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Analysis of gene networks in white adipose tissue development reveals a role for ETS2 in adipogenesis

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SUMMARY

Obesity is characterized by an expansion of white adipose tissue mass that results from an increase in the size and the number of adipocytes. However, the mechanisms responsible for the formation of adipocytes during development and the molecular mechanisms regulating their increase and maintenance in adulthood are poorly understood. Here, we report the use of *leptin-luciferase* BAC transgenic mice to track white adipose tissue (WAT) development and guide the isolation and molecular characterization of adipocytes during development using DNA microarrays. These data reveal distinct transcriptional programs that are regulated during murine WAT development in vivo. By using a de novo cis-regulatory motif discovery tool (FIRE), we identify two early gene clusters whose promoters show significant enrichment for NRF2/ETS transcription factor binding sites. We further demonstrate that *Ets* transcription factors, but not *Nrf2*, are regulated during early adipogenesis and that *Ets2* is essential for the normal progression of the adipocyte differentiation program in vitro. These data identify ETS2 as a functionally important transcription factor in adipogenesis and its possible role in regulating adipose tissue mass in adults can now be tested. Our approach also provides the basis for elucidating the function of other gene networks during WAT development in vivo. Finally these data confirm that although gene expression during adipogenesis in vitro recapitulates many of the patterns of gene expression in vivo, there are additional developmental transitions in pre and post-natal adipose tissue that are not evident in cell culture systems.

KEY WORDS: FIRE, Adipogenesis, ETS2, Fat, In vivo, Mouse

INTRODUCTION

In mammals, white adipose tissue (WAT) functions both as a depot for the storage of lipid and as an endocrine organ, controlling energy homeostasis and metabolism through secretion of leptin and additional adipokines (Otto and Lane, 2005; Rosen, 2005). Increased energy intake relative to energy expenditure results in excess accumulation of WAT, eventually leading to obesity (Friedman, 1997). The increase of WAT mass in obesity results from an increase in both adipocyte number and adipocyte size (Tontonoz and Spiegelman, 2008). However, the molecular mechanisms responsible for the accretion of adipose mass in vivo are poorly understood, and elucidating the mechanisms that control WAT formation would advance our understanding of obesity.

Enormous progress in adipocyte biology has derived from experiments using established immortal preadipocyte cell lines (Lefterova and Lazar, 2009). In vitro studies using these cell lines have elucidated a transcription factor 'cascade' involving the sequential induction of transcription factors, including *Krox20* (*Egr2* – Mouse Genome Informatics), *Klf4*, *Cebpb*, *Cebpd* and *Pparg*, that are necessary for adipocyte differentiation (Lefterova and Lazar, 2009). However, little is known about the regulation of adipocyte growth and proliferation in vivo both during development and in adults. Specifically, it is not entirely clear to what extent adipogenesis studied in vitro fully recapitulates adipocyte determination and differentiation in vivo. Although gain and loss of specific adipogenic genes identified via in vivo studies in mouse models partially reflect phenotypes observed in vitro, it is intrinsically difficult to study the function of genes that play a role in early stages of adipogenesis in vivo using adipocyte-specific knockouts (Bluher et al., 2002; Jones et al., 2005; Miyoshi et al., 2010; Rohl et al., 2004; Tanaka et al., 1997; Wang et al., 1995). To date, studies generally use promoters of genes that are expressed late in adipocyte differentiation, such as *Fabp4*, in order to study the role of transcription factors expressed earlier in the adipogenic cascade. Furthermore, there is evidence that in vitro temporal expression of some adipogenic transcription factors, such as PPARγ, occurs differently in vivo during embryonic development (Tang et al., 2008). Thus, in-depth analysis of the mechanisms involved in WAT development in vivo would provide a basis for comparison of gene expression during adipogenesis in vivo and in vitro. In addition to their general importance, such studies could also identify promoters that are expressed early in adipocyte development allowing an assessment of the effect of adipocyte precursor specific knockouts of genes that play a key role in the early phases of adipocyte commitment and/or differentiation.

Here, we report the use of previously described *leptin-luciferase* transgenic mice (Birsoy et al., 2008) to visualize and isolate WAT early in development. This strategy enabled us to study gene expression in the adipocyte lineage during prenatal and early postnatal stages of embryonic development. We then used a novel

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mutual information algorithm (FIRE) (Elemento et al., 2007) to identify transcription factors that can coordinately regulate clusters of early adipogenic networks. FIRE analysis identified the nuclear respiratory factor 2 (NRF2/GABPA) sequence motif, to which the NRF2 and ETS transcription factors are known to bind. Further analysis revealed that ETS2, an ETS transcription factor known to play a role in the development of other mesodermal tissues, including cartilage and bone (Sumarsono et al., 1996), exerts a functional role during adipogenesis. *Ets2* is induced early during adipogenesis in vitro and its expression is enriched in the stromavascular fraction of WAT in vivo, consistent with its expression in early adipogenesis in vitro. We conclude that suppression of *Ets2* during adipogenesis blocks adipocyte differentiation in vitro, possibly by perturbing clonal mitotic expansion.

MATERIALS AND METHODS

Mouse experiments and imaging

Leptin-luciferase animals were previously described (Birsoy et al., 2008). Luciferase activity in adipose tissues was measured using Luciferase Assay Kit (Promega). Protein normalization was performed after protein measurements using the BCA (Pierce) kit. In vivo imaging of transgenic animals was performed using the Xenogen IVIS Lumina imaging system (Caliper). Briefly, luciferin (200 μl of 15 mg/ml in PBS) was intraperitoneal injected into anesthetized animals. After 15-20 minutes, photon emission from the luciferin/luciferase reaction was detected with a sensitive CCD camera and photon image was superimposed on a normal video image of the mouse with Living Image 3.0 software (Caliper).

Adipose tissue fractionation and FACS sorting

Epididymal fat pads from male C57BL/6 and/or *leptin-luciferase* mice were minced and digested with collagenase in Krebs-Ringer bicarbonate buffer as previously described (Rodeheffer et al., 2008). Isolated adipocytes were separated from SVF fraction by centrifugation at 300 ***g*** for 3 minutes. For qPCR analysis cells were FACS sorted directly into TRIzol LS reagent (Invitrogen) for immediate RNA isolation.

Whole-mount staining

Embryonic adipose tissue pieces were stained with LipidTOX and isolectin GS-IB4 conjugated with Alexa Fluor 488 (Invitrogen) without any fixatives. Nuclei were counterstained with DAPI (Invitrogen). A confocal laser-scanning microscope (LSM510 Meta; Carl Zeiss) was used for imaging.

Virus infection, cell differentiation and proliferation assays

Knockdown of *Ets1*, *Ets2* or *Nrf2* in 3T3-L1 cells was achieved by lentiviral vector-mediated shRNA expression (Open Biosystems). Lentivirus was generated in 293T cells following the manufacturer's instructions using Lipofectamine 2000 (Invitrogen). Viral supernatants were supplemented with 8 μg/ml polybrene and added to cells for infections for 24-36 hours. Cells were selected with 2 μg/ml puromycin (Sigma), expanded and seeded for differentiation experiments.

3T3-L1 cells were maintained in DMEM with 10% FBS (Invitrogen) in 5% CO_2. Stable cell lines from retroviral transduction were cultured to confluence, starved and exposed to the differentiation cocktail (1 μg/ml insulin, 0.25 μg/ml dexamethasone, 0.5 mM IBMX). After 2 days, cells were maintained in 1 μg/ml insulin-containing medium until day 8 for harvest. Oil Red O staining was performed as described (Chen et al., 2005). For BrdU analysis, 3T3-L1 cells were differentiated with standard cocktail and 16-hour post-induction cells were pulsed with 30 μg/ml BrdU for 2 hours. Cells were then fixed with 70% ethanol and stained with a BrdU primary antibody anti-BrdU-FITC (Abcam; 1:1000) and DAPI.

Real-time PCR analysis and immunoblotting

Total RNA was isolated from cells and tissues by QIAGEN RNeasy kit (Qiagen). Real-time PCR (qPCR) was performed by the SyBr green or TaqMan system (Applied Biosystems). Expression was normalized to cyclophilin for cell culture experiments and to β-actin or *Tbk1* for cell fraction and sorting experiments. Sequences of the qPCR primers and probes were as described by Chen et al. (Chen et al., 2005). Other primers for qPCR are included (see Table S1 in the supplementary material). Student's *t*-test (unpaired, two-tailed) was used to compare two groups. Western blotting was performed as described previously (Chen et al., 2005). ETS2 antibody was a kind gift from Michael Ostrowski (Ohio State University, USA).

Microarray and clustering analysis

RNA from each sample was labeled using the Illumina TotalPrep RNA Amplification kit (Ambion) and cRNA was hybridized to Illumina's MouseRef-8 Expression BeadChips (Illumina). The gene expression data was clustered using the *k*-means algorithm (Hartigan, 1973) as implemented in MATLAB software (Mathworks, Natick, MA). Raw data are available from Gene Expression Omnibus (GEO dataset GSE29502).

Pathway enrichment and de novo cis-regulatory motif analysis and concordance

In order to discover pathways that are significantly enriched (or depleted) in the gene expression clusters, the iPAGE software (Goodarzi et al., 2009) was used to identify pathways whose distribution across the genome are informative of the clustering partition. De novo cis-regulatory motif discovery was performed on the expression clusters through the application of the FIRE algorithm, using default parameters (Elemento et al., 2007). Functionally enriched annotation groupings within the cluster 7 and 12 gene sets were identified and assigned a statistical enrichment score using the online functional annotation tool NIAID/NIH DAVID (http://david.abcc.ncifcrf.gov).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on 3T3-L1 fibroblasts using the Millipore EZ-Magna ChIP G kit (17-409). Formaldehyde crosslinked DNA was sheared to 200-1000 bp fragments through sonication with a Misonix S400 and 431a cup horn for 30 minutes (10 second pulses, 10 second rest, amplitude 20). ETS-2 antibody (10 μg) (Santa Cruz Biotechnology, sc-351) was added to each immunoprecipitation. Immunoprecipitated and input DNA was quantified through Thermo Scientific Pico Green assays on a Nanodrop 3300 (Thermo Scientific). DNA (250 ng) was used to determine enrichment of potential ETS2 target genes in immunoprecipitated versus input fractions via qPCR. Primers for qPCR (see Table S1 in the supplementary material) were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify an ~100 bp sequence of each promoter sequence containing the putative Nrf2-binding domain identified via FIRE analysis. Statistical analysis was performed through one-way ANOVA followed by post-hoc Tukey multiple comparison testing.

RESULTS

Visualization of murine adipocyte development in vivo

To visualize developing WAT in mouse embryos, we used a *leptin-luciferase* BAC transgenic mouse model (Birsoy et al., 2008; Rodeheffer et al., 2008). *Leptin-luciferase* BAC transgenic mice express luciferase with fidelity exclusively in adipocytes under the control of leptin gene regulatory sequences (Birsoy et al., 2008). We have previously shown that luciferase is produced in the adipose tissue in these animals with expression being highest in the adipocyte fraction. However, we also measured a low but detectable luciferase signal (above background; $P<0.01$) in the stromal-vascular fraction (SVF), which contains the adipocyte precursors, suggesting adipocyte precursor cells from *leptin-luciferase* mice also express low levels of leptin (see Fig. S1 in the supplementary material). Prior to lipid accumulation in adipocyte precursors early in post-natal life, there is little distinct morphology for embryonic WAT (Hooper et al., 1990). Thus, we reasoned that we could use the leptin reporter mice to circumvent this issue and identify and isolate developing WAT in vivo. In this context, the

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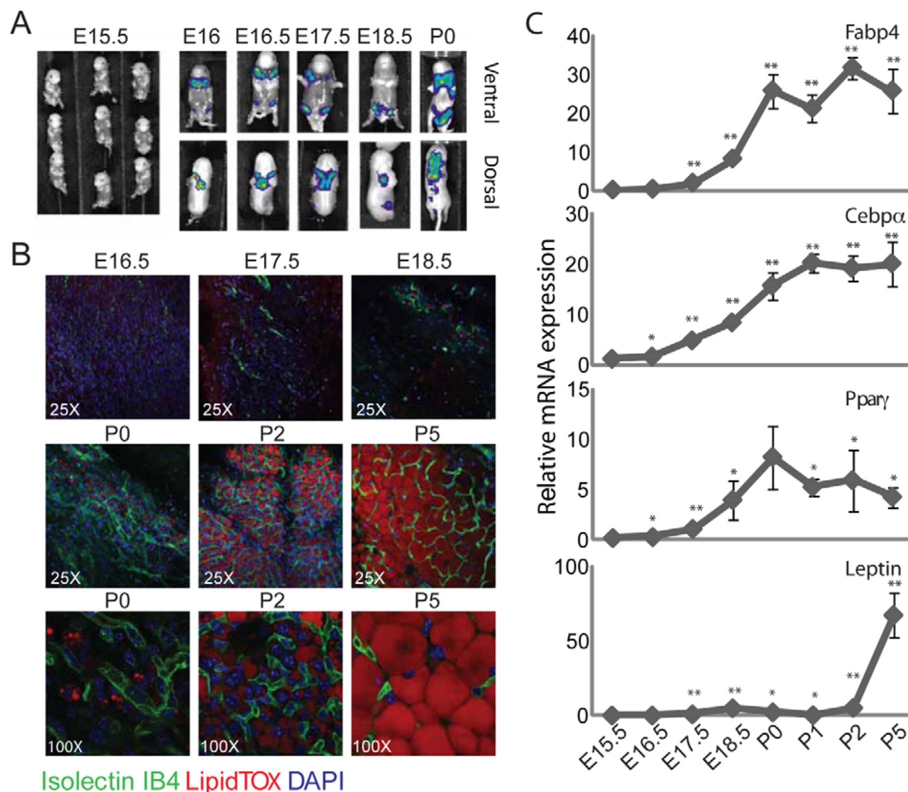

Fig. 1. Characterization of white adipose tissue development. (**A**) Bioluminescent imaging of *leptin-luciferase* transgenic mice at indicated embryonic developmental time points. Luciferase signal was detected using IVIS illumina imager. (**B**) Whole-mount confocal imaging of posterior subcutaneous white adipose tissue. Adipose tissue fragments were stained for lipidTOX (red/neutral lipids) and the endothelial marker isolectin $GSIB_4$ (green/vasculature). (**C**) Real-time PCR analysis of developing posterior subcutaneous adipose tissue for adipocyte markers Fabp4, Pparg, Cebpα and leptin. Error bars indicate s.e.m. **$P<0.01$, *$P<0.05$ (compared with E15.5).

complete specificity of luciferase expression in adipose tissue in the *leptin-luciferase* mice is advantageous compared with other adipocyte markers, which in almost all other instances are expressed in sites other than adipose tissue (Barak et al., 1999; Urs et al., 2006).

Luciferin was injected into *leptin-luciferase* mice at various embryonic time points and a CCD camera was used to image luciferase activity. At E15.5, none of the embryos showed detectable luciferase activity (Fig. 1A). Starting with E16.0 embryos, a low-intensity signal was detected in the posterior subcutaneous and intrascapular regions, both of which are known to become the first sites of adipogenesis at later stages of murine development in vivo (Fig. 1A). We used this anatomical information to guide the dissection of tissues from these regions at specific embryonic and postnatal time points (Fig. 1), with care taken to ensure that the isolates did not include epithelial tissue. Whole-mount samples were then analyzed by confocal microscopy after staining for vascular tissue and the presence of intracellular lipid using isolectin $GSIB_4$ and lipidTOX dye, respectively. These studies failed to show significant intracellular lipid accumulation at these anatomic sites in the embryo. By contrast, developing adipocytes rapidly accumulate lipid early in postnatal life, becoming easily identifiable by postnatal day 2 and with the subsequent development of a clear unilocular structure by P5 (Fig. 1B).

In order to provide evidence that the tissue dissected from sites of prenatal leptin expression was composed of adipocyte precursors, real-time quantitative PCR (qPCR) analysis for genes known to regulate and/or serve as markers for adipogenesis was performed on RNA isolated from the developing subcutaneous WAT at all of the pre- and postnatal time points. The adipocyte genes *Pparg*, *Cebpa* and *Fabp4* are expressed at significant levels in the dissected tissues, even in prenatal stages, in advance of the observed lipid accumulation (Fig. 1C). Even though *Pparg*, *Cebpa* and *Fabp4* can individually be expressed in other cell types, their co-expression provides strong evidence that we had accurately dissected an adipocyte precursor population. At these prenatal times, leptin was expressed at much lower levels compared with lipid-laden mature adipocytes (Birsoy et al., 2008; Maffei et al., 1995). Leptin remained relatively low in embryonic and early postnatal time points, but its expression was greatly increased at P5 (Fig. 1C), coincident with a marked increase in cellular lipid accumulation (Fig. 1B). These data are consistent with previous observations that adipocyte lipid stores are highly correlated with leptin gene expression (Birsoy et al., 2008; Maffei et al., 1995).

Identification of distinct gene expression signatures during adipocyte development in vivo

Having validated the isolation of tissue expressing canonical adipocyte marker genes indicative of prenatal and postnatal WAT depots, we next generated global transcriptional profiles using microarray technology. RNA from developing WAT were isolated from four independent samples of embryos at each embryonic and postnatal time point and pooled. The RNA was then labeled and hybridized to Illumina Mouse Ref8 BeadChips (GSE29502). The resulting gene expression matrix was clustered using the *k*-means algorithm (Hartigan, 1973), which organized ~70% of expressed genes into 15 groups that display temporally distinct expression patterns, each containing between 743 and 2849 genes. In five of the 15 clusters, genes were induced (three clusters) or repressed (two clusters) during the developmental time course, while all other clusters were characterized by transient expression at specific intermediate time points or more complex expression patterns (Fig. 2A, left panel).

We next tested the extent of correlation between the expression profiles during in vivo and in vitro adipocyte differentiation. For this approach, the 15 temporal profiles identified by *k*-means

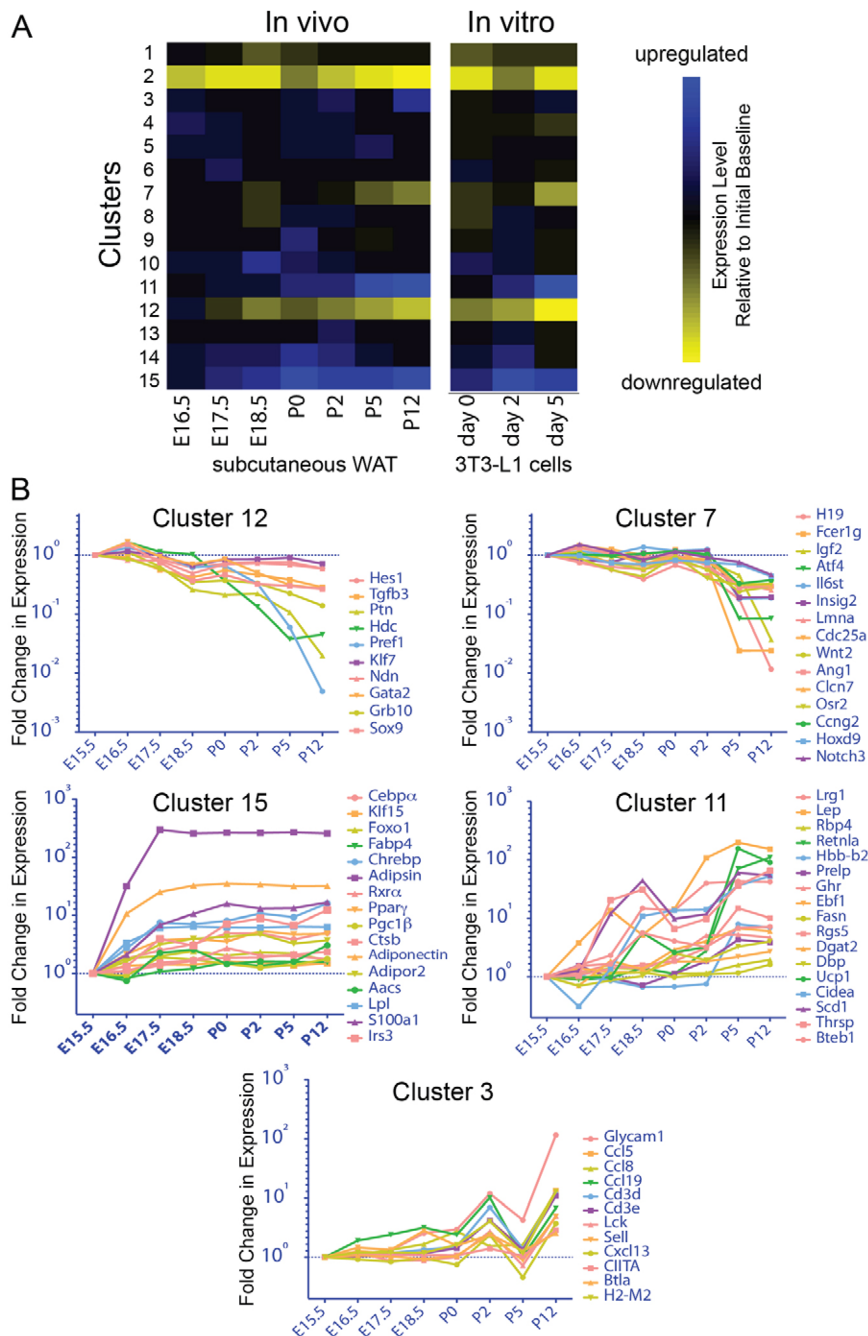

Fig. 2. Gene expression analysis of developmental adipogenesis in vivo and comparison to adipogenesis in 3T3L1 cells. (**A**) *k*-means cluster analysis of genes during in vivo adipocyte development. (Left) All genes are grouped into 15 clusters based on expression profiles across 8 time points. Fold change relative to E15.5 is shown using a heatmap as indicated on the far right. (Right) In vivo clusters are compared with a publicly available microarray dataset in which confluent 3T3-L1 cells were induced to differentiate into adipocytes in culture. Log2 expression fold change compared with initial baseline values (E15.5 and day 2 in vivo and in vitro, respectively) was calculated for all genes for all subsequent time points and averaged across all genes in a cluster. Heatmaps were generated by limma Bioconductor package. (**B**) Fold change relative to E15.5 RNA expression levels is shown for selected genes in clusters 3, 7, 11, 12 and 15 during the WAT development time course.

clustering using data from in vivo microarray analysis of developing WAT (GSE29502) was compared with a publicly available microarray dataset downloaded from GEO (GSE20696) in which confluent preadipocyte 3T3-L1 cells were induced to differentiate into adipocytes in culture. Log2 expression fold change compared with initial baseline values (E15.5 and day 2 in vivo and in vitro, respectively) was calculated for all genes for all subsequent time points and averaged across all genes in a cluster. This analysis showed strikingly similar patterns for almost all clusters in vivo versus in vitro (see quantitative analysis below, Fig. 2A; see Fig. S2B in the supplementary material). These data provide strong evidence that we indeed isolated WAT at a series of early developmental times thus enabling the characterization of adipogenesis in vivo, despite the fact that the dissected samples contained multiple cell types.

In order to further assess the concordance between in vitro (3T3-L1) and in vivo (developmental) temporal expression patterns, expression fold change between the endpoints (P12 and day 7 in vivo and in vitro, respectively) to initial baseline values (E15.5 and day 2 in vivo and in vitro, respectively) was calculated for all genes with a 1:1 mapping between the probes of the two microarray platforms (see Fig. S2A in the supplementary material). Subsequently the number of up- or downregulated genes in a given cluster was determined and the overlap between the in vivo and in vitro genes was quantified. The results show that there is a significant intersection between in vivo and in vitro expression trends. However, we also noted several in vivo specific expression networks suggesting that there are also other specific transcriptional programs in vivo that are not recapitulated in vitro (see Fig. S2A in the supplementary material).

Two of the clusters, Cluster 15 and 11, include genes that are induced during embryonic development prior to the accumulation of intracellular lipid. Many of these genes have previously identified roles in adipocyte differentiation in vitro. For example, cluster 15 contains several factors involved in the late stages of adipogenesis, including *Cebpa*, *Fabp4*, *Pparg*, Rxr genes, adiponectin, adipsin and *Klf15* (Fig. 2B). Consistent with the qPCR analysis of these late adipogenic genes and previously reported in vitro gene expression analysis (Soukas et al., 2000; Soukas et al., 2001) (Fig. 1C), the in vivo induction of these genes is also observed in the late prenatal/early postnatal stage (from E17.5 to P0), just prior to lipid accumulation (Fig. 1B). By contrast, the genes in cluster 11 are induced later in development, concomitant with appearance of lipid-filled unilocular adipocytes (Fig. 1B) and include leptin, retinol binding protein (*Rbp4*) and fatty acid synthase (*Fasn*), all of which are expressed in mature adipocytes (Kern et al., 2004; Lee et al., 2007; Menendez et al., 2009). Cluster 11 also contains transcription factors that have been implicated in adipogenesis, such as *Ebf1* (Jimenez et al., 2007), which, based on these data, could have additional roles in regulating or responding to lipid accumulation in vivo.

A set of genes in cluster 3 is also upregulated during adipocyte formation include genes that are induced even later in WAT development, first appearing at P12 (Fig. 2B). Cluster 3 comprises genes involved in inflammation, such as leukocyte-specific protein tyrosine kinase (*Lck*), B and T lymphocyte attenuator (*Btla*), MHC class Ib gene (*H2-M2*) and CC chemokine ligands (*Ccl5*/*Ccl8*/*Ccl19*). However, it is not clear whether these genes are expressed from developing adipocytes themselves versus other cell types, such as immune cells that can infiltrate WAT.

Finally, two other clusters, cluster 7 and cluster 12, include genes that are downregulated during WAT development in vivo (Fig. 2B). These genes include previously identified genes, including *Gata2*, *Pref1* (*Dlk1* – Mouse Genome Informatics), *Sox9*, *Ang* and *Igf2* that are expressed in 3T3-L1 preadipocytes and progressively downregulated through differentiation in vitro (Jing et al., 2009; Tong et al., 2000; Wang and Sul, 2009). This result adds further evidence that the tissue we had isolated was in fact early developing WAT by confirming that it expresses a set of validated preadipocyte markers.

The other clusters show more complicated irregular patterns of expression (Fig. 2B).

NRF2/GABPA-binding sites are enriched in the promoters of preadipogenic genes in vivo

Having identified clusters of genes that are coordinately regulated during the early development of WAT in vivo and in vitro, we next set out to use a set of novel computational tools to identify DNA sequence motifs enriched in the promoter regions and/or 3′UTRs of genes within the expression clusters. Any DNA motifs that are enriched regulatory sequences in the genes within a cluster represent candidate cis-regulatory elements for regulating the expression of genes within that specific cluster. To accomplish this, finding informative regulatory elements (FIRE) analysis, a recently described algorithm designed to identify candidate cis-regulatory elements from microarray data (Elemento et al., 2007) was employed. The FIRE method scores the correlation between gene expression levels and potential regulatory motifs using a mutual information algorithm and identifies high-confidence DNA motifs whose presence in the regulatory regions is most informative about the expression of the corresponding expression clusters.

FIRE identified five upstream sequence motifs and one 3′UTR motif that were enriched in a subset of the clusters. The highest scoring motif was for the highly conserved seed sequence for nuclear respiratory factor 2 (NRF2/GABPA), CCGGAAG (z score: 60), which is enriched in the regulatory sequences of genes in both clusters 7 and 12 (Fig. 3A). Note that genes in these clusters are progressively downregulated during WAT development. Another enriched sequence, albeit one with a lower z score was for EVI1 (z score: 22.1), which was modestly enriched in cluster 6, a cluster that comprises genes that are transiently upregulated in WAT at E17.5. FIRE also identified an SP1 motif as enriched in cluster 2 genes, but with an even lower z score (z score: 8.4). Finally, FIRE returned motifs with low scores are not known to be associated with transcription factors, such as the 3′UTR motif ‘UUUUUUA’ and two other promoter motifs CTTTGCC and ACCTGAC in a number of gene clusters (Fig. 3A). To date, none of the candidate regulatory motifs, including NRF2/GABPA, have been suggested to play a role in adipocyte development.

We chose to study the NRF2/GABPA motif further, as it was the motif with the highest score in FIRE analysis, and it was enriched in two clusters, 7 and 12, in which gene expression peaks during early development and decreases as adipocytes fill with lipid. Furthermore, genes in cluster 7 and cluster 12 are of particular interest because preadipocyte markers such as *Pref1* (*Dlk1*), *Sox9* and *Gata2* are enriched in these clusters (Jing et al., 2009; Tong et al., 2000; Wang and Sul, 2009), as well as genes involved in metabolic homeostasis and WAT mass regulation, such as *Igf2*, *Insig2*, *Lmna* and *Angpt1* (Krapivner et al., 2008; Longo et al., 2002; Verseijden et al., 2009; Wojtanik et al., 2009). In addition to previously characterized preadipocyte markers, many of the genes in these clusters also play a role in cell cycle regulation, as shown by gene ontology (GO) term enrichment analysis (Fig. 3B). The developmental decrease in the expression of these cell cycle genes is consistent with mature adipocytes being post-mitotic (Birkenmeier et al., 1989; Christy et al., 1989).

These data predict that a transcription factor recognizing this motif should play a functional role to control key aspects of adipogenesis in vitro and in vivo, and that studying this factor may enable us to identify bona fide in vivo regulators of adipocyte differentiation. To identify the transcription factors binding to the NRF2/GABPA seed motif, CCGGAG, we employed the TFBIND transcription factor binding program (Tsunoda and Takagi, 1999). TFBIND analysis yielded two candidate transcription factor families that are known to bind to the CCGGAG seed motif, ETS and NRF2/GABPA (Fig. 3A). As none of these factors has previously been implicated in adipogenesis or WAT mass regulation (Sharrocks, 2001; Tymms and Kola, 1994; Watabe et al., 1998), we next set out to analyze the expression and function of the three candidate transcription factors that are known to bind CCGGAG, NRF2/GABPA, ETS1 and ETS2.

Expression of *Nrf2/Gabpa*, *Ets1* and *Ets2* during adipogenesis

The SVF of WAT includes the adipocyte precursors that express pre-adipocyte markers and factors involved in the early stages of adipogenesis. To assess whether *Nrf2/Gabpa* element binding factors are enriched in the SVF, we separated WAT into mature adipocytes and the SVF and assayed gene expression levels of *Ets1*, *Ets2* and *Nrf2/Gabpa* using qPCR. Leptin and *Gata2* were used as adipocyte and preadipocyte control markers, respectively

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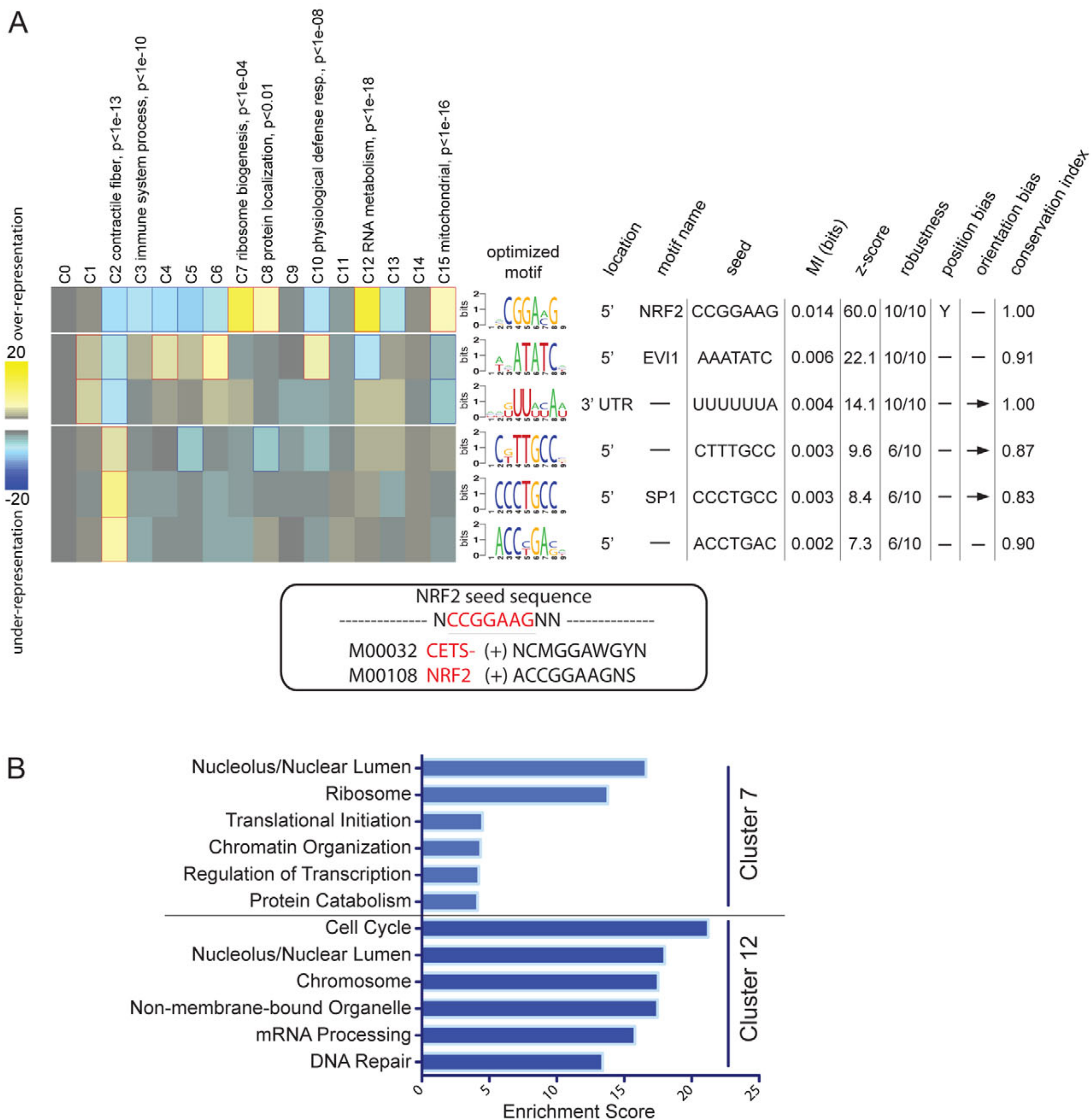

Fig. 3. Identification of potential regulatory sequences and functional annotation of genes within WAT clusters. (**A**) Identification of potential cis-regulatory elements in WAT development. FIRE analysis discovered over-represented patterns of cis-regulatory elements for specific in vivo gene clusters. Rows correspond to the discovered motifs by FIRE analysis. Columns represent the 15 clusters that were determined by *k*-means clustering (Fig. 2A). At the top of specific clusters is the pathway determined to be over-represented by iPAGE. For each discovered motif, location, statistical significance, score, conservation index, sequence and name of the putative known motif is shown. (Below) TFBIND analysis of the highest scoring motif (NRF2) reveals ETS and NRF2 as the candidate transcription factors binding to this motif. Transcription factor binding site for each transcription factor is indicated. (**B**) Gene Ontology categories over-represented in clusters 7 and 12. Functionally enriched annotation groupings within the cluster 7 and 12 gene sets were identified and assigned a statistical enrichment score using NIAID/NIH DAVID.

(Fig. 4A). The qPCR analysis showed that *Ets1* and *Ets2* were highly enriched (7-fold and 6.5-fold, respectively, $P<0.01$) in the SVF compared with the adipocyte fraction. There was also a significant, but lesser enrichment of *Nrf2/Gabpa* in the SVF ($P<0.05$) (Fig. 4A), suggesting that all of three of these genes are expressed in the SVF. Analysis of gene expression in isolated adipocyte progenitor cells (APs) (Rodeheffer et al., 2008), demonstrates that all three genes are expressed in APs, but only Ets2 is significantly enriched compared with total SVF (Fig. 4C).

In order to determine the temporal expression of these NRF2/GABPA motif-binding factors during adipogenesis, we assayed their expression during differentiation of 3T3-L1 cells in culture. Adipogenesis was induced using the standard cocktail of IBMX, dexamethasone and insulin. Analysis of gene expression over a differentiation time course via qPCR revealed that *Ets2* mRNA is upregulated 1 hour post-induction, peaking at 2 hours post-induction, then decreased at day 1 and remaining low for the remainder of the time course. The level of *Ets1* mRNA is similar

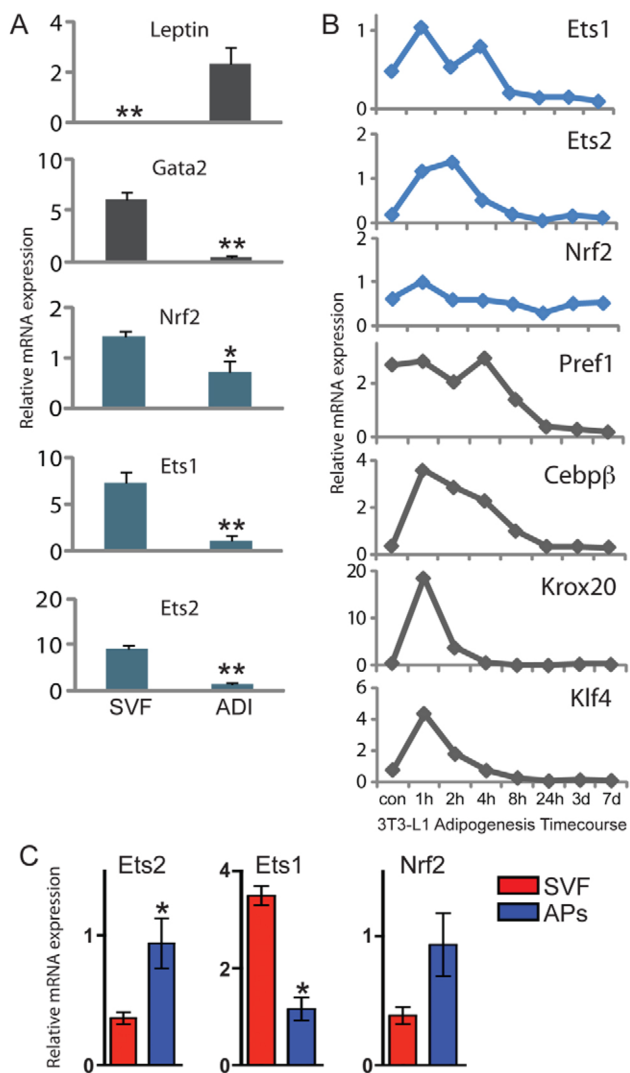

Fig. 4. Expression analysis of *Ets* and *Nrf2* transcription factors. (**A**) Real-time PCR expression analysis of *Ets1*, *Ets2* and *Nrf2* in stromavascular and adipocyte fractions from posterior white adipose tissue. Leptin and *Gata2* levels were used as controls for purity of adipocyte and stromavascular fractions, respectively. Error bars indicate s.e.m. $**P<0.01$, $*P<0.05$. (**B**) Real-time PCR analysis of *Ets1*, *Ets2* and *Nrf2* during differentiation of 3T3-L1 cells. Cells were harvested at the indicated times for analysis. Cyclophilin was used as an internal control for Taqman analysis. Transcript levels for early genes *Pref1*, *Cebpb*, *Klf4* and *Krox20* are shown for comparison. (**C**) Real-time PCR analysis of *Ets1*, *Ets2* and *Nrf2* in adipocyte progenitors. RNA was isolated from FACS sorted CD45^{-}:CD31^{-}:CD29^{+}:CD34^{+}:Sca1^{+}:CD24^{+} adipocyte progenitors and SVF singlet cells that were collected from the sorter. *Tbk1*, a gene that is similarly expressed in SVF and all isolated adipogenic cell populations, as determined via global gene expression analysis, was used as an internal control for qPCR. Error bars represent s.e.m. $*P<0.05$.

to *Pref1* in that it is elevated in undifferentiated 3T3-L1 cells and is progressively downregulated during the time course of differentiation (Fig. 4B). *Nrf2/Gabpa* mRNA levels did not change during differentiation. These results indicate that both *Ets1* and *Ets2* are regulated early during adipocyte differentiation in vitro, but that only *Ets2* in enriched in APs in vivo, suggesting a possible role for *Ets2* in adipogenesis.

Effect of *Ets1*, *Ets2* and *Nrf2/Gabpa* knockdown on adipogenesis

In order to determine the functional role of the *Ets1*, *Ets2* and *Nrf2* in adipocyte differentiation, we generated 3T3-L1 cell lines stably expressing shRNAs targeting each of these three genes, or expressing a non-specific control hairpin construct. 3T3-L1 cells were then infected with lentiviruses expressing two independent hairpin shRNA sequences for each gene and stable cell lines with constitutive shRNA expression were established by selection with puromycin. These stable cell lines were then cultured to confluence and differentiated using the standard induction protocol for 8 days. The extent of the knockdown by the targeted shRNAs for *Ets1*, *Ets2* and *Nrf2* was confirmed by western and qPCR analysis (Fig. 5A; see Fig. S3A in the supplementary material), with qPCR showing that each of the shRNA constructs effectively knocks down the intended target. We then tested the effect of these knockdowns using Oil Red O staining, which reveals the extent of lipid accumulation. Although knockdown of *Nrf2* and *Ets1* did not affect lipid accumulation (see Fig. S3B in the supplementary material), knockdown of *Ets2* resulted in a marked diminution of lipid accumulation in two independent *Ets2* shRNA cell lines compared with control cells (Fig. 5B).

To assess further the effect of *Ets2* knockdown on adipogenesis, we determined the expression levels of adipogenic markers by qPCR (Fig. 5C). Consistent with the oil red O phenotype, the levels of *Fabp4*, *Pparg* and adipsin (*Cfd*) were markedly decreased at all the points examined in the *Ets2* shRNA cell lines, indicating that *Ets2* knockdown inhibits the expression of adipogenic marker genes (Fig. 5C).

After treatment with the induction cocktail, 3T3-L1 preadipocytes undergo two rounds of mitosis (referred to as mitotic clonal expansion), which is required for progression of adipogenesis (Tang et al., 2003). The previously described role of *Ets2* in proliferation (Gutierrez-Hartmann et al., 2007), combined with its early expression in adipogenesis and requirement for proper differentiation suggest that effect of *Ets2* on adipogenesis might be explained by altered mitotic clonal expansion. In order to test this hypothesis, we performed BrdU incorporation studies. Control and *Ets2* shRNA cells were differentiated and pulsed with BrdU between 16 and 18 hours post-differentiation, which corresponds to S phase of mitotic clonal expansion. Consistent with our finding that cluster 12 is enriched for cell cycle-related genes and for Nrf2 motif-associated genes, *Ets2* shRNA 3T3-L1 cells proliferated significantly less than control-shRNA cells, as demonstrated by a lower percentage of BrdU-positive cells (Fig. 5D).

Furthermore, quantitation of mRNA levels for the top downregulated GO cell cycle genes in cluster 12 indicates that most cell cycle genes containing NRF2/GABPA motifs are significantly altered during adipogenesis in the absence of *Ets2* (see Fig. S4A in the supplementary material). In addition, chromatin immunoprecipitation (ChIP) with an anti-ETS2 antibody indicates that ETS2 binds to the promoters of several cell cycle genes that are dynamically regulated during adipogenesis (Fig. 5E; see Fig. S4B in the supplementary material). These findings suggest that impaired differentiation upon Ets2 knockdown is caused by a defect in the mitotic clonal expansion phase of adipocyte differentiation. Overall, these findings identify an important novel transcriptional program in vivo that is of crucial importance for adipocyte differentiation in vitro. With this information, the role of this essential program can now be tested in vivo.

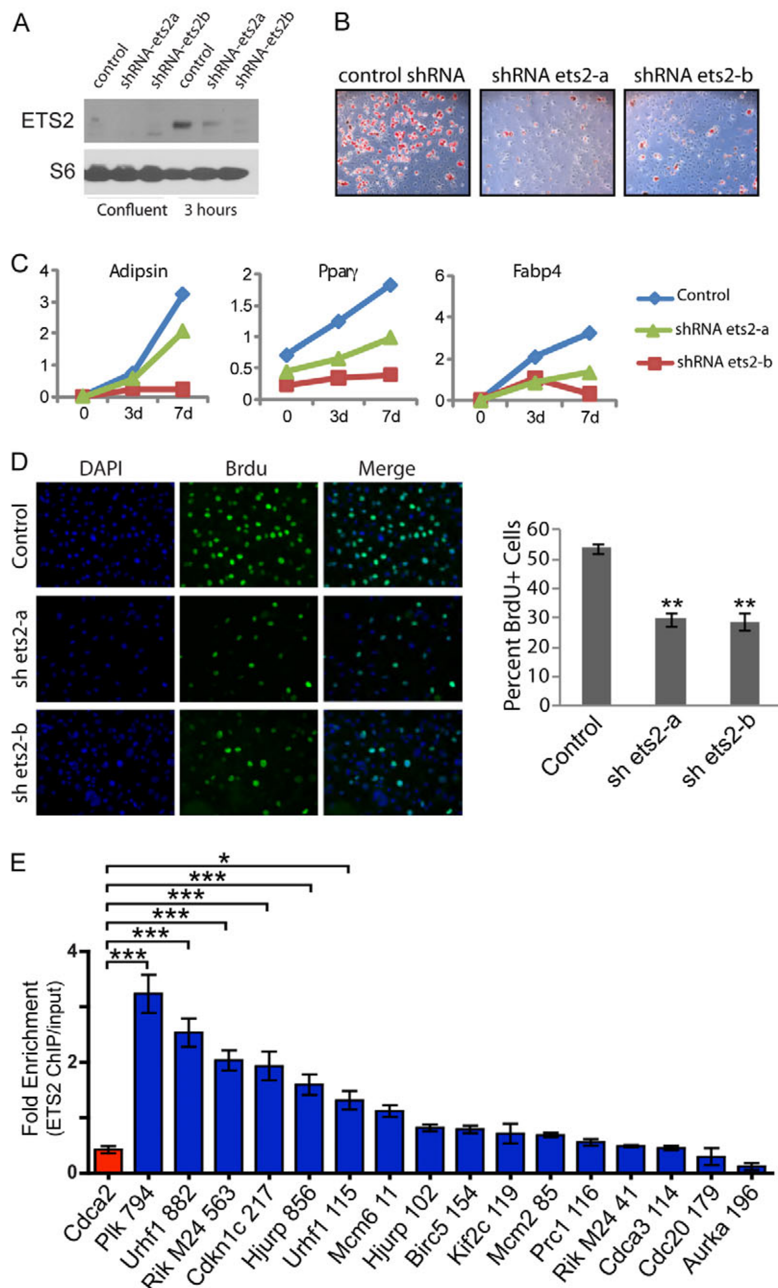

Fig. 5. *Ets2* knockdown impairs adipogenesis through an effect on clonal expansion. 3T3-L1 cells were infected with lentivirus containing shRNAs targeted to either *Ets2* or control. (**A**) ETS2 levels were determined in control and knockdown cells by immunoblotting protein from untreated confluent cells and 3 hours after induction with the differentiation cocktail. S6 antibody is used as a loading control. (**B**) Oil red O staining of *Ets2* knockdowns and control at day 7. (**C**) Expression levels of adipocyte markers *Fabp4*, *Pparg* and adipsin at confluence (0), 3 days (3d) and 7 days (7d) after differentiation. (**D**) Effect of *Ets2* knockdown on clonal expansion. At 16-18 hours after induction of differentiation, cells were pulsed with BrdU and immediately fixed with 70% ethanol. Cells were then stained with a BrdU antibody and DAPI and photographed with a fluorescence microscope. BrdU-positive cells were then counted and percentage of BrdU-positive cells in the total cell population are shown in the graph. Error bars represent s.e.m. (n=5). $^{**}P<0.01$. (**E**) ETS2 chromatin immunoprecipitation from 3T3-L1 cells. ETS2 ChIP was performed on confluent 3T3-L1 cells. Enrichment of predicted NRF2 promoter elements from GO cell cycle genes from cluster 12 (blue bars) was determined and compared with *Cdca2*, a cluster 12 GO cell cycle gene without a predicted NRF2 promoter (red bar). The genes interrogated are indicated below the graph. Numbers indicate position of putative NRF motif relative to transcription start site. Rik M24 indicates Riken 6720463M24. Error bars represent s.e.m. $^{***}P<0.001$, $^{*}P<0.05$.

DISCUSSION

Although there has been enormous progress in understanding the differentiation of adipocytes in cell culture, the molecular mechanisms controlling the development of white adipocytes in vivo is poorly understood. In an effort to both visualize WAT development and study the molecular processes that control adipocyte differentiation in vivo, we used *leptin-luciferase* transgenic mice to define the locations of WAT formation in the developing mouse embryo. We then used this information to isolate embryonic and neonatal WAT tissues, even in advance of cells having deposited lipid, the canonical feature of adipocytes. Transcription profiles generated using RNA from these depots confirmed that many genes previously identified during adipogenesis in vitro display similar expression patterns during formation of WAT, providing direct evidence that differentiation of adipocytes in cell culture recapitulates many of the transcriptional programs that are functional during development in vivo. However, these developmental studies identified additional in vivo specific programs of gene expression during WAT development that display different kinetics compared with in vitro adipocyte differentiation, including a specific cluster whose temporal expression coincides with the accumulation of intracellular lipid. These data provide an opportunity to broaden our understanding of the molecular mechanisms controlling WAT development and also provide a basis for studying the extent to which these in vivo programs underlie the increase in WAT mass as obesity develops.

Several distinct networks of genes were identified during adipocyte differentiation in vivo. One cluster (Cluster 15) contains previously characterized genes that are upregulated during WAT development. This group of genes include factors such as *Pparg*, *Cebpa*, *Klf15*, adiponectin, *Foxo1*, *Chrebp*

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(*Mlxipl* – Mouse Genome Informatics) and adipsin, which are also known to be induced during adipocyte differentiation in vitro (Rosen and MacDougald, 2006) and are commonly used as mature adipocyte markers. These genes are induced by E17.5 in vivo, well in advance of the onset overt lipid accumulation, suggesting that these genes are involved in priming the adipocyte precursors to accumulate lipid. The remarkable overlap between these in vivo genes and the genes induced during in vitro adipogenesis (50%; see Fig. S2A in the supplementary material) suggests that assaying these early stages of WAT development does indeed provide a valid means for the study of adipogenesis in vivo, and confirms that, to a considerable extent, in vitro adipogenesis faithfully represents the early adipogenic processes that occur in vivo.

Another group of genes, cluster 11, comprises genes whose expression levels increase through the early postnatal time points, and more closely correlates with the accumulation of lipid in adipocytes in vivo. Cluster 11 includes leptin, *Rbp4*, *Fasn*, resistin-like alpha (*Retnla*), growth hormone receptor (*Ghr*), leucine-rich glycoprotein 1 (*Lrg1*) and perilipin. Several of these genes are known to play a role in lipid transport or lipid droplet function and leptin expression has repeatedly been shown to tightly correlate with the amount of lipid stores (Birsoy et al., 2008; Maffei et al., 1995). There are also reports of a correlation between adiposity and expression of *Rbp4* (Kelly et al., 2010; Lee et al., 2007) and perilipin, a protein that is localized to the surface of the lipid droplets in adipocytes (Kern et al., 2004). These data further suggest that distinct transcriptional mechanisms may regulate genes that control adipocyte differentiation versus those associated with lipid accumulation and that these processes are regulated independently during development in vivo. Furthermore, the majority of genes present in cluster 11 are specifically expressed in vivo, indicating that in vitro models of adipogenesis may not be suitable for the study of gene programs that play a role in increasing lipid accumulation. Further study of the factors correlated with the accumulation of lipid may lead to the identification of novel genes/pathways that have roles in lipid accumulation/sensing in vivo, possibly providing new targets for therapeutic treatment of obesity and other disorders. It should be noted that similar lipid-sensing systems might be recapitulated in other cell types such as tumor cells, some of which are heavily dependent on lipids as a source of nutrient (Knowles et al., 2004).

The ability to cluster groups of WAT genes during development also provided an opportunity to use computational tools to identify previously unappreciated transcriptional mechanisms accounting for aspects of the differentiating adipocyte phenotype. To achieve this, we used an information-theoretic algorithm, FIRE (Elemento, 2007; Elemento et al., 2007), to identify putative cis regulatory elements that are enriched in the promoters and 3′UTRs of specific gene clusters (networks). Our goal in these studies was to identify candidate transcription factors that coordinately regulate specific classes of genes during adipocyte development. The highest scoring motif from FIRE analysis was a highly significant match for the binding site of NRF2/GABPA, which is preferentially enriched in cluster 7 and 12. These clusters contain genes for a set of previously identified preadipocyte factors and are also enriched for genes that may play a role in clonal expansion (Fig. 3B). This implies that factors binding to this motif might play a functional role in regulating adipocyte development. Several factors are known to bind to the consensus sequence for the 'CCGGAAG' NRF2 site, including NRF2/GABPA, ETS1 and ETS2. We thus measured the levels of expression of these factors during adipocyte differentiation in vitro and found that *Ets* factors, but not *Nrf2/Gabpa*, are expressed in the preadipocytes and their expression is decreased early during adipogenesis in vitro. However, only *Ets2* was found to play a role in adipogenesis, as *Ets2* knockdown in 3T3-L1 cells represses adipogenesis, possibly via an effect on mitotic clonal expansion.

The ETS transcription factor family has been shown to regulate several endocrine and metabolically active tissue types, including pituitary, prostate, mammary gland and thyroid gland (Gutierrez-Hartmann et al., 2007), and several hundred genes regulating cell growth, differentiation and development have been identified as ETS targets. ETS expression has also been shown to be increased in many human tumors (Turner and Watson, 2008). Overexpression of *Ets1* and *Ets2* abolishes the serum requirements of fibroblast cells in culture and leads to a neoplastic transformation, and both proteins were shown to regulate cellular senescence (Ohtani et al., 2001). The transient increase and suppression of *Ets* during adipogenesis in parallel with a decrease in the expression of cell cycle genes is consistent with this documented ability to promote cellular growth. Our present findings show that *Ets2* plays a role to regulate adipogenesis in vitro through its role in clonal expansion. This finding, combined with enrichment of *Ets2* in APs in vivo, suggests that ETS2 plays a functional role in adipocyte differentiation in vivo.

Ets2 knockout mice display early perinatal lethality (Higuchi et al., 2007; Yamamoto et al., 1998). The complex phenotype of these animals, with effects on many organ systems, requires that an adipocyte progenitor-specific knockout be generated to assess a role for this factor in adipocyte development in vivo. At present, the only adipocyte lineage-specific promoters that are available for the generation of WAT-specific conditional expression mouse models include the *Fabp4* (*aP2*) promoter, the adiponectin and leptin regulatory regions. These genes are only induced in late stages of adipogenesis and would induce the expression of a WAT specific cre-recombinase at later stages in differentiation than probable *Ets* function. Thus studies of the role of Ets and other early factors in adipogenesis in vivo are limited by the fact no specific promoters expressed early in adipogenesis have been characterized. The only widely accepted marker of preadipocytes is *Pref1*, which is expressed in many tissues (Raghunandan et al., 2008; Sun et al., 2008). It would be advantageous to identify a specific early adipocyte precursor promoter to generate traditional transgenic mice or conditional models. The data presented from these studies provides potential candidates for such promoters, especially in clusters 7 and 12, which include genes that are expressed earlier in WAT development and are downregulated postnatally.

In conclusion, we report the transcriptional and morphological characterization of WAT at a series of early developmental time points. These data allowed us to compare the differences and similarities between in vitro and in vivo adipocyte development, and provided the basis for the elucidation of new factors involved in the regulation of WAT mass in vivo. Studies of these genes led to the identification of *Ets2* as a factor involved in the early stages of adipogenesis. By providing promoters that could be used to drive cre-recombinase or other reporter genes early in adipocyte development, information derived from these studies may also prove useful for the control of genes expressed during early

adipocyte development, allowing the generation of new models for adipocyte precursor cell-specific manipulation of *Ets2* and similar early regulatory factors in vivo.

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Competing interests statement
The authors declare no competing financial interests.

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