic stem cells were cultured and differentiated as described in [19].

siRNA knockdown. Synthetic double stranded siRNA against C/EBPβ or C/EBPδ mRNAs were purchased from Ambion. 3T3-L1 preadipocytes were plated at a density 1 x 10^5 cells per well in 12-well plates the day before siRNA transfection. siRNA/ liposome mixes containing 2 µg of Lipofectamine 2000 (Invitrogen) and 100 nM of siRNA/well were incubated with cells for 6 hours in the absence of serum. Medium was replaced with serum containing 3T3-L1 growth medium for 18 hours before the induction of differentiation. 3T3-L1 preadipocytes stably expressing shRNA sequences targeting C/EBPα were generated using the pSiren retroQ kit (BD Biosciences) according to the manufacturer's instructions. Retrovirus production, 3T3-L1 infection and selection were essentially as described in [18].

RNA Isolation, cDNA Synthesis and Real Time PCR. Total RNA was extracted from cell cultures using an RNeasy kit (Qiagen). Adipose tissue was isolated from C/EBPβ null mice or their wild type littermates and RNA isolated as previously described [20]. All procedures were approved by the UCHSC Animal Care and Use Committee. Primer Express, version 1.0 software (Perkin Elmer Applied Biosystems) was used to design the probes and primers for real time quantitative PCR to determine SREBP1c, SREBP1a, DGAT2, PPARγ_2, aP2, Glut-4, C/EBPβ and C/EBPδ mRNA expression. Primer/probe mix to assay C/EBPα was obtained from Applied Biosystems. RNA was reverse transcribed using Moloney murine leukemia virus-reverse transcriptase and random hexamer primers (Promega). The resulting cDNA was used in 12 µl PCR reactions, in which 300 nmol/l of forward and reverse primers and, where applicable, 150 nmol/l of fluorogenic probe were used in combination with ABI Taqman or Sybr green master mix (Applied Biosystems). Reactions were carried out in duplicate for each sample on an ABI 7900 sequence detection system (Perkin Elmer Biosystems) according to the manufacturer's instructions. The relative quantities of amplified cDNAs were analyzed by the SDS software (Applied Biosystems) and target values were normalised to 18S rRNA (tissue samples) or cyclophilin A mRNA (cell culture samples).

ChIP Assay. 3T3-L1 preadipocytes in 35-mm wells were differentiated for various times as indicated. The DNA and protein were cross-linked *in situ* with 0.5 % (v/v) formaldehyde at 37 °C for 5 min. Soluble chromatin was prepared using a ChIP assay kit (Upstate Biotechnology, Inc.). The lysate was sonicated four times for 10 s at 4 °C. The lysates were precipitated with either 5 µl of anti-C/EBPβ, anti-C/EBPδ or anti-C/EBPα antibody (Santa Cruz Biotechnology) overnight before protein A-agarose beads were added. DNA was recovered by digesting with 10 µg/ml proteinase K at 45 °C for 30 min, purification using a QIAquick PCR purification kit (Qiagen). The presence of DNA sequences associated with the immunoprecipitated proteins was determined using specific primers amplifying DNA sequences including the binding sites being assayed and Sybr green master mix according to the manufacturer's instructions. Values obtained from immunoprecipitated samples were normalised to those from input samples.

Western Blotting. Protein samples were extracted by scraping in lysis buffer containing 1% NP40, followed by sonication as described previously [18]. After centrifugation for 10 min at 13,000 x g, samples of supernatant containing 30 μg of protein were denatured and analysed by western blotting. All antibodies were from Santa Cruz Biotechnology.

Statistics. Statistical analyses were performed using t-tests or ANOVA for multiple comparisons.

RESULTS

The gene *srebpf1*, encodes the proteins SREBP1a and SREBP1c which have highly overlapping specificities for target sequences but appear to differ in their transactivation capacities [21]. Whilst both *SREBP1a* and *SREBP1c* are expressed in adipose tissue *in vivo*, SREBP1c is the more highly expressed in this tissue in both mice and humans [22]. However, initial studies suggested that only the SREBP1a isoform was expressed in differentiating 3T3-L1 cells [22], although other investigators have reported substantial expression of SREBP1c [12]. We therefore examined the expression of each isoform in differentiating 3T3-L1 preadipocytes. As shown in Fig. 1A, SREBP1c mRNA expression was almost undetectable in preadipocytes but dramatically induced during adipogenesis and abundant in 3T3-L1 adipocytes. In contrast SREBP1a was clearly detectable in preadipocytes and, whilst also induced further during differentiation did not show the dramatic increases observed for SREBP1c (Fig. 1B). These assays do not permit quantitative comparison of SREBP1a versus SREBP1c levels, however they do show that SREBP1c is highly induced and abundant during adpogenesis making 3T3-L1 cells suitable to study the transcriptional regulation of SREBP1c. When searching for candidate pathways that might regulate SREBP1c we noted that the time course of its induction was similar to that observed for known C/EBPβ targets such as C/EBPα, 11βHSD1 and DGAT2 [9, 23, 24]. In addition we found that SREBP1c and the C/EBPβ target DGAT2 were induced with similar time-courses in both murine ES cells undergoing adipogenic differentiation (Fig S1A and S1B) and differentiating isolated human preadipocytes (Fig S1C and S1D).

To further investigate whether C/EBPβ might regulate SREBP1c expression we examined its expression in cells overexpressing the inhibitory LIP isoform of C/EBPβ and cells constitutively expressing the C/EBPβ inhibitor ETO [9]. As shown in Fig. 1C and 1D respectively, LIP expression inhibited not only C/EBPα expression but also the induction of SREBP1c mRNA. Likewise, inhibition of C/EBP activity with ETO impaired the induction of C/EBPα (Fig. 1E) and SREBP1c expression (Fig. 1F) to a similar degree. Together these data support a role for C/EBPβ in the regulation of SREBP1c during adipogenesis.

LIP is known to inhibit multiple C/EBP isoforms whilst the specificity of ETO is not fully defined. Therefore we next used specific siRNA oligos to knockdown individual C/EBP isoforms to examine their contribution to the regulation of SREBP1c. Cells were transfected with siRNAs targeting C/EBPβ or C/EBPδ or a mixture of both prior to induction of differentiation. As shown in Fig.2A the increase in C/EBPβ expression following the induction of differentiation was inhibited by transfection with C/EBPβ siRNA but not C/EBPδ siRNA. A similar degree of inhibition was observed when C/EBPβ siRNA were co-transfected with C/EBPδ siRNA. Likewise C/EBPδ siRNA significantly impaired the

induction of C/EBPδ expression but did not prevent C/EBPβ induction (Fig. 2B). C/EBPδ siRNA were similarly effective when co-transfected with C/EBPβ siRNA, substantially inhibiting C/EBPδ expression.

Lysates from identically transfected cells differentiated for various times were western blotted to determine either C/EBPβ or C/EBPδ protein expression (Fig 2C). Knockdown of C/EBPβ or C/EBPδ with siRNA resulted in an almost complete loss of the induction of C/EBPβ (upper panel) or C/EBPδ (lower panel) protein respectively, consistent with the effects observed at the RNA level. Again, co-transfection of cells with siRNA targeting both C/EBPβ and C/EBPδ led to almost complete inhibition of the expression of both these proteins. As we have previously reported, knockdown of C/EBPβ led to an almost complete inhibition of lipid accumulation in differentiating 3T3-L1 cells as assessed by oil-red O staining (Fig 2D). Knockdown of C/EBPδ expression had a less marked effect on lipid accumulation in these cells. The knockdown of both C/EBPβ and C/EBPδ together led to the most dramatic inhibition of lipid accumulation.

We next examined the effect of C/EBPβ and C/EBPδ knockdown on a well-characterised C/EBP target gene, C/EBPα. As predicted by previous cellular and *in vivo* studies C/EBPα expression was most strongly affected by inhibition of C/EBPβ although C/EBPδ knockdown also substantially inhibited its induction during adipogenesis (Fig 2E). Knockdown of both C/EBPβ and C/EBPδ in the same cells further impaired the induction of C/EBPα mRNA. Having shown that C/EBPβ regulates DGAT2 induction during adipogenesis in a previous study [9] we also examined whether C/EBPδ could regulate DGAT2 expression. Indeed, knockdown of C/EBPδ significantly inhibited DGAT2, which was reduced to 45% and 50% of the levels seen in control cells at day 3 and day 5 of differentiation, respectively (Fig.2F). In addition, the combined knockdown of both C/EBPβ and C/EBPδ caused an even greater reduction in DGAT2 expression. These data suggest that, in addition to C/EBPβ, C/EBPδ makes an important contribution to the regulation of DGAT2 expression during early adipogenesis.

Analysis of SREBP1c expression in these samples revealed that the regulation of SREBP1c was very similar to that of C/EBPα and DGAT2. Knockdown of C/EBPβ alone led to inhibition of SREBP1c expression to approximately 30% and 50% of the levels seen in control cells at day 3 and day 5, respectively, following differentiation of these cells (Fig. 2G). Inhibition of C/EBPδ using siRNA gave very similar results suggesting that both C/EBPβ and C/EBPδ are important for the induction of SREBP1c during adipogenesis. Moreover, siRNA knockdown of both C/EBPβ and C/EBPδ in the same cells reduced expression of SREBP1c at day 3 and day 5 further to approximately 20% and 35% of those in control cells, respectively.

To determine the specificity of these effects the effect of C/EBPβ and C/EBPδ inhibition on SREBP1a expression was also determined. As shown in Fig. 2H the inhibition of C/EBPβ, C/EBPδ or combined inhibition of both factors by siRNA did not significantly affect SREBP1a expression at any of the time points tested. These data show that, as SREBP1a is normally induced in these cells, C/EBPβ/δ knockdown does not inhibit all changes associated with adipogenesis but rather is specific to genes downstream of these factors. This data also indicates that the two isoforms of SREBP are regulated by different factors during adipogenesis.

To determine whether our observations in 3T3-L1 cells may extend to *in vivo* adipocyte development, we examined gene expression in white adipose tissue isolated from

C/EBPβ knockout mice. C/EBPβ mRNA was undetectable in these samples (Fig. 3A), whilst the expression of C/EBPα was significantly reduced (Fig. 3B), as previously reported [9]. Consistent with our data from cultured cells, SREBP1c expression was significantly decreased in the white adipose tissue of these mice (Fig. 3C). This suggests that C/EBPβ is also involved in the expression of SREBP1c in adipocytes *in vivo*. In addition, the expression of SREBP1a was not affected in mice lacking C/EBPβ (Fig. 3D), providing *in vivo* support for our previous findings in cultured preadipocytes that SREBP1c, but not SREBP1a, is selectively regulated by C/EBP factors.

We next sought to determine whether C/EBPβ and C/EBPδ could directly regulate SREBP1c through binding to its promoter. Examination of the putative promoter of SREBP1c revealed several potential C/EBP consensus binding sites within 4kb upstream of the transcriptional start site. To assess binding to these putative sites we performed ChIP analysis, immunoprecipitating C/EBPβ or C/EBPδ and using real-time PCR to quantify binding to specific DNA sequences. Of nine putative binding sites identified, three showed significant binding of either C/EBPβ or C/EBPδ which was responsive to the induction of adipogenesis, whilst the other six did not (data not shown). The three responsive sites were designated site 1, site 2 and site 3 and are schematically represented in Fig. 4A. As shown in Fig. 4B, 4C and 4D, C/EBPβ binding to all three sites was increased during early differentiation, as C/EBPβ expression increases. Similarly these sites also bound C/EBPδ with similar time courses in identically treated cells (Fig. 4E, 4F and 4G). Together these data strongly suggest that both C/EBPβ and C/EBPδ are direct upstream regulators of SREBP1c expression directly binding to the SREBP1c promoter during early adipogenesis.

As has been previously described for the C/EBPα and DGAT2 promoters, amongst others, the maximal binding of C/EBPβ and C/EBPδ to the SREBP1c promoter lags behind the induction of these factors by several hours, likely due to the binding of inhibitory factors such as CHOP10 and ETO. Similarly the maximal binding of C/EBPβ and C/EBPδ precedes the peak of SREBP1c expression as has previously been observed for other well characterised C/EBPβ/δ targets, probably due to the formation of inactive promoter bound complexes that must be de-repressed for SREBP1c expression to occur.

We have previously observed that C/EBPα replaces C/EBPβ as a major regulator of DGAT2 expression when the expression of the latter diminishes in the later stages of adipogenesis [9]. Thus we investigated whether C/EBPα might similarly take over the regulation of SREBP1c expression from C/EBPβ and C/EBPδ as adipogenesis progresses. To test this we generated 3T3-L1 cells stably expressing shRNA targetting C/EBPα to inhibit its expression. This led to a significant inhibition of C/EBPα induction, which was reduced by 80% or greater following induction of differentiation (Fig 5A). Consistent with the reciprocal regulation of PPARγ and C/EBPα during adipogenesis, C/EBPα inhibition also led to significant reductions in the expression of PPARγ in cells differentiated for 5 days (Fig. 5B). The expression of key markers of the maturing adipocyte including the fatty acid binding protein aP2, and the insulin sensitive glucose transporter Glut-4, were also suppressed in cells in which C/EBPα had been knocked down. Similarly, the expression of DGAT2 was significantly inhibited in these cells consistent with our previous study using preadipocytes transiently transfected with siRNA targeting C/EBPα [9]. In the same cells SREBP1c expression was found to be inhibited by approximately 70% at day 5 of differentiation. In

contrast, the loss of C/EBPα had no significant effect on the expression of the SREBP1a isoform.

To determine whether the regulation of SREBP1c by C/EBPα could occur through direct binding of the same sites in the SREBP1c promoter occupied in early adipogenesis by C/EBPβ and C/EBPδ we performed ChIP assays. C/EBPα was immunoprecipitated from 3T3-L1 preadipocytes differentiated for various times and bound DNA sequences corresponding to putative C/EBP binding sites were assayed by real-time PCR. This revealed that site 1 inducibly binds C/EBPα as adipogenesis proceeds, replacing the binding by C/EBPβ and C/EBPδ 96 hours after induction of differentiation (Fig. 5C). In contrast, relatively weak binding was observed for C/EBPα to sites 2 and 3 and the marginal increases detected as adipogenesis progressed were not significant (Fig. 5D and 5E).

Together these data suggest that during the later stages of adipogenesis, C/EBPα may substitute for C/EBPβ and C/EBPδ in the control of SREBP1c expression and that this involves binding to site 1 approximately 3.5 kb upstream of the transcriptional start site in the SREBP1c promoter.

DISCUSSION

SREBP1c plays a central role in lipid metabolism, particularly in the liver and adipose tissue. Whilst the regulation of SREBP1c in the liver has been extensively studied, the factors regulating the induction of SREBP1c in developing adipocytes has received less attention, despite the important role acknowledged for this protein in adipocyte development. Our data are the first to show a key direct role for C/EBP factors in the regulation of SREBP1c with C/EBPβ and C/EBPδ initially binding to the SREBP1c promoter and subsequent regulation by C/EBPα. Selective siRNA or shRNA mediated knockdown demonstrate that the loss of any of these C/EBP factors significantly impairs the induction of SREBP1c. In addition our data suggests that, at least for C/EBPβ, this regulatory mechanism is likely to operate *in vivo* as mice lacking C/EBPβ also have reduced SREBP1c levels in adipose tissue.

Whilst our work places C/EBPβ upstream of SREBP1c during adipogenesis, the C/EBPβ promoter has conversely been described as a direct target of SREBP1c in mature adipocytes [25]. In this instance SREBP1c appears to be at least in part responsible for the induction of C/EBPβ in response to insulin. This illustrates that, rather than acting in a fixed canonical cascade, these genes function in a complex inter-regulatory network, the order of which will depend on factors including extracellular signals and the differentiation status of the cells involved.

From our experiments it is difficult to discriminate the relative importance of C/EBPβ and C/EBPδ activity directly versus the effects of consequent reduced expression of C/EBPα in the control of SREBP1c and lipogenesis. Forced expression of PPARγ in fibroblasts from C/EBPα null mice permits appropriate induction of adipocyte genes such as aP2, Glut-4 and adiponectin during adipogenesis but with significantly reduced lipid accumulation [26, 27]. We have previously shown that DGAT2, a key enzyme of lipogenesis, is a direct target of C/EBPα in adipocytes [9]. We now show that the effect of DGAT2 loss in cells with impaired C/EBP function may be exacerbated by the decreased expression of SREBP1c and the panoply of lipogenic genes it regulates. The precise relative importance of direct C/EBPα mediated vs indirect SREBP1c mediated pathways in the control of lipogenesis will require the specific knockdown of SREBP1c.

Given the complex interacting network of transcription factors involved in adipocyte differentiation it is also difficult to determine the contribution of direct versus indirect actions of C/EBP factors in regulating the SREBP1c promoter. C/EBPs have important roles in inducing the expression of many proadipogenic transcription factors, notably PPARγ, and several of these are likely to regulate SREBP1c expression themselves. However, our ChIP assay data clearly show physical binding of C/EBPs to the SREBP1c promoter in intact differentiating adipocytes, strongly suggests that the C/EBPs make a significant contribution to SREBP1c expression as primary regulators. Moreover, the observation that knockdown of C/EBPβ expression does not affect C/EBPδ induction and vice versa, strengthens the case that SREBP1c inhibition in each case does not result from an overall impairment of adipogenesis but rather a selective inhibition of downstream genes.

It is interesting that C/EBP inhibition selectively inhibited SREBP1c but not SREBP1a expression. The relative importance of the SREBP1a and SREBP1c isoforms in different cultured models of adipogenesis has been controversial. Although specific inhibition of these two isoforms has not been compared in cultured preadipocytes our data is consistent with a more important role for SREBP1c in adipogenesis. In the absence of C/EBPβ, C/EBPδ and/or C/EBPα induction lipogenesis, adipogenesis and SREBP1c expression are co-ordinately impaired whilst SREBP1a expression is unaffected. Although this does not demonstrate that loss of SREBP1c alone would replicate this phenotype it does suggest that, of the two isoforms, SREBP1c is more tightly linked to adipocyte development and lipogenesis. Given that both SREBP1a and SREBP1c appear to have near identical target specificities and that SREBP1a is the more potent, at least in cultured hepatocytes [21] it is not clear why this is the case. Cellular studies selectively inhibiting the SREBP1 isoforms during adipogenesis using shRNA will be valuable in dissecting their relative importance, particularly as attempts to understand specific SREBP1 function *in vivo* using animal models has given confusing results. Loss of both SREBP1 isoforms caused significant embryonic lethality and upregulation of SREBP2 in surviving mice [28], whilst selective ablation of SREBP1c led to reduced hepatic expression of lipogenic gene expression but no overt adipose phenotype although epididymal fat mass was reduced [29]. Paradoxically, adipose specific overexpression of constitutively active nuclear SREBP1 in mice led to a complex syndrome of lipodystrophy [30]. Other important regulators of adipogenesis, such as PPARγ and C/EBPα, have required more complex *in vivo* models, including chimeric and hypomorphic mice and inducible post-natal knockouts to circumvent mortality *in utero* [31-33]. Similar models may be required to clarify the true importance of SREBP1c in adipose tissue development *in vivo*.

From a pathophysiological perspective SREBP1c levels may be decreased in obese and type 2 diabetic subjects [34, 35] whilst altered SREBP1c levels have also been reported in lipodystrophic HIV patients undergoing antiretroviral therapy [36]. Furthermore, altered SREBP1c function may underlie or exacerbate lipodystrophy in patients with mutations in lamin A/C [37, 38]. Thus, a fuller understanding of how SREBP1c expression and/or activity may be modulated could be of significant therapeutic benefit in these conditions.

In summary, we have demonstrated for the first time that SREBP1c is regulated directly by C/EBP factors during adipocyte differentiation. This provides novel insight into the poorly defined transcriptional regulation of SREBP1c in developing adipose tissue, a key site of its action. Given the pleiotropic effects of SREBP1c, it identifies an additional mechanism via which C/EBP factors can indirectly influence lipid homeostasis and insulin action. Such further delineation of the complex network controlling adipocyte development is

important for the development of therapeutic strategies to treat diseases featuring altered adipose tissue mass or function including obesity and lipodystrophies.

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FIGURE LEGENDS

Figure 1. Inhibtion of C/EBPβ activity by constitutive LIP or ETO expression inhibits the induction of SREBP1c during adipogenesis.

3T3-L1 preadipocytes were induced to differentiate for various times up to 8 days (D8), RNA was isolated and the expression of SREBP1c (***A***) or SREBP1a (***B***) expression was determined by real-time PCR. Data shown are normalised to cyclophilin A +/- SEM, n=4. ***C***, 3T3-L1 preadipocytes were stably transfected with LIP (black bars) or empty vector (white bars) and confluent cells were induced to differentiate for the times shown. RNA was isolated and the expression of C/EBPα mRNA determined by real-time PCR. ***D***, Identical samples were also assayed for SREBP1c mRNA. ***E***, C/EBPα mRNA expression was determined in differentiating 3T3-L1 preadipocytes stably transfected with empty vector (white bars) or ETO (black bars). ***F***, SREBP1c mRNA expression was also determined in the same samples. All data shown are normalised to cyclophilin +/- SEM, n=4. * indicates statistically significant difference in expression compared with mock-transfected cells at the same time-point ($p<0.05$).

Figure 2. siRNA knockdown of C/EBPβ and/or C/EBPδ activity inhibits SREBP1c induction during adipogenesis.

A, 3T3-L1 preadipocytes were transfected with control siRNA (white bars) or siRNA targeting C/EBPβ (light gray bars), C/EBPδ (dark gray bars) or a mixture of siRNA targeting both C/EBPβ and C/EBPδ (black bars) and subsequently induced to differentiate for various times. C/EBPβ mRNA expression was assayed by real-time PCR. ***B***, C/EBPδ mRNA expression was assayed in the same samples. In both cases data are +/- SEM, n=4. * indicates statistically significant difference in expression compared with control siRNA transfected cells at the same time-point ($p<0.05$). ***C***, control, C/EBPβ, C/EBPδ and C/EBPβ+C/EBPδ siRNA transfected cells were also assayed for C/EBPβ (LIP/LAP) and C/EBPδ protein expression by western blotting. ***D***, lipid accumulation was assessed by oil-red O staining in control, C/EBPβ, C/EBPδ and C/EBPβ+C/EBPδ siRNA transfected cells following differentiation for 8 days.

Control (white bars), C/EBPβ (light gray bars), C/EBPδ (dark gray bars) and C/EBPβ+C/EBPδ (black bars) siRNA transfected cells were also differentiated for various times, RNA isolated and assayed for the expression of C/EBPα (***E***), DGAT2 (***F***), SREBP1c (**G**) and SREBP1a (**H**) by real-time PCR. Data shown are normalised to cyclophilin +/- SEM, n=4. * indicates statistically significant difference in expression compared with control siRNA transfected cells at the same time-point ($p<0.05$).

Figure 3. SREBP1c but not SREBP1a expression is reduced in adipose tissue of mice lacking C/EBPβ.
RNA was isolated from white adipose tissue of wild-type (WT) or C/EBPβ knockout (KO) mice, reverse transcribed and the expression of C/EBPβ (***A***), C/EBPα (***B***), SREBP1c (***C***) and SREBP1a (***D***), was assayed by real-time PCR. Results are the mean of 3 independent samples normalised to 18S +/- SEM, * indicates statistically significant expression compared with wild-type mice ($p<0.05$).

Figure 4. Identification of C/EBPβ and C/EBPδ binding sites in the SREBP1c promoter.
A, analysis of 4kb upstream of the first exon of SREBP1c by TESS revealed three potential C/EBPβ binding sites, denoted site 1 to site 3. 3T3-L1 preadipocytes were induced to differentiate for various times and ChIP analysis performed to assess binding of C/EBPβ to the putative site 1 (***B***), site 2 (***C***), site 3 (***D***). C/EBPδ binding to the same sites was also determined (***E***,***F***,***G***). C/EBPβ and C/EBPδ bound DNA in immunoprecipitates was quantified by real-time PCR. Values in each sample were normalised to total genomic DNA of the same sequence in the "input" starting sample prior to immunoprecipitation. Data are the average of 4 independent experiments +/- SEM. * indicates statistically significant difference compared with binding at 0h ($p<0.05$).

Figure 5. C/EBPα regulates SREBP1c expression in the later stages of adipogenesis.
A, 3T3-L1 preadipocytes stably expressing a control shRNA (white bars) or shRNA targeting C/EBPα (black bars) were induced to differentiate for various times, RNA extracted and the expression of C/EBPα mRNA determined by real-time PCR. ***B***, Day 5 differentiated samples from cells expressing control (white bars) or C/EBPα targeting shRNA (black bars) were assayed for the expression of PPARγ_2 (Pγ2), aP2, Glut-4 (GLT4), DGAT2 (DGT2), SREBP1c (SR1c) and SREBP1a (SR1a). Data shown are normalised to cyclophilin +/- SEM, n=3. * indicates statistically significant difference in expression compared with control shRNA transfected cells at the same time-point ($p<0.05$). 3T3-L1 preadipocytes were induced to differentiate for various times and subjected to ChIP analysis to assess binding of C/EBPα to the putative site 1 (***C***), site 2 (***D***), site 3 (***E***) indicated in figure 4A. C/EBPα bound DNA in immunoprecipitates was quantified by real-time PCR. Values in each sample were normalised to total genomic DNA of the same sequence in the "input" starting sample prior to immunoprecipitation. Data are the average of 4 independent experiments +/- SEM. * indicates statistically significant difference compared with binding at 0h ($p<0.05$).

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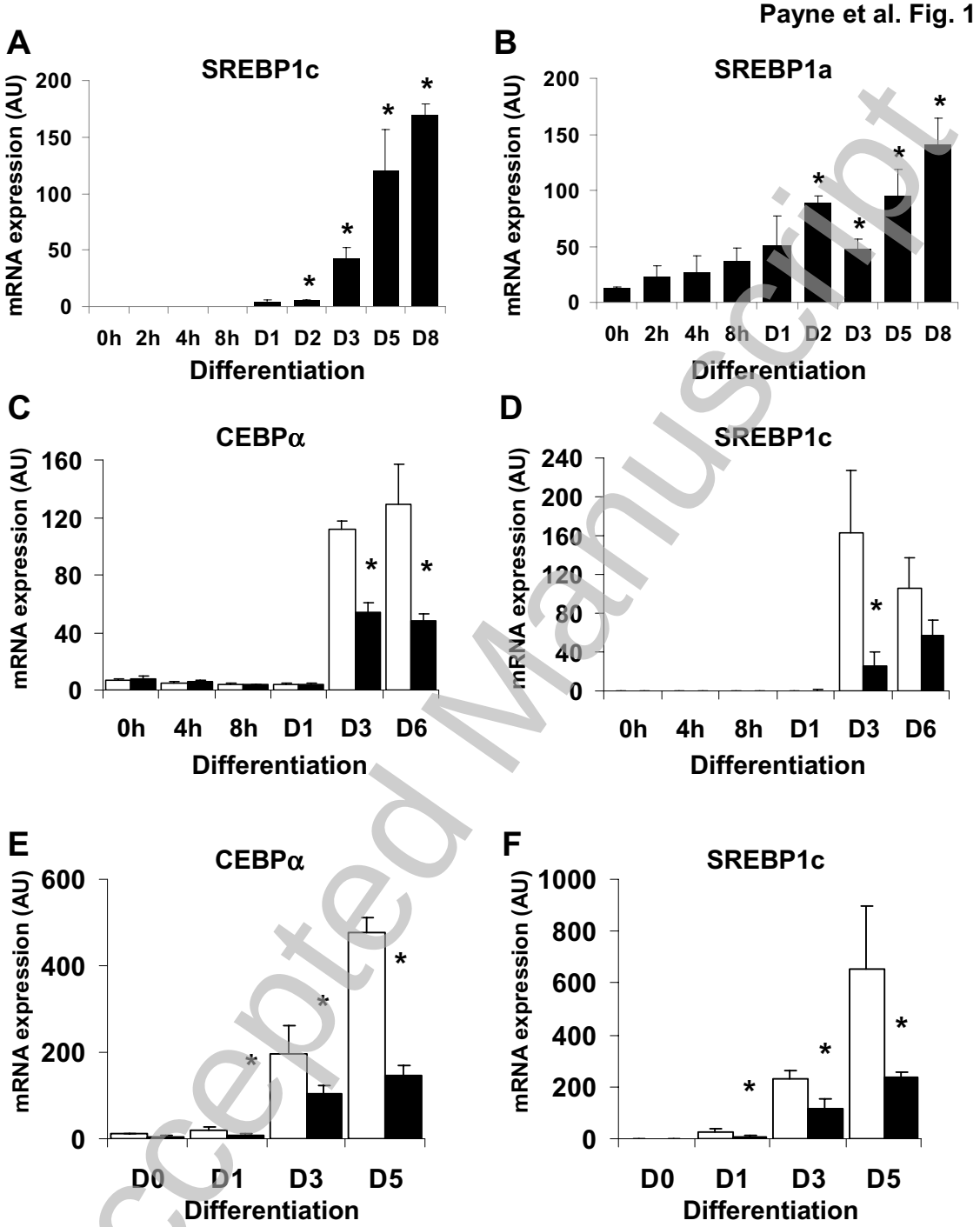

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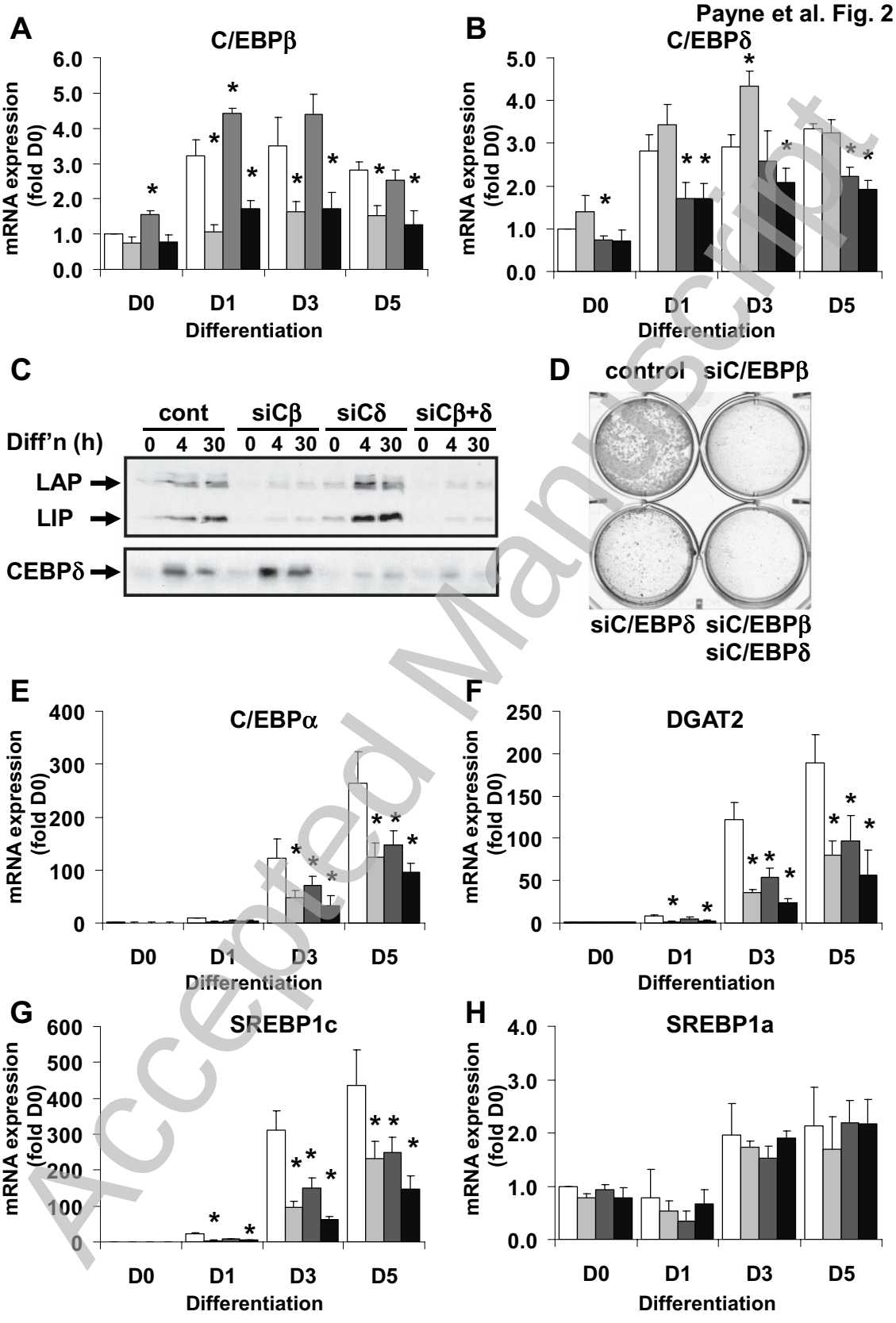

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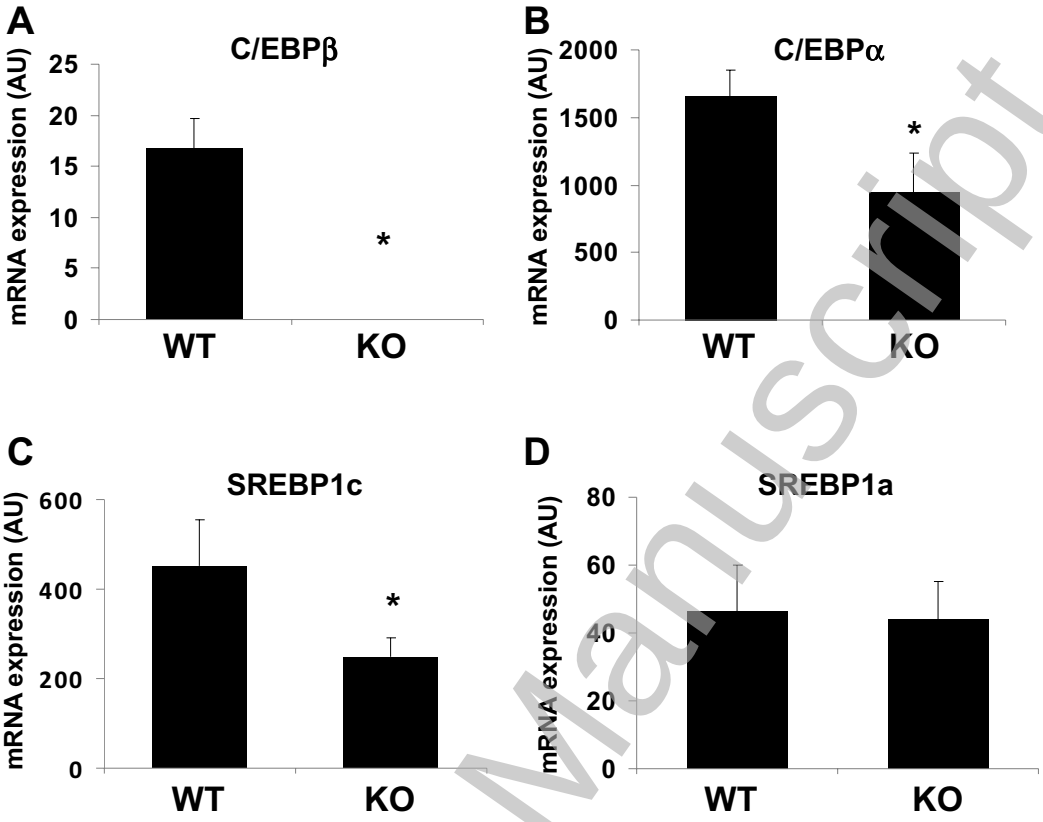
Payne et al. Fig. 3



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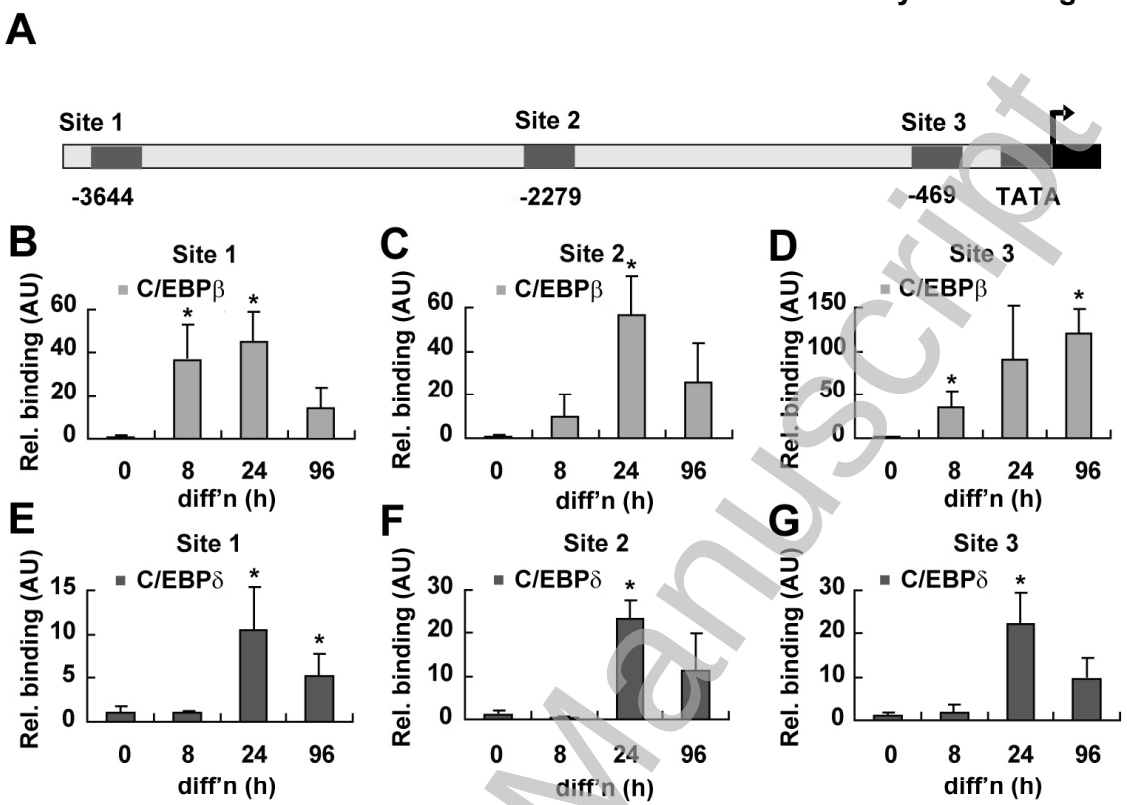

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