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Transcriptional Regulation of Adipogenesis by KLF4

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SUMMARY

While adipogenesis is known to be controlled by a complex network of transcription factors, less is known about the transcriptional cascade that initiates this process. We report here the characterization of KLF4 as an essential early regulator of adipogenesis. KLF4 is expressed in 3T3-L1 cells within 30 minutes after exposure to a standard adipogenic cocktail of insulin, glucocorticoids and IBMX. A knockdown of KLF4 inhibits adipogenesis and downregulates C/EBPβ levels. KLF4 binds directly to the C/EBPβpromoter as shown by CHIP and gel shift assays, and together with Krox20, cooperatively transactivates a C/EBPβ reporter. A C/EBPβ knockdown increases the levels of KLF4 and Krox20 suggesting that C/EBPβ normally supresses Krox20 and KLF4 expression via a tightly controlled negative feedback loop. KLF4 is specifically induced in response to cAMP, which by itself can partially activate adipogenesis. The data suggest that KLF4 functions as an immediate-early regulator of adipogenesis to induce C/EBPβ.

INTRODUCTION

Obesity, defined as an excess amount of body fat relative to lean body mass, is a major health concern in the United States and an increasing problem in the developing world. (Friedman, 2004) A fuller understanding of the molecular processes governing adipose tissue formation is of basic and potentially clinical importance.

Two decades of research have revealed that adipogenesis is controlled by a complex network of transcription factors, including numerous transcriptional activators, coactivators and repressors (Farmer, 2006). When confluent 3T3-L1 and 3T3-F442A cell lines are exposed to a cocktail of hormonal stimulants, these cell lines accumulate lipid and develop the characteristic morphology of mature adipocytes (Green and Meuth, 1975). The earliest inductive event in this process is transcriptional activation of two of the C/EBP family of transcription factors, C/EBPβ and C/EBPδ, which then stimulate expression of PPARγ, the late major transcription factor (Cao et al., 1991; Lane et al., 1999; Wu et al., 1995; Yeh et al., 1995). PPARγ is necessary and sufficient for adipocyte differentiation (Tontonoz et al., 1994). However, less is known about what controls C/EBP gene expression. The identification of the factors that regulate these important early genes in preadipocytes would provide additional insight into the mechanisms regulating the initiation of fat cell differentiation.

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In an earlier paper, we showed that Krox20 is expressed early in adipogenesis and that it could transactivate C/EBPβ (Chen et al., 2005). However, we were unable to show direct binding of Krox20 to the C/EBPβ promoter despite the fact that it potently transactivated a luciferase reporter driven by an element between −1.45kb to −1.1kb upstream of the C/EBPβ transcription initiation site. These findings suggested that Krox20 might interact with another factor to transactivate the C/EBPβ promoter. We considered the possibility that this additional factor might be included in a panel of transcription factors that are expressed at a much higher level in white adipose tissue in vivo than in 3T3-L1 cells in vitro (Soukas et al., 2001). It was this same panel that led to the identification of Krox20.

One of the transcription factors that is induced in vivo is KLF4, a member of a large family of zinc-finger proteins transcription that is known to play important roles in differentiation and proliferation (Ghaleb et al., 2005). Several other KLFs have previously been implicated in the regulation of adipogenesis. KLF6 and KLF15 have both been shown to promote adipogenesis and KLF15 also upregulates GLUT4 expression (Li et al., 2005; Mori et al., 2005). In addition, another KLF factor, KLF5, is necessary for adipocyte differentiation and acts by transactivating the Pparg2 promoter (Oishi et al., 2005). All these KLF factors are thought to function through different coactivators or repressors(Farmer, 2006).

However, a role of KLF4 in adipogenesis has not been previously demonstrated. KLF4, also known as GKLF/ZIF, is highly expressed in differentiated, post-mitotic cells of the skin and the gastrointestinal tract and it has been suggested to have variety of roles as differentiation-proliferation switch and regulator of the cell cycle (Garrett-Sinha et al., 1996; Shields et al., 1996; Takahashi and Yamanaka, 2006; Yoon et al., 2003). KLF4 knockout mice die around 12 hours after birth due to defects in skin development with a failure of normal basement membrane formation. (Segre et al., 1999) In these mice, the skin's fat layer is disrupted, causing the loss of skin barrier function and rapid loss of body fluids. (Segre et al., 1999) We thus considered the possibility that KLF4 might play a role in regulating the development of adipose tissue and tested this possibility by studying the functional role of KLF4 during the differentiation of 3T3L1 adipocytes.

RESULTS

KLF4 is an early-expressed and necessary factor during 3T3-L1 differentiation

We have previously shown that KLF4 is highly enriched in white adipose tissue, compared to fully differentiated 3T3-L1 adipocytes in vitro (Soukas et al., 2001). Because adipose tissue in vivo includes stromal elements and pre-adipocytes, this raised the possibility that KLF4 might be expressed in the stromal preadipocyte fraction and plays a role in the early stages of adipocyte differentiation. To assess this, we assayed KLF4 gene expression at numerous times early during 3T3-L1 differentiation that was induced using the standard IBMX, dexamethosone, and insulin cocktail. Quantitative RT-PCR revealed that KLF4 mRNA appeared 30 min post-induction and peaked at 2 hr post-induction. Protein levels for KLF4 were consistent with the mRNA levels (Figure 1A). This result shows that KLF4 is expressed very early in the course of 3T3-L1 differentiation with a similar pattern to other previously described early transcription factors including Krox20, C/EBPβ and C/EBPδ. (Figure S1) These same factors were also enriched in the stromal/pre-adipocyte fraction of the adipose tissue compared to the adipocyte fraction (Figure 1B).

The role of KLF4 in adipogenesis was tested using retroviruses carrying siRNA for KLF4, to knock down its expression. Two independent hairpin siRNA sequences for KLF4 (cloned into pSIREN-RetroQ-puro retroviral plasmid) were effective for knocking down KLF4 expression. (Figure 1C) 3T3-L1 cells were infected with the two KLF4 siRNA-containing retroviruses along with a control hairpin. After selection with puromycin, we established stable cell lines

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with constitutive siRNA expression. Note, these cell lines were a mixture of all cells expressing the selectable marker and were not clonal. These stable cell lines were cultured to confluence and differentiated using the standard induction cocktail for 8 days. Oil red O staining revealed that both KLF4 knock down cell lines accumulated significantly less lipid than the control cell line (Figure 1C). RNA levels of adipogenic regulators such as adipsin, ap2 and PPARg2 were also reduced consistent with the oil red-O staining. In addition, early C/EBPβ expression was also reduced in KLF4 knock down cell lines compared to control cells (Figure 1D). These results suggest that KLF4 is necessary for normal differentiation of 3T3-L1 cells and acts upstream of C/EBPβ and PPARγ. Of note however, the reduction of PPARγ gene expression was not proportional to the decrease in adipogenesis suggesting that KLF4 might partially promote the differentiation of adipocytes independent of PPARγ.

KLF4 transactivates C/EBPβ at −1.45kb to −1.1 kb upstream region

The data shown above suggested that KLF4 might regulate C/EBPβ transcription. To test this hypothesis, a plasmid expressing a KLF4-HA protein or a control plasmid were cotransfected into 293T cells with a C/EBPβpromoter-luciferase reporter plasmid (B3K), which contains 3kb of the C/EBPβ promoter (This construct was generously provided by Daniel Lane). This data showed that KLF4 transactivated the C/EBPβ promoter (B3K) by ~10 fold, in a dose dependent manner (Figure S2).

To further narrow down the region(s) responsible for the transactivation of KLF4, we generated a series of 5′ deletions of the C/EBPβ promoter, and analyzed the induction in response to KLF4. Transactivation by KLF4 dropped by more than 5 fold, when a region from −1.45kb to −1.1kb was deleted (Figure 2A). Deletion of sequences from −3kb to −1.5kb region resulted in a further 2-fold decrease in the activation.

To assess whether KLF4 directly activated the C/EBPβ gene, we next assayed the binding of KLF4 to the endogenous C/EBPβ promoter in 3T3-L1 cells using chromatin immunoprecipitation assays 3T3-L1 cells were differentiated for 2.5 hours at which time DNA-protein complexes were cross-linked and CHIP assay was performed by using an anti-KLF4 antibody, or rabbit IgG as a control. Chromatin immunoprecipitation using the anti-KLF4 antibody caused a significant enrichment of the C/EBPβ promoter sequence indicating that the KLF4 transcription factor is directly bound to the C/EBPβ promoter (Figure 2B). Further characterization of the region between −1.45 to −1.1 kb of the C/EBPβ promoter was performed using gel shift assays with fragments of the promoter and bacterially expressed KLF4. Seven 55bp oligos spanning the 1.45kb–1.1kb region were incubated with recombinant GST-KLF4 protein. GST-KLF4 shifted 3 of these oligos and this was correlated with the results of a cotransfection of Klf4 with a series of C/EBPβ promoter deletions between −1.45kb to−1.1 kb (Figure S3) Taken together, these findings suggest that KLF4 transactivates C/EBPβ gene by binding directly to several sites on 1.45kb–1.1kb region of the promoter. However the sequences of these oligonucleotides were different and DNAse footprinting failed to reveal a clear consensus sequence (data not shown). This suggests that the Klf4 binding site may be highly degenerate. Nonetheless, a cross-species comparison of the C/EBPβ promoter further indicated that the 1.45kb–1.1kb region is the only region that is significantly conserved among mammals (Figure S4).

KLF4 and Krox20 bind together and transactivate the same site of the C/EBPβ promoter

It was previously reported that Krox20 can also transactivate the C/EBPβ promoter via sequences between 1.45kb–1.1kb which is identical to that reported above for Klf4. (Chen et al., 2005) The fact that KLF4 and Krox20 also have similar expression kinetics led us to examine whether the ability of these transcription factors to activate C/EBPβ promoter is synergistic. Plasmids directing the expression of Krox20 and KLF4 were co-transfected into

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293T cells using the same C/EBPβ promoter-luciferase reporter. Transfection of KLF4 or Krox20 alone led to a 6-fold and 4-fold induction respectively, while simultaneous transfection of both plasmids increased the luciferase activity 10-fold (Figure 2C).

Since Krox20 (Chen et al., 2005) and KLF4 bind to the same region and can both activate transcription of C/EBPβ, we considered the possibility that these proteins might interact. FLAG-tagged KLF4 and HA-tagged Krox20 were ectopically expressed in 293T cells for 2 days. The lysate was immunoprecipitated using anti-FLAG M2 agarose beads (Sigma), washed extensively with BA300 (300mM NaCI) and immunoblotted using an anti-HA antibody. In this assay, immunoprecipitation of KLF4 using an anti-FLAG antibody pulled down Krox20 (Figure 2D). Consistent with this result, purified GST-KLF4 pulled down in vitro translated Krox20 in in-vitro pull-down assays. (Figure 2E) In addition studies using a standard mammalian 2-hybrid assay provided functional evidence that Krox20 and Klf4 can bind to one another and activate gene expression. (Figure S5).

Krox20 and C/EBPβ overexpression partially overcomes the knockdown of KLF4

The data suggested that Klf4 acts upstream of C/EBPβ. If true, we would expect that overexpression of C/EBPβ or Krox20 should bypass the effect of KLF4 knockdown. To test this, 3T3-L1 cells were infected with retroviruses carrying KLF4 siRNAs and either Krox20-HA or C/EBPβ. These data confirmed that the negative effect of a KLF4 knockdown on adipogenesis can be ameliorated by the overexpression of either Krox20 or C/EBPβ as assessed using Oil red O staining and measuring the level of expression of aP2, adipsin and PPARg2. (Figure 3A,B)

KLF4 expression is dependent on IBMX but not on DEX or Insulin

Our data show that KLF4 and Krox20 cooperate to activate C/EBPβ transcription and establish them as among the earliest if not the earliest factors induced during adipogenesis. If this is true, both factors might be expected to respond directly to one of the components of the adipogenic cocktail and not the others. To test this, confluent 3T3 cells were exposed individually to each of the three components of the induction cocktail: insulin, dexamethasone, and the phosphodiesterase inhibitor, IBMX. Insulin and dexamethasone concentrations did not change KLF4 levels at 2 hr post-induction, the time at which KLF4 is maximally induced in response to the three-component cocktail. On the other hand, IBMX increased KLF4 levels 4 to 5 fold (Figure 3C) and increasing concentrations of IBMX proportionally increased C/EBPβ and KLF4 gene expression (Figure 3C, Figure S6).

To further assess the effect of IBMX on KLF4, we transfected 293T cells with a luciferase reporter driven by 2kb KLF4 promoter. When the transfected cells were exposed to IBMX, 3 to 4-fold increase in luciferase activity was observed (Figure 3D).

KLF4 and Krox20 are regulated through a negative feedback loop

KLF4, Krox20 and C/EBPβ compose a network of transcription factors that play a key role in orchestrating the early events of adipogenesis. We explored the interaction among these factors by analyzing their expression in cells in which only one of these transcription factors was knocked down by stable expression of their corresponding siRNA. The C/EBPβ knockdown cell lines showed a robust 2 fold increase in Krox20 and KLF4 expression levels at 2 hours post-induction, suggesting that C/EBPβ expression normally inhibits its upstream activators via a negative feedback mechanism. These data add further evidence that it is a downstream target of KLF4 and Krox20 (Figure 4A).

Consistent with the hypothesis, a 3T3L1 cell line that over expressed C/EBPβ showed a robust early decrease of KLF4, and to a lesser extent in Krox20 expression levels (Figure 4B). A

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negative effect of C/EBPβ on KLF4 expression was also seen using the KFL4-luciferase reporter. Co-expression of the KLF4-luciferase reporter with C/EBPβ in 293T cells reduced KLF4 expression as much as 30% and was dose-dependent. This inhibition was even more robust, i.e. 70%, when these cells are incubated with IBMX (Figure 4C). These results suggest a potential negative feedback mechanism whereby C/EBPβ actively down-regulates its upstream regulators and add further evidence to the possibility that KLF4 and Krox20 act upstream of C/EBPβ.

Overall, these data indicate that the level of expression of Klf4 is tightly controlled, a finding that is consistent with the observation that overexpression of Klf4 leads to cell cycle arrest in preadipocytes (and other cell types) further suggesting that Klf4 must be expressed at tightly regulated levels in a narrow window during adipogenesis.

Discussion

Adipogenesis is controlled by a cascade of transcription factors that act to induce the expression of gene products necessary for the acquisition of the characteristic morphology and specialized functions of adipocytes (Farmer, 2006). Previously C/EBPβ has been shown to be among the first transcription factors activated during adipogenesis and it is considered to be one of the initiators of a transcriptional cascade, which in turn activates C/EBPα and PPARγ2 (Rosen et al., 2002). These latter genes then activate the genes that mark the fully differentiated adipocyte phenotype. However, the upstream factors regulating C/EBPβ expression have not been characterized. We have previously reported that Krox20 can partially activate C/EBPβ (Chen et al., 2005). Here we report that KLF4, another transcription factor expressed early during adipogenesis can also transactivate C/EBPβ and that the combination of KLF4 and Krox20 can markedly induce C/EBPβ expression.

KLF4 gene expression is induced early during adipogenesis in response to IBMX, one of the components of the standard induction cocktail. KLF4 knockdown cell lines showed a marked decrease in adipogenesis as assessed using Oil Red O and reduced expression of differentiated fat markers such as aP2, adipsin and PPARg2. However, the decrease in PPARg2 expression seems to be less prominent than for the other two markers suggesting that Klf4 might have PPARγ2-independent effects. KLF4 knockdown cells also showed reduced expression of C/EBPβ, adding functional evidence to support the conclusion that KLF4 is upstream factor of C/EBPβ in the transcriptional network controlling adipogenesis. Consistent with this, KLF4 is able to transactivate a luciferase reporter driven by 3kb C/EBPβ promoter in a dose dependent manner, and a deletion series further localized the critical region to between −1438 and −1134 base pairs. CHIP assays and gel shift assays confirmed the binding of KLF4 to C/EBPβ promoter at this conserved critical region. KLF4 seems to bind several sites along this region and we failed to find a clear consensus sequence for KLF4 binding as assessed by footprinting studies (data not shown). However the extensive homology of this region of the C/EBPβ promoter suggests that these promoter sequences are functionally important.

Krox20 has previously been shown to activate C/EBPβ promoter in the same promoter region. (Chen et al., 2005) Klf4 and Krox20 transactivation is additive with KLF4, suggesting that both factors cooperate to activate C/EBPβ expression in adipogenesis. In addition, overexpression of either Krox20 or C/EBPβ seems to bypass KLF4 knockdown phenotype, further supporting the role of Krox20 and KLF4 to activate C/EBPβ. We have also shown that, in cooperation with p300 these two factors potently transactivate C/EBPβ promoter. (Figure S9) p300 has been previously shown to interact directly with KLF4 by using pull-down assays (Feinberg et al., 2005). These data suggest that p300 might play a role in early phases of adipogenesis via interactions with Krox20 and KLF4 potentially with other proteins to form a

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protein complex that induces C/EBPβ and coordinates some (though not all, see below) of the key early events during adipogenesis.

A knockdown of C/EBPβ increases the expression of KLF4 and the over expression reduces KLF4 expression. These data support that C/EBPβis downstream of KLF4 and reduces its expression by feedback inhibition. The need to down regulate KLF4 is not clear though it could be related to the known effect of KLF4 to arrest the cell cycle in other cell types in some contexts. Ectopic expression of KLF4 results in cell-cycle arrest in several cell lines including NIH3T3 cells and its transcriptional targets are involved in differentiation and cell-cycle inhibition (Chen et al., 2001; Chen et al., 2003; Shields et al., 1996). Consistent with this, we found that marked over-expression of KLF4 in 3T3L1 cells also leads to slow proliferation even before differentiation and these cells differentiate extremely poorly. (Figure S7) These results suggest that the levels of KLF4 must be tightly controlled either quantitatively and/or temporally.

KLF4 is specifically activated by IBMX, an inducer of cAMP, but not by dexamethosone or insulin, the other two components of the adipgenic cocktail. IBMX by itself can partially induce adipocyte differentiation in 3T3L1 cells in vitro but IBMX treatment does not fully recapitulate the effects of the entire cocktail (Yeh et al., 1995). This suggests that other factors activated by dexamethosone and insulin, in combination with KLF4, are necessary for the activation of the fully differentiated adipocyte phenotype. It is not known whether the failure of IBMX to fully activate adipogenesis is the result of a failure to fully induce the quantitative expression of downstream mediators such as C/EBPs or if additional, as yet unidentified factors are required. The identification of these dexamethosone and insulin induced target genes would resolve this issue and advance our understanding of the earliest events of adipogenesis. It is not known however whether KLF induction by cAMP is a direct effect via PKA phosphorylation of CREB family transcription factors or if another mechanism contributes (Reusch et al., 2000; Zhang et al., 2004). CREB phosphorylation is not effected by a Klf4 knockdown (Figure S8), which suggests either that KLF4 might act downstream of CREB or that other cAMP-responsive transcription factors are involved. Cotransfection of a KLF4-promoter-luciferase reporter construct with CREB in the presence of forskolin, did not show any increase in KLF4 expression (data not shown). This result suggests that either the responsive element for CREB is not represented in the promoter or cAMP regulation of KLF4 expression is mediated by a different transcriptional activator other than CREB.

The role of KLF4 in vivo could be further tested in fat specific knockouts of KLF4 however the interpretation of such data could be complicated by the fact that the expression of KLF4 might not be required at later times in adipogenesis such as when the aP2 promoter is turned such as in aP2-cre or leptin-cre mice (unpublished data) (Jones et al., 2005). In other words, an aP2 directed knockout of KLF4 would not be informative, if KLF4 is not required for the maintenance of adipose tissue. The low levels of KLF4 in fully differentiated adipocytes are consistent with this possibility. Thus promoters that direct cre expression early in adipogenesis i.e.; in preadipocytes are likely to be required to assess the inductive role of KLF4 in tissue specific knockouts. Because such promoters are not known or available, the delineation of a role for KLF4 in vivo might require an ES cell complementation strategy analogous to that recently developed to study pancreatic development (Stanger et al., 2007).

In summary, we report the identification of KLF4 as an essential player in adipocyte differentiation. Our findings put KLF4 among a group of key proadipogenic early transcription factors, including Krox20 and CREB(Reusch et al., 2000; Zhang et al., 2004). It seems that there is an earlier transcriptional regulatory network before C/EBPβ expression at least in vitro. KLF4 is induced by cAMP and in turn controls C/EBPβ expression, linking the hormonal cocktail response to C/EBPβ. KLF4 can now be added to the growing list of KLF family of

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transcription factors that have roles as proadipogenic and anti-adipogenic mediators. KLF transcription factors act as transcriptional activators or repressors, depending on the their interaction with various co-activators or co-repressors and the context of the cell. In vivo studies of Klf4 function will be necessary to evaluate the precise roles of these early transcription factors in adipogenesis and to determine whether there is functional redundancy. Since these genes are expressed very early during differentiation of adipocytes, well before differentiated markers typically used to drive cre expression in adipocyte specific knockouts, there is a requirement to develop new in vivo systems to study early genes in the adipogenic program.

Experimental procedures

Plasmids—For over expression studies, KLF4 cDNA was cloned by RT-PCR and an HA epitope was added at the C terminus. It was subsequently inserted into pMSCVpuro (Clontech). Other pMSCV vectors are as used in Chen et al. (Chen et al., 2005). FLAG-KLF4 is courtesy of Douglas Boyd. Retroviral infections and 3T3-L1 differentiations are explained in supplementary methods.

RT-PCR analysis—Total RNA was isolated from cells by QIAGEN RNA prep kit following TRIZOL reagent (Invitrogen). Real Time PCR was performed by the Taqman system (Applied Biosystems) according to the manufacturer's instructions. Oligos were designed by the Primer Express software. Amount of expression was normalized with cyclophilin for cell culture experiments and with ELK3 for preadipocyte fraction experiments. Sequences of the Taqman primers and probes are described in Chen et al., 2005 and supplementary methods.

Transfections, reporter assays and immunoblotting—C/EBPβ reporter was kindly provided by Daniel Lane. Deletions were made by PCR and cloned into pGL3 plasmid. (Primers are available upon request.) The reporter plasmids along with effector plasmids were transfected using Fugene6 into 293T cells at 80–90% confluence. The pRL-TK (Promega) that carries rennila luciferase(Promega) was also cotransfected as an internal control for transfection efficiency. Cells were harvested after 20–24h and luciferase activities analyzed using the Promega Dual-Luciferase assay kit as recommended by the manufacturer. Western blot has been performed as described (Boyer-Guittaut et al., 2005).

GST pull-down and Coimmunoprecipitation—293T cells grown in DMEM supplemented with 10% FBS were transiently transfected using Fugene6 (Roche) according to the manufacturer's recommendations. Transfected cells were harvested in BA300 buffer (BA0 with 300mM NaCl) after 36h, and lysates were used for coimmunoprecipitation as described (Dou et al., 2005). KLF4 was cloned by PCR from pMSCVpuro-KLF4 and subsequently inserted into pGEX-T1 plasmid to express GST-KLF4 protein. GST-KLF4 protein is isolated as described (Morlon and Sassone-Corsi, 2003). In vitro transcription and translation of the Krox20 protein was performed by using the TNT T7 quick-coupled transcription-translation kit (Promega). A detailed GST pull-down assay is explained in the Supplementary methods section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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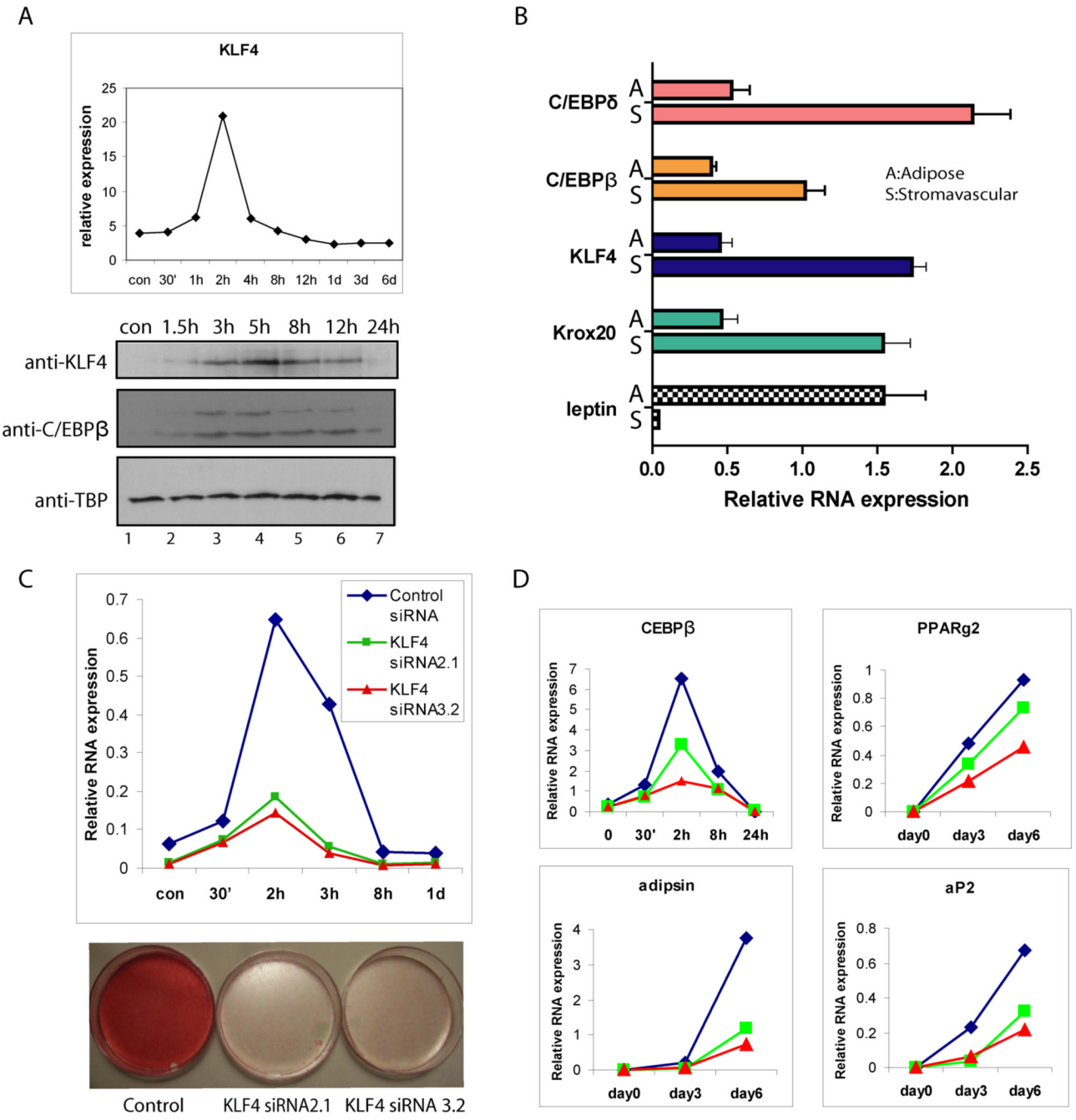

Figure 1. Expression and Function of KLF4

A) The expression of KLF4 during differentiation of 3T3-L1 cells was analyzed using Taqman RT-PCR and Western blots. Cells were harvested at the indicated times. Cyclophilin was used as an internal control for Taqman analysis.

B) Expression of early genes in stromavascular fraction and adipocyte fraction. Taqman RT-PCR was performed in stromavascular and adipocyte fractions of the adipose tissue disintegrated by collagenase. Leptin levels (enriched in the adipose fraction) were checked as a control of the preparation. Error bars are SEM.

C)) KLF4 knock-down impairs adipogenesis. 3T3-L1 cells were infected with pSIREN-RetroQ-derived retroviruses carrying either siRNA oligos for KLF4 or control oligo, selected

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for puromycin resistance, expanded as mixed population and induced to differentiate using MDI cocktail. The upper panel shows the efficiency of the 2 knock-down plasmids against KLF4 by Taqman assays. Lower panel shows ORO staining of KLF4 knockdown and control hairpin cell lines at day 8.

D) Expression levels of fat markers aP2, PPARg2, adipsin and an early gene, C/EBPβ in control and KLF4 knock-down 3T3-L1 cell lines at different time points during differentiation.

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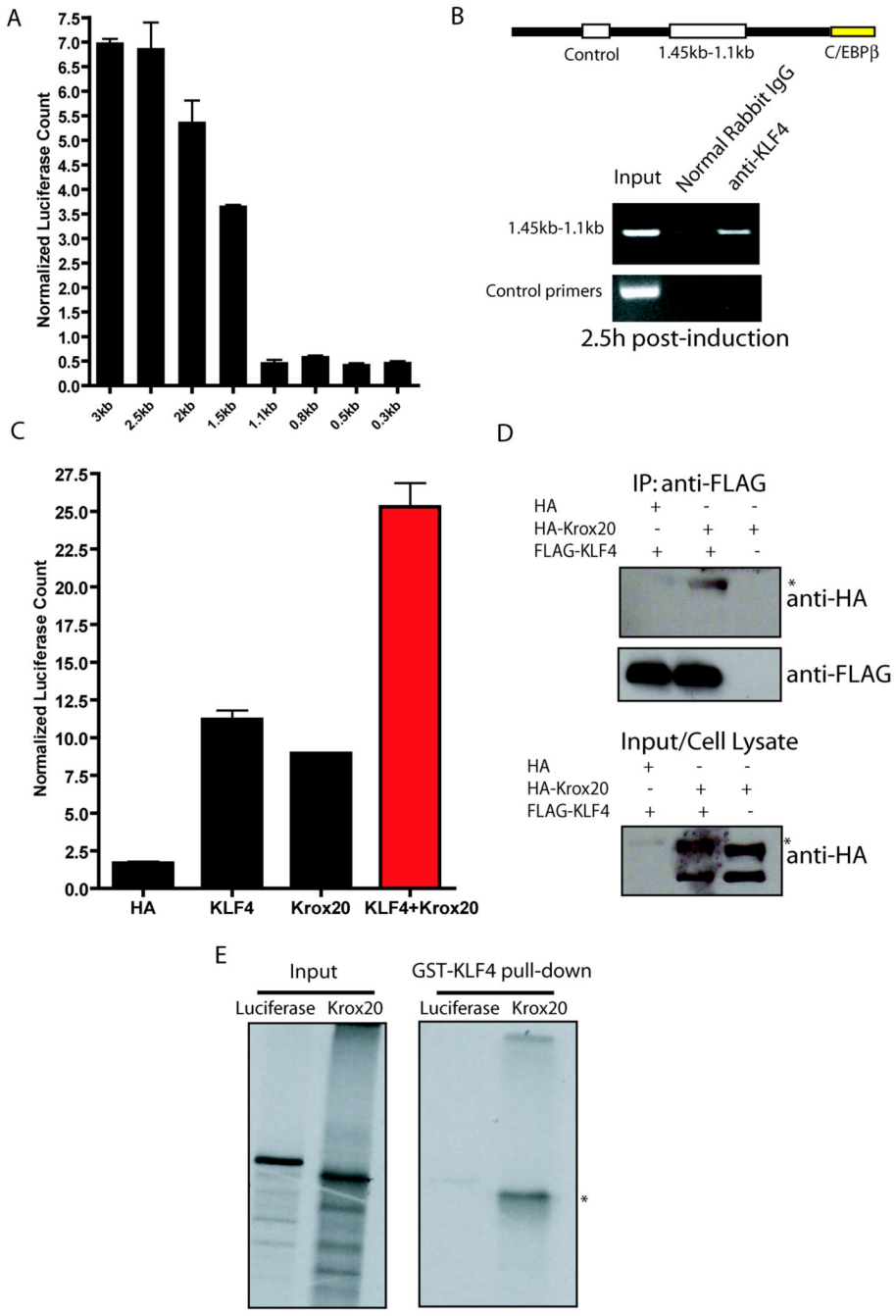

Figure 2. KLF4 transactivates the C/EBPβ promoter by binding to a conserved 1.45–1.1kb region

A) A deletion series derived from of a luciferase reporter construct (B3K), carrying 3kb promoter region of C/EBPβ (generously provided by Daniel Lane) was cotransfected with 250ng of pMSCV-KLF4 and pRL-TK(Renilla). Results were expressed as firefly luciferase activity normalized for renilla luciferase activity.

B) CHIP analysis of KLF4 binding to the target region on C/EBPβ promoter. 3T3-L1 cells were fixed 2.5h post-differentiation and chromatin samples were subjected to CHIP assays by using a KLF4 antibody or normal rabbit IgG as a control. An upstream region (−2.5kb) in C/EBPβ promoter was checked as a negative control.

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C) KLF4 and Krox20 cooperatively transactivate C/EBPβ promoter. KLF4 and Krox20 expression plasmids were cotransfected with the C/EBPβ luciferase reporter construct. An additive effect for transactivating C/EBPβpromoter was shown.
D) Coimmunoprecipitation of KLF4 with Krox20. FLAG-KLF4 and Krox20-HA were cotransfected into 293T cells. After 2 days, cells were lysed and co-immunoprecipitated using an anti-FLAG antibody and immunoblotted against HA. The data shows the specific binding of KLF4 to Krox20.
E) GST pull-down assays. Krox20 and luciferase plasmids were in vitro translated in presence of S35, mixed with purified GST-KLF4 and pulled-down by GST beads. Krox20 but not luciferase was pulled down by GST-KLF4.

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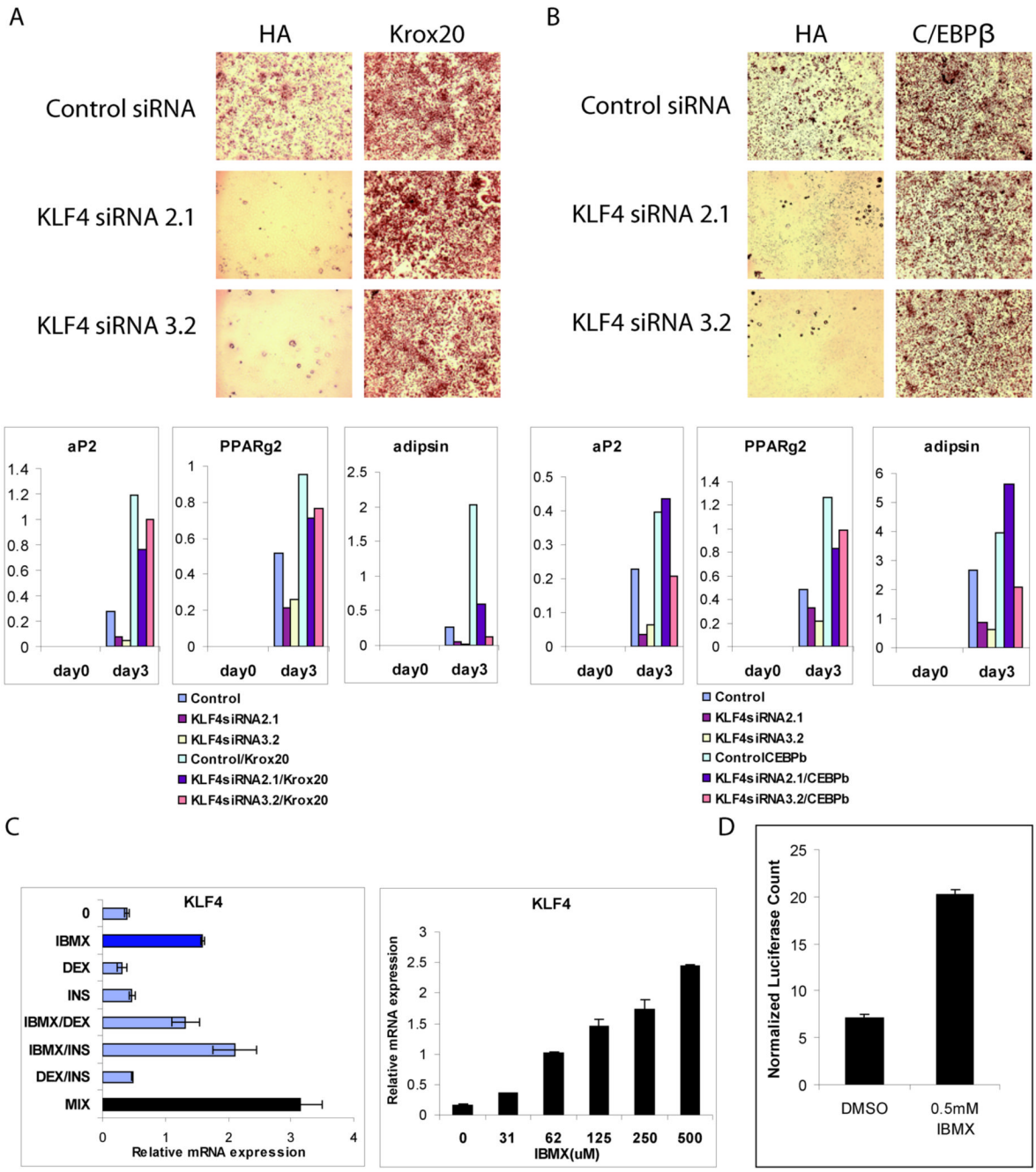

Figure 3. Krox20 and C/EBPβ overexpression partially overcomes the knockdown of KLF4

3T3-L1 cells were coinfected with pMSCVhyg-derived retroviruses carrying either Krox20-HA/C/EBPβ or control insert and pSIREN-RetroQ-derived retroviruses carrying either siRNA oligos for KLF4 or control oligo, selected for hygromycin and puromycin resistance, and induced to differentiate till day 7.

A) Overexpression of Krox20 overcomes the knockdown of KLF4. Upper panel shows the oil red O staining and lower panel shows the TAQMAN analysis of 3 late genes, aP2, adipsin and PPARg2.

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B) Overexpression of C/EBPβ overcomes the knockdown of KLF4. Upper panel shows the oil red O staining and lower panel shows the TAQMAN analysis of 3 late genes, aP2, adipsin and PPARg2.

KLF4 expression is dependent on IBMX but not on Dexamethasone or Insulin.

C) Individual components of the induction cocktail (IBMX, dexamethasone and insulin) were added onto confluent 3T3-L1 cells alone or in combination. Cells were harvested 2h post-treatment for Taqman analysis of KLF4. Left panel shows the effect of each component on KLF4 expression. The middle panel shows dose-dependent induction of KLF4. Confluent 3T3-L1 cells were treated by increasing amounts of IBMX (0 to 0.5 mM). Cells were harvested 2h-post induction for Taqman Analysis.

D) IBMX can transactivate C/EBPβ promoter in 293T cells in vitro. 293T cells were transfected with KLF4 luciferase reporter plasmid carrying 2kb upstream of KLF4 promoter. 16 hours later, the cells were either treated with DMSO or 0.5mM IBMX for 8 hours. After treatment, cells were harvested for luciferase assays.

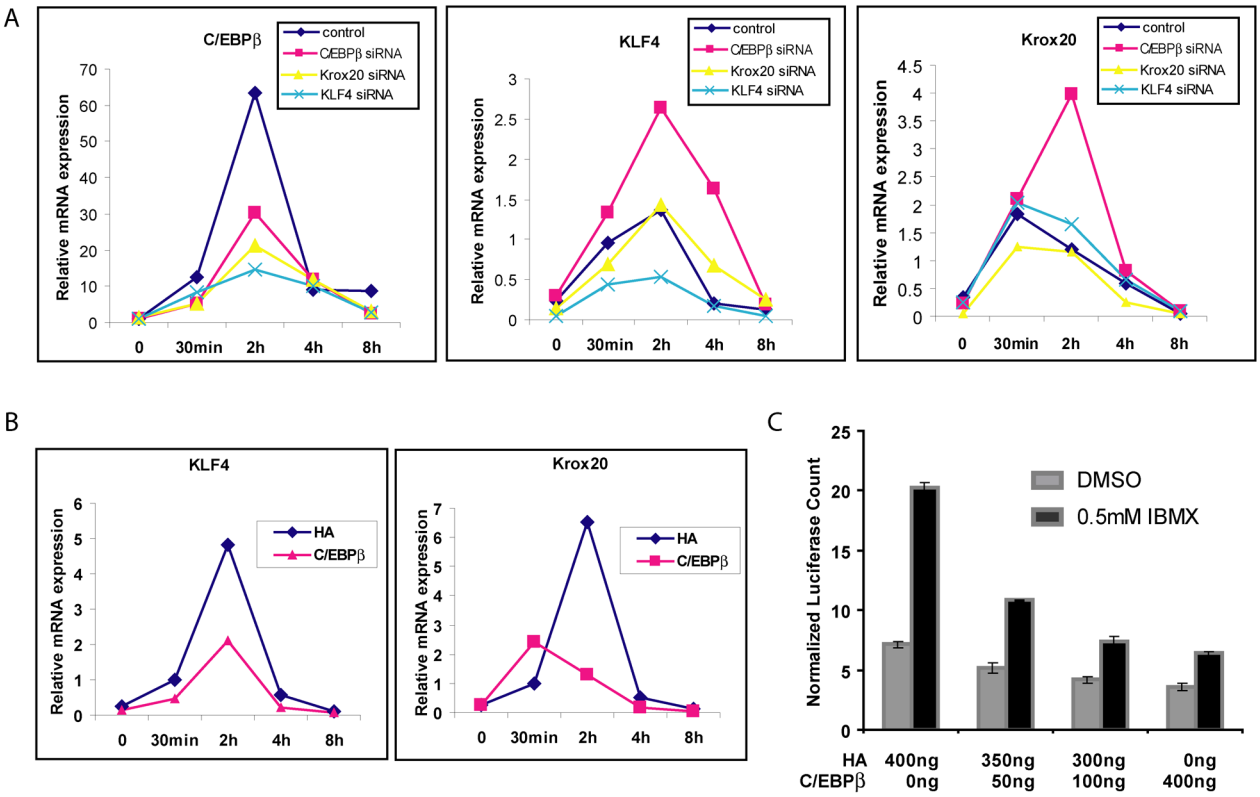

Figure 4. KLF4 and Krox20 are regulated by C/EBPβ through a negative feedback loop

3T3-L1 cells were infected with retroviruses expressing C/EBPβ knockdown or overexpression constructs. Cells were selected, differentiated and the effect of C/EBPβ expression on KLF4 was observed.

A) KLF4 and Krox20 levels are upregulated in C/EBPβ knockdown 3T3-L1 cells.

B) KLF4 and Krox20 levels are downregulated in C/EBPβ overexpressing 3T3-L1 cells

C) KLF4 transcription is inhibited by C/EBPβ. 293T cells were cotransfected with increasing amounts of C/EBPβplasmid along with a luciferase reporter plasmid carrying 2kb promoter of KLF4. After 16 hours the cells were treated with DMSO or 0.5mM IBMX. Cells were harvested after 8 hours for luciferase assays. Error bars are SEM.

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