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Long noncoding RNAs regulate adipogenesis

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The prevalence of obesity has led to a surge of interest in understanding the detailed mechanisms underlying adipocyte development. Many protein-coding genes, mRNAs, and microRNAs have been implicated in adipocyte development, but the global expression patterns and functional contributions of long noncoding RNA (lncRNA) during adipogenesis have not been explored. Here we profiled the transcriptome of primary brown and white adipocytes, preadipocytes, and cultured adipocytes and identified 175 lncRNAs that are specifically regulated during adipogenesis. Many lncRNAs are adipose-enriched, strongly induced during adipogenesis, and bound at their promoters by key transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (CEBPα). lncRNA-mediated loss of function screens identified functional lncRNAs with varying impact on adipogenesis. Collectively, we have identified numerous lncRNAs that are functionally required for proper adipogenesis.

Obesity is a major source of morbidity and mortality and is increasingly prevalent in many areas of the world. By 2008 the incidence of this disease was 32.2% among adult men and 35.5% among adult women in United States (1). Excess fat accumulation is a contributing factor in such severe human diseases as type 2 diabetes, certain cancers, and cardiovascular disease (2). Understanding the detailed mechanisms controlling adipogenesis and energy homeostasis is a critical part of the effort to combat obesity.

Adipogenesis is governed by a transcriptional cascade driven, in large part, by peroxisome proliferator-activated receptor γ (PPARγ), an adipocyte-enriched nuclear receptor (3). PPARγ is both necessary and sufficient for adipogenesis (4, 5); no other factor has been found to be able to induce adipogenesis in the absence of PPARγ. PPARγ cooperates with CCAAT/enhancer-binding proteins (CEBP), including Cebpα, Cebpβ, and Cebpγ, to induce the expression of many genes important for terminal differentiation, such as Fabp4/p2, Cd36, Lipe/Hsl, Olr1, and Me1 (6). Unlike transcriptional factors that bind to DNA directly, transcriptional cofactors often serve as molecular scaffolds to link the basal transcription machinery to either active or inactive complexes (7–9). Many cofactors, such as CBP and p300, have enzymatic activity in histone modification and can adjust chromatin environments to be more or less accessible to the transcription machinery. Indeed, dramatic changes in epigenetic signatures have been observed during adipogenesis, indicating an important role of epigenetic modifications in regulating adipogenesis (10). Noncoding RNAs are known to play a regulatory role in many developmental contexts, including adipogenesis, and several microRNAs have been identified that positively or negatively regulate adipogenesis (11, 12). Recently we and others have identified a class of long noncoding RNAs (lncRNAs) (13–15). Previous studies have demonstrated that lncRNAs are essential regulators in a variety of biological processes, including, but not limited to, X chromosome inactivation (16), p53-mediated apoptosis (17, 18), cancer metastasis (19), and reprogramming of induced pluripotent stem cells (20), among many others (21–23). We therefore hypothesized that lncRNAs participate in the regulatory network governing adipogenesis.

To test this we used deep RNA sequencing (RNA-Seq) to identify mRNAs and polyadenylated lncRNAs that are regulated during adipogenesis. To survey the functional contributions of lncRNAs during adipogenesis we performed RNAi mediated loss of function (LOF) experiments for 20 candidate lncRNAs. In scoring each LOF assay for functional relevance, we present here a unique method that uses the Jensen-Shannon distance metric to quantify the lncRNA-dependent “transcriptome shift” from mature adipocyte to reversion back to the precursor state. Our combined results establish that lncRNAs constitute an as yet unexplored and important layer in adipogenesis regulation.

Results

Global Identification of lncRNAs Regulated During Adipogenesis. We first set out to identify global transcriptional regulation during adipogenesis for both coding and noncoding genes alike. To this end we used massively parallel RNA sequencing as previously described (24) to sequence polyA-selected RNAs from in vitro cultured brown and white preadipocytes, in vitro differentiated mature brown and white adipocytes, and primary brown and white mature adipocytes directly isolated from mice. RNA-Seq reads were mapped to the mouse genome (mm9) using TopHat (25), to which we provided gene annotations for known and/or cloned and previously undescribed transcripts to maximize spliced alignment accuracy. All annotated transcripts, corresponding to known University of California at Santa Cruz (UCSC) genes and available RIKEN cloned sequences (26), were quantified across each condition and differential expression analysis between preadipocytes and mature adipocytes using Cuffdiff (27). We identified 1,734 coding genes and 175 lncRNAs that were significantly up- or downregulated greater than twofold during differentiation of both brown and white adipocytes [false discovery rate (FDR) <5%] (Fig. L4). To further explore the biological validity of our derived adipocytes we examined the expression of several well-known adipogenesis markers, such as fatty acid binding protein 4 (Fabp4), adiponectin (AdipoQ), and glucose transporter type 4 (Glut4) (Fig. L8) as well as several additional markers, such as preadipocyte factor 1 (Pref1), cell death-inducing DFFA-like effector a (Cidea), and uncoupling protein 1 (Ucp1) (Fig. S1), all of which are regulated as expected for appropriately differentiating white and/or brown preadipocytes. We next examined the protein coding gene sets up-regulated during adipogenesis by global gene...
ontology analysis. Consistent with bona fide adipocytes and many previous studies, we observed significant enrichments in lipid metabolism and adipocyte terms, whereas down-regulated genes are enriched in cell cycle and fibroblast terms (Fig. S2), demonstrating that our RNA-Seq data accurately reflect the known hallmarks of adipogenesis.

Expression of lncRNAs Is Tightly Regulated During Adipogenesis.
Hierarchical clustering of the RNA-Seq expression profiles determined that both lncRNAs and protein coding genes alike accurately distinguished precursors from adipocytes (Fig. L1A). Specifically, precursor cells are clustered together yet distinctly different from the gene expression patterns of both cultured adipocytes and primary adipocytes (Fig. L1A). Importantly, cultured and primary brown and white adipocytes exhibited similar expression patterns. Together these observations demonstrate that our in vitro cell cultured adipocytes share similar gene expression signatures to their primary counterparts across both protein-coding and noncoding lncRNA genes.

Several recent studies have identified ncRNAs directly regulated by key transcription factors that define and drive cellular differentiation (14, 18, 20, 28–30). Therefore, we explored whether lncRNAs regulated during adipogenesis are controlled by known adipogenic transcription factors, such as Pparγ and Cebpα. To this end we analyzed data from previous genome-wide binding site localizations of Pparγ (10) and Cebpα (31). Pparγ is physically bound within the promoter region (2 kb upstream of the transcriptional start site) of 23 (13%) of 175 lncRNAs up-regulated during adipogenesis; Cebpα was bound upstream of 34 up-regulated lncRNAs (19%). Up-regulated protein coding genes were similarly enriched for Pparγ and Cebpα binding for (215 (14%) and 352 (20%) of 1,734 genes, respectively). A permutation analysis confirmed these enrichments to be statistically significant (P < 10<sup>-5</sup>) over random chance. Collectively, these computational and experimental analyses suggest that a similar portion of regulated lncRNAs and protein coding genes are bound and activated by transcription factors responsible for coordinating adipogenesis.

To focus our functional validation efforts, we first ranked candidate lncRNAs according to their level of up-regulation in brown and white fat, and the presence of PPARγ or CEBPα binding at the lncRNA promoter. These criteria resulted in 32 top candidates for further analysis. We performed quantitative RT-PCR (qRT-PCR) to independently measure the regulation of these 32 lncRNAs during differentiation of independently derived precursors (Fig. 2 A and B). As a control, we monitored the adipogenesis marker Fabp4 as well. As assessed by qRT-PCR, 26 of 32 selected lncRNA genes were confirmed as up-regulated during adipogenesis in both primary brown and white adipocyte cultures (Fig. 2 A and B), similar to Fabp4.

To determine whether these 26 lncRNAs were specifically regulated in adipose tissue we monitored their expression levels using qRT-PCR across 10 additional tissues (Fig. 2C). Intriguingly, many of these lncRNAs were greatly enriched in but not totally specific to other brown adipose tissue and white adipose tissue compared with 10 other organs. Collectively these experiments identified 26 lncRNAs that are induced during adipogenesis and exhibit adipose-enriched expression patterns; several exhibit promoter binding of Pparg and Cebpα, suggestive of a functional role in adipogenesis.

LOF Screening Reveals Functional lncRNAs During Adipogenesis. To identify lncRNAs that are functionally important for adipogenesis, we used RNAi-mediated LOF to screen the top 20 lncRNA genes identified by the above criteria of significant up-regulation in both brown and white fat cultures, PPARγ and CEBPα promoter binding, and independent validation of adipose-specific expression. Briefly, we separately transfected three siRNAs targeting each lncRNA into s.c. preadipocyte cultures 1 d before differentiation. Two siRNAs targeting Pparg mRNA were used as positive controls and a nontargeting scrambled siRNA as a negative control. After 4 d of differentiation, lipid accumulation was evaluated by Oil Red O (ORO) staining. In addition, total RNA was subsequently isolated to monitor the siRNA knockdown efficiency of each construct. As expected, siRNA constructs targeting Pparg showed a marked reduction in lipid accumulation (Fig. 3A). Ten of the targeted lncRNAs were not effectively knocked down or
did not result in detectable changes in ORO staining relative to nontargeting controls. In contrast, we identified 10 lncRNAs exhibiting moderate to strong reductions in lipid accumulation upon LOF (Fig. 3A). To further investigate the effect of lncRNA LOF on the adipogenesis process we performed qRT-PCR on four adipocyte markers: Pparg, Cebpα, Fabp4, and AdipoQ across the subset of the lncRNAs with moderate to strong depletion in lipid accumulation (Fig. 3B). Each of the tested siRNAs caused a reduction in the expression of at least three key adipogenic marker genes relative to the scrambled control. Collectively our LOF screen of 20 lncRNAs identified 10 lncRNAs that seem to play key roles in proper differentiation of adipocyte precursors. For simplicity, we refer to these lncRNAs as Regulated in AdiPogenesis (lnc-RAP-n).

Information-Theoretic Metric Scores Cellular Phenotype. We next set out to quantify the LOF phenotypes by scoring the effect of each lncRNA perturbation on the global white adipocyte transcriptome. Such a scoring method should accurately measure how the expression profile from a knockdown differs from the precursor and adipocyte states, and the score should correlate with the qualitative ORO staining. Commonly used scoring functions, such as the Pearson correlation or the Euclidean distance, have been used for similar purposes. However, neither of these methods accurately corresponded with the ORO staining results (Fig. S3). This is likely due to some inherent limitations of these approaches. For example, (i) Pearson correlation is capable of identifying similar expression patterns across a range of different intensities; however, correlation is not a true distance metric and is sensitive to outliers, which can interfere with meaningful hierarchical clustering of samples; and (ii) Euclidean distance is greatly influenced by the absolute level of expression; differences in a few abundant genes may cause two profiles that are otherwise qualitatively very similar to appear distant.

To overcome these limitations, we turned to a metric of similarity between two frequency profiles based on Shannon Entropy, termed Jensen-Shannon distance (JSD) (Experimental Procedures). This metric has been used previously for simple purposes in quantifying differential splicing in high-throughput data (27, 32) and is increasingly used in machine learning applications. To test the hypothesis that JSD was a more quantitative measure to examine phenotype relative to ORO staining, we first identified the gene signature that best distinguishes the precursor state (D0) from mature cultured white adipocytes (D4). We collected total RNA from three biological replicates of D0 and D4 cells and performed

**Fig. 2.** Validation of selected lncRNAs. Primary brown preadipocytes (A) and primary white preadipocytes (B) were isolated, cultured, and differentiated as described in Experimental Procedures. Total RNA was extracted from D0 and D6 cells, and qRT-PCR was performed to examine the expression of selected lncRNAs. Twenty-six lncRNAs (n = 3) were validated as significantly differentially up-regulated during both brown and white fat differentiation (P < 0.05; means ± SEM). (C) RT-PCR was performed to examine the expression of lncRNAs across 12 adult mouse tissues. Their expression profiles, presented here as differences from the row mean, are plotted as a heatmap. Red represents a higher expression and blue a lower expression. BAT, brown adipose tissue; Epi, epididymal white fat.
toward the mean values for the adi-

To further elucidate a role for lncRNAs in adipogenesis, we analyzed the expression of selected lncRNA transcripts in the lncRNA depletion samples and the scramble control. The resulting gene expression pattern comprised 1,727 genes that discretely distinguished precursors from mature adipocytes.

We thus quantified the “transcriptome shift” toward the mean D0 (precursor) expression profile across all 1,727 significant genes for each of the 10 lncRNA depletion expression profiles and the scrambled controls that exhibited significant to moderate ORO staining phenotypes. The mean-centered expression profiles are presented as a heatmap, where conditions are ordered by JSD to the D0 profile (Fig. 4). As expected, the gene profile of the scramble control shRNA most closely resembled the D4 differentiated adipocytes. Similarly, one lncRNA knockdown, RAP-10, had a differentiation profile similar to the scramble control and the D4 differentiated adipocytes, suggesting little to no effect on adipogenesis by depleting this lncRNA.

Importantly, upon knockdown, nine lncRNAs exhibited a partial or near-complete reversion of the mature adipocyte (D4) to precursor (D0) expression signature. This indicates that these lncRNAs are required for proper regulation of the transcriptional network implicated in adipogenesis. Remarkably, JSD scores closely correspond to the observed levels of lipid accumulation (ORO staining) for each lncRNA knockdown as measured by the Oil Red staining (Fig. 4). Thus, the JSD metric of gene expression phenotypes proves to be a valuable means of quantifying adipogenic phenotypes that is universally applicable to other cell systems and comparisons between biological conditions.

**Discussion**

Thousands of lncRNAs have been identified in mammalian genomes (13–15, 29), but the functions of the vast majority remain elusive. Our study has identified hundreds of lncRNAs that are regulated during adipogenesis, a substantial fraction of which are under the control of the same key transcription factors that activate and repress coding genes during adipocyte differentiation. Of those
lnc-RAP5 and lnc-RAP2

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that we tested, most are specific to adipose tissue, and nearly half of these adipogenic up-regulated lncRNAs that we tested by knockdown are important for the development of mature adipocytes. These data suggest that lncRNAs, like protein coding genes, are likely regulated by the same mechanisms and protein factors that control protein-coding genes in adipogenesis and other developmental processes. Interestingly, loss of several lncRNAs each globally perturbed large portions of the collective adipogenic gene expression signature, suggesting that each of these plays a key role in induction of multiple adipocyte-specific genes.

Although large-scale LOF screens of lncRNAs will offer a wealth of useful information about the importance of lncRNAs in developmental processes, some inherent pitfalls of the RNAi method should be acknowledged. Some antisense lncRNAs may form RNA:RNA intermediates with their sense transcripts, and RNAi at times should be acknowledged. Some antisense lncRNAs may form RNA:DNA antisense oligo (100 nm) were transfected by DharmaFect 2 (final 5 μL/mL according to the manufacturer’s instruction (Dharmacon). Twelve hours after transfection, cells were recovered in full culture media and grown to confluence, and then cells were induced to differentiate. Four days after differentiation, RNAs were harvested for downstream analysis. siRNAs were purchased from GenePharma. siRNA sequences are provided in Fig. 56.

Cell Isolation and Tissue Culture. Primary adipocytes were isolated according to published methods, with few modifications (21, 22). Interscapular brown adipose tissues, epididymal fat pads, and s.c. fat pads were harvested from five 8-wk-old male mice. Fat tissues were minced, digested in collagenase, and fractionated by centrifugation. Adipocytes were collected from the top layer. Brown preadipocytes were isolated from interscapular brown fat, and white preadipocytes were isolated from s.c. fat from young mice (2 wk old). After collagenase digestion and fractionation, preadipocytes, enriched in bottom stromal vascular fractions, were resuspended and cultured to confluence in DMEM supplemented with 10% (vol/vol) newborn bovine serum. The cells were then exposed to differentiation medium: 10% (vol/vol) FBS DMEM, insulin 850 nM (Sigma), dexamethasone 0.5 μM (Sigma), 3-isobutyl-1-methylxanthine (IBMX) 250 μM (Sigma), and rosiglitazone 1 μM (Cayman Chemical) (brown adipocyte cultures, T3 1 nM and indomethacin 125 nM, were also added into the medium). After 2 d, cells were incubated in one new medium containing insulin 160 nM (supplemented with T3 for brown adipocyte cultures) for another 2 d, and then were switched to 10% FBS DMEM.

siRNA Knockdown of lncRNAs. When cultured primary preadipocytes reached 70–80% confluence, siRNAs (200 nM) or modified DNA antisense oligo (100 nm) were transfected by DharmaFect 2 (final 5 μL/mL according to the manufacturer’s instruction (Dharmacon). Twelve hours after transfection, cells were recovered in full culture media and grown to confluence, and then cells were induced to differentiate. Four days after differentiation, RNAs were harvested for downstream analysis. siRNAs were purchased from GenePharma. siRNA sequences are provided in Fig. 56.

Library Preparation and Sequencing. Total RNAs were extracted using a QiaGen kit, and 10 μg of total RNAs for each sample were used to prepare the mRNASeq library according to the manufacturer’s instruction (illumina). cDNA libraries were prepared and sequenced by illumina GAII according to the manufacturer’s instructions.

Differential Expression Analysis of RNA-Seq. Reads from each sample were mapped against the mouse genome (mm9 build) using TopHat (version 1.1.0), using options “–novel-juncs -a -F 0.0”. A splice junction index derived from the combined UCSC and RefSeq mm9 annotations together with previously discovered lincRNA transcript models built using RNA-Seq from several cell lines (39). This set of annotated transcripts was quantitated in each sample using Cuffdiff (27), which estimates transcript and gene expression in each condition using a generative statistical model of RNA-Seq. Cuffdiff displays a key role in induction of multiple adipocyte-specific genes.
calculated the abundances in each condition for all transcripts that maximize the likelihood of observing the reads in the experiment under this model. Cuffdiff allows for adjustment to observed changes to gene expression, and we restricted analysis to genes significantly differentially expressed by at least twofold between preadipocytes and adipocytes. We also required that differentially expressed genes used in downstream analysis were supported by at least 10 reads in either condition.

**ORO Staining.** To prepare ORO solution, 0.5 g ORO (Sigma, catalog no. 0-0625) was dissolved in 100 mL isopropanol, mixed with H₂O at a ratio 6:4, and filtered with Whatman #1 filter paper. Primary preadipocytes were differentiated in six-well plates. Cells were washed with PBS twice and fixed with formalin for 15 min at room temperature. After formalin fix, cells were washed with PBS and stained with freshly prepared ORO solution for 1 h in room temperature. Cells were washed with H₂O to remove the residual ORO.

**Expression Microarray Profiling.** Three replicate RNA samples each from D0 undifferentiated preadipocytes, D4 PPARG-induced adipocytes, and D4 scramble siRNA controls were labeled and hybridized to Affymetrix Mouse 430a2 arrays. Additionally, two replicate RNA samples from each of the 10 IncRNA siRNA studies were labeled and hybridized as well using standard Affymetrix protocols. Data were collected and analyzed using the “affy” package in R/Bioconductor. Briefly, probes were background corrected using the “maa” algorithm. Probe values were quantile normalized, and probe sets were summarized using the average difference of perfect matches only. All significance tests were performed using significance analysis of microarrays (SAM) (40) with Benjamini-Hochberg multiple hypothesis testing correction (MTC). Significant gene lists were selected with a Δ that constrained the FDR <0.5%.

Significant gene expression data were biclustered using the JSD, which is derived from the Shannon entropy

\[ H(p) = - \sum_{i=1}^{n} \ln(p_i) \]

where \( p \) is a discrete probability distribution. The JSD is

\[ \text{JSD}(p, q) = \frac{1}{2} \sum_{i=1}^{n} \left( H(p) + H(q) - H(p+q) \right) \]

where \( p \) and \( q \) are two discrete probability distributions. In our array analysis, these distributions each represent either the relative abundance of all genes in a condition (for condition-level clustering) or the density of a gene's expression across all conditions (for gene-level clustering). Pairwise condition distances reflect an information-theoretic summary of how similar the program of gene expression in two cell states (e.g., preadipocyte and adipocyte). Pairwise gene distances reflect the information-theoretic similarity of expression across all conditions (potentially implying coordinated regulation).

Because only two replicates were available for each knockdown vs. scramble control analysis, rank-ordered lists of all genes were generated and used as input for the nonparametric preranked GSEA analysis (33). Rank-ordered gene lists were compared with all “curated gene sets” (C2) in MSigDB with the following parameters: collapse = True, nom = meanfold, scoring_scheme = weighted, mode = Max probe, set_min = 12, set_max = 500, nperm = 1000, chip = Mouse430a_2.chip.

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