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Interrogation of nonconserved human adipose lincRNAs identifies a regulatory role of *linc-ADAL* in adipocyte metabolism

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SUPPLEMENTARY MATERIALS

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Data and materials availability: The human adipose and ASC adipocyte RNA-seq data files are deposited at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE76404 and GSE95173. Published adipose/adipocyte RNA-seq, ChIP-seq, and mass spectrometry data sets used in bioinformatic analysis are available through Gene Expression Omnibus (GSE27450, GSE54652, GSE65540, and GSE56747), ArrayExpress (E-MTAB-2624), and ProteomeXChange (PXD003095).

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Abstract

Long intergenic noncoding RNAs (lincRNAs) have emerged as important modulators of cellular functions. Most lincRNAs are not conserved among mammals, raising the fundamental question of whether nonconserved adipose-expressed lincRNAs are functional. To address this, we performed deep RNA sequencing of gluteal subcutaneous adipose tissue from 25 healthy humans. We identified 1001 putative lincRNAs expressed in all samples through de novo reconstruction of noncoding transcriptomes and integration with existing lincRNA annotations. One hundred twenty lincRNAs had adipose-enriched expression, and 54 of these exhibited peroxisome proliferator-activated receptor γ (PPAR γ) or CCAAT/enhancer binding protein α (C/EBP α) binding at their loci. Most of these adipose-enriched lincRNAs (~85%) were not conserved in mice, yet on average, they showed degrees of expression and binding of PPAR γ and C/EBP α similar to those displayed by conserved lincRNAs. Most adipose lincRNAs differentially expressed ($n = 53$) in patients after bariatric surgery were nonconserved. The most abundant adipose-enriched lincRNA in our subcutaneous adipose data set, *linc-ADAL*, was nonconserved, up-regulated in adipose depots of obese individuals, and markedly induced during in vitro human adipocyte differentiation. We demonstrated that *linc-ADAL* interacts with heterogeneous nuclear ribonucleoprotein U (hnRNP U) and insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2) at distinct subcellular locations to regulate adipocyte differentiation and lipogenesis.

INTRODUCTION

Obesity and related metabolic dysfunction in adipose tissue increase the risk of type 2 diabetes and cardiometabolic disorders. Previous studies using human adipose tissue and cultured human adipocytes coupled to mechanistic studies in rodent models have provided fundamental insights into regulatory pathways that alter adipocyte metabolism, including brown adipose thermogenesis (1, 2). Yet, a lack of complete understanding of human adipose biology and pathophysiology has limited clinical translation and therapeutic progress in obesity and related disorders. Addressing these knowledge gaps may require functional studies of human-specific, nonconserved regulatory elements in complex human disease. Genome-wide association studies (GWAS) in humans have revealed loci implicated in human obesity and related metabolic traits, many of which are intergenic and likely regulatory (3), that are not apparent from rodent models. Such human discoveries serve as reminders of the known differences between mouse and human adipose biology in the context of genetic regulation such as peroxisome proliferator-activated receptor γ (PPAR γ) genome-wide binding sites during adipogenesis (4) and metabolic functions such as brown adipose thermogenesis (5). Furthermore, recent evolutionary profiling at a genome-wide level reveals marked separation between primates and other species in the presence and

diversity of alternative splicing (6) and noncoding RNA (ncRNA) species (7, 8), regulatory elements now recognized to play species-specific roles in cellular biology and metabolic pathophysiology.

Long intergenic ncRNAs (lincRNAs) are increasingly implicated as regulators of cellular processes and human diseases (9, 10). LincRNAs, a subclass of ncRNAs, are usually more than 200 nucleotides (nt) in length and, like protein-coding mRNAs, are often spliced and polyadenylated at their 3' ends. Compared to mRNAs, lincRNAs are, on average, shorter in length, are more tissue-specific, and have lower expression (11, 12). Through nuclear and cytoplasmic interactions with a variety of proteins and other RNA species, lincRNAs can modulate gene expression and protein function through epigenetic and posttranscriptional mechanisms. They are involved in a variety of biological processes, such as X chromosome inactivation (13), cell pluripotency (14), and differentiation (15). In the context of adipose biology, ~1500 lincRNAs have been detected in mouse white and brown adipose tissue (16). Hundreds of mouse lincRNAs were recently discovered to be regulated during in vitro differentiation of mouse white and brown adipocytes (17), as well as brown adipose activation and white adipose browning (18). Furthermore, adipose lincRNAs may regulate the differentiation of white adipocytes (*Firre*, *ADINR*, and *Paral1*) and brown adipocytes (*Blnc1*, *lnc-BATE1*, and *lnc-BATE10*) by interacting with transcription factors, histone-modifying proteins, or nuclear matrix factors such as heterogeneous nuclear ribonucleoprotein U (hnRNP) (17–23).

Despite these discoveries, there has been comparatively little primary focus on lincRNA discovery and their biology in human adipose tissue. This represents a notable biological and potential clinical blind spot because lincRNAs have rapidly evolved relative to other small ncRNAs and protein-coding genes. Two studies systematically evaluated the evolutionary conservation of lincRNAs across mammals and other vertebrates (7, 8). Strikingly, only ~2000 of a total of ~14,000 human lincRNAs were estimated to be conserved across nonprimate species, with most lincRNA families unique to primates (7, 8). The vast number of nonconserved lincRNAs in humans raises the important question of whether these recently evolved lincRNAs are functional. To date, the few functionally studied adipose lincRNAs have mostly been derived from rodent-based discovery (*lnc-BATE1* and *lnc-BATE10*) or prioritized because of conservation in mouse (*Firre* and *Blnc1*).

Overall, little is known about the functional and clinical relevance of nonconserved, human adipose lincRNAs. Here, we performed deep RNA sequencing (RNA-seq) of subcutaneous adipose biopsy samples from 25 healthy and lean humans. We defined 1001 unique putative lincRNAs present in the adipose tissue of all study participants, most of which were not conserved between humans and mice. We examined these lincRNA loci for signs of functionality including active histone modifications, binding by adipocyte master regulator transcription factors PPAR γ or CCAAT/enhancer binding protein α (C/EBP α), and differential expression after bariatric surgery. As a proof of principle of biological roles for nonconserved lincRNAs, we established a functional role for the most highly expressed adipose-enriched lincRNA we detected, *linc-ADAL* (lincRNA for adipogenesis and lipogenesis), in human adipocyte differentiation and de novo lipogenesis.

RESULTS

Deep RNA-seq identifies unannotated adipose lincRNAs in humans

To establish a comprehensive profile of the human adipose lincRNA transcriptome, we performed deep RNA-seq of gluteal subcutaneous adipose samples biopsied from 25 healthy and lean volunteers [average body mass index (BMI), ~23.9] as previously described (tables S1 and S2 and Fig. 1A) (24). We implemented de novo transcriptome assembly using Cufflinks (25) and identified previously unannotated lincRNAs as well as those previously annotated in GENCODE and the Human BodyMap (11). We then excluded monoexonic transcripts and transcripts shorter than 200 nt in length as these may represent transcriptional artifacts (26). We identified 244 previously unannotated lincRNA loci after removing transcripts with coding potential or which overlapped with any known protein-coding genes, already annotated lincRNAs, small RNAs, or pseudogenes. To identify lincRNAs reliably expressed in adipose tissue, we excluded lincRNAs with expression lower than the first percentile of all adipose tissue coding and noncoding transcripts. We identified 1001 lincRNAs (144 not previously annotated) in subcutaneous adipose tissue of all 25 subjects. In the context of this paper, we term these 1001 lincRNAs reliably expressed in human adipose tissue as “adipose lincRNAs,” recognizing that many of these adipose-expressed lincRNAs may also be expressed in other tissues.

The anatomic distribution of human adipose tissue exhibits strong sexual dimorphism and racial differences (27, 28). Sex-linked differences in the adipose protein-coding transcriptome have been well documented (29). We found that adipose lincRNAs showed specific profiles by sex and race. Of ~2900 lincRNAs found in more than 25% of subjects, 37 and 23 lincRNAs (Fig. 1B and table S3) were differentially expressed between the sexes in individuals of European and African ancestry, respectively [fold change > 2; false discovery rate (FDR)-adjusted $P < 0.05$]. Sex-differentially expressed lincRNAs included those located on autosomal and sex chromosomes. Specific lincRNAs, such as *linc-NUDT10*, on the X chromosome were expressed about twofold higher in females, thus appearing to escape X chromosome inactivation (table S3). Furthermore, 15 and 12 lincRNAs differed (fold change > 2; FDR-adjusted $P < 0.05$) by self-reported race in female and male subjects, respectively (Fig. 1C and table S4). Thus, in support of their physiological relevance (27, 29, 30), the expression of subsets of adipose lincRNAs is influenced by sex and race.

For the remaining analyses, we focused on the 1001 adipose lincRNAs expressed in adipose tissue across all 25 participants (tables S5 and S6). Consistent with previous reports, the average expression of lincRNAs was lower than that of the mRNAs in adipose samples (Fig. 1D). In previously published RNA-seq data from human white and brown adipocytes (31), ~31% of our 1001 adipose lincRNAs were detected, possibly an underestimate of overlap due to the lower sequencing depth of these published data (~35 million reads per sample compared to ~400 million reads per sample in our study) (32). Subcutaneous adipose lincRNAs in our data set had, on average, about twofold higher expression in white versus brown adipocytes (Fig. 1E).

Next, we sought to validate and explore the clinical relevance of these 1001 adipose lincRNAs in an independent human adipose tissue RNA-seq data set (33) that had not yet been analyzed for lincRNA expression. Mapping all known lincRNAs from GENCODE and Human BodyMap along with our newly identified adipose lincRNAs onto this data set, we identified 1175 putative lincRNAs (155 newly annotated) expressed in the abdominal subcutaneous adipose tissue of 22 severely obese patients (Fig. 1F and table S7) (33). Despite differences in adipose depot location, RNA-seq depth, and patient characteristics, ~60% of these abdominal subcutaneous lincRNAs were expressed in our gluteal adipose samples (Fig. 1F). Expression of 53 (~5%) of these lincRNAs (16 newly annotated) was modulated (FDR-adjusted $P < 0.05$; tables S8 and S9, fig. S1A, and Fig. 1G) in the abdominal adipose tissue of patients 3 months after bariatric surgery (46.5 ± 5.6 and 39.9 ± 4.9 , average BMI before and after the surgery, respectively). Coexpression analysis revealed 100 lincRNA-mRNA pairs involving 21 lincRNAs and 56 mRNAs (fold change > 1.5) that were modulated after bariatric surgery in either similar or opposite directions (table S10). Gene ontology analysis demonstrated that the mRNAs in these lincRNA-mRNA pairs associated with bariatric surgery-induced weight loss were enriched in fatty acid metabolism and fibrosis pathways, suggesting potential roles in adipose lipid metabolism and matrix remodeling for weight loss-related lincRNAs (Fig. 1H).

Human nonconserved adipose lincRNAs have active regulatory features

To assess the tissue-specific enrichment of adipose lincRNAs, we calculated the fractional expression of each adipose-expressed transcript (lincRNA or mRNA) by calculating its expression in one specific tissue (for example, adipose) relative to the sum of its expression over 15 tissues in Human BodyMap data as previously described (11, 16). Consistent with previous reports of lincRNA tissue specificity (11, 16), adipose lincRNAs exhibited greater adipose enrichment than mRNAs (Fig. 2A). We identified a set of 120 adipose-enriched lincRNAs (85 previously annotated and 35 newly annotated; table S11) that showed similar or greater fractional expression in adipose tissue than the key adipose transcription factor *PPARG* (adipose fractional expression, ~0.2). In human adipose chromatin immunoprecipitation sequencing (ChIP-seq) data available from the Roadmap Epigenomics Project (34), adipose lincRNAs had enriched active histone modification markers H3K4me3, H3K4me1, and H3K27ac at transcription start sites (TSSs) and lincRNAs with higher expression had stronger histone modification signals (fig. S2A). Using published human adipocyte ChIP-seq data (4), we found 254 *PPARγ* and 321 *C/EBPα* binding sites near the TSS (± 1.5 kb) of 218 (38 previously unannotated) and 262 (40 previously unannotated) adipose lincRNAs, respectively. Of these transcription factor-bound adipose lincRNA loci, 145 exhibited occupancy by both *PPARγ* and *C/EBPα*. Adipose-enriched lincRNAs with *PPARγ* or *C/EBPα* binding had higher expression than other adipose-expressed lincRNAs (Fig. 2B). The distances between these transcription factor binding sites to lincRNA TSSs were inversely correlated with the adipose fractional expression of these transcription factor-bound lincRNAs but not their abundance in adipose tissue (fig. S3). *PPARγ* and *C/EBPα* binding sites were more abundant around adipose-enriched lincRNA loci (45.0%) compared to adipose lincRNAs that were less tissue-specific (31.9%; $P = 0.0053$). Collectively, these findings suggest that adipogenic transcription factors regulate the tissue-enriched expression of a subset of adipose lincRNAs.

Human and primate lincRNAs are undergoing rapid evolution and only ~1000 to 2000 of 14,000 human lincRNAs are estimated to have homologs in nonprimate species including mouse (7, 8). To evaluate species conservation, we focused on synteny, or relative positional conservation in the genome (16), because most homologous lincRNAs exhibit poor sequence conservation but appear in conserved syntenic genomic regions (7, 8). Although ~59% of adipose lincRNA loci have syntenic regions in the mouse genome (Fig. 2C), only ~15% of adipose lincRNA loci had evidence of transcription in mouse adipose tissue at syntenic regions (Fig. 2C) based on public mouse adipose lincRNA annotations (16). We thus defined “nonconserved human adipose lincRNAs” as those without synteny or those with synteny but without evidence of transcription at the mouse syntenic genomic region. Of 53 human adipose lincRNAs that were modulated by bariatric surgery (fig. S1A and Fig. 1G, subset), only eight (15%) appear conserved in mouse, that is, syntenic in the mouse genome as well as having an adipose lincRNA expressed in these regions (table S9). For three of these conserved and likely weight loss–modulated lincRNAs (*SNHG15*, *linc-SLC25A45-5*, and *linc ZCCHC13-2*), the adipose expression of their putative mouse orthologs was modulated in brown adipose activation or white adipose browning (fig. S1B). Although these three lincRNAs are of specific pathophysiological interest, the majority (~85%) of weight loss–modulated and potentially functional lincRNAs (table S9) are not conserved in mouse, including adipose-enriched, PPAR γ -bound *linc-OXCT1-1*, and sex–differentially expressed *linc-NUDT10* (Fig. 1G). This predominant lack of conservation in mouse is also true for sex- and race-associated human lincRNAs (tables S3 and S4).

Nonconserved human lincRNAs also constitute most adipose lincRNAs with PPAR γ or C/EBP α occupancy near their TSS (Fig. 2C). Active chromatin modifications at nonconserved lincRNA loci were generally comparable to those at conserved lincRNAs (fig. S2, B and C), particularly for adipose-enriched lincRNAs with PPAR γ or C/EBP α binding (Fig. 2, D and E). Examples of such nonconserved lincRNAs are shown in Fig. 2F, with expression of these nonconserved lincRNAs validated by quantitative polymerase chain reaction (qPCR) in human adipose stromal cell (ASC)–derived adipocytes (fig. S4, A and B). Similar to known PPAR γ target gene *PLIN1* (perilipin 1), both *linc-OXCT1-1* (a bariatric surgery–modulated lincRNA) and *linc-SWAT-2* (a newly annotated lincRNA) exhibited positive histone modification markers as well as PPAR γ and C/EBP α binding (Fig. 2F). Published CAGE (cap analysis of gene expression) profiling of human adipocytes (35) revealed a progressive increase in the 5' CAGE tag at these nonconserved lincRNAs during adipocyte differentiation (Fig. 2F), suggesting regulated induction of their transcription. Consistent with their proposed regulation by PPAR γ , these lincRNA examples were markedly induced during adipocyte differentiation in vitro and suppressed in mature adipocytes by short-term PPAR γ antagonism (fig. S4C).

***Linc-ADAL*, an abundant adipose-enriched lincRNA, is regulated by PPAR γ in human adipocytes**

Linc-ADAL (Fig. 3A and fig. S5A) was selected for adipocyte functional studies because it is (i) the most abundant adipose-enriched, nonconserved lincRNA in gluteal adipose tissue (table S5) and also ranked among the top first percentile of lincRNAs expressed in abdominal subcutaneous adipose tissue of obese patients (33); (ii) markedly induced during

ASC differentiation to adipocytes (Fig. 3B and fig. S5B); (iii) not detected in human monocytes, macrophages, or endothelial cells (fig. S6A); and (iv) elevated in subcutaneous adipose tissue of obese compared to lean humans (Fig. 3D). *Linc-ADAL* is located between protein-coding genes *AQPEP* and *AP3S1* on chromosome 5. RNAseq, 3' RACE (rapid amplification of cDNA ends), and the 5' TSS identified by CAGE (35) in human adipocytes suggest that *linc-ADAL* has two alternatively spliced isoforms sharing the same promoter (fig. S5A), and isoform 2 is the main adipose transcript (fig. S5B). Phylogenetic information-based codon substitution frequency (PhyloCSF) analyses suggested that the two *linc-ADAL* transcripts had low probabilities of containing open reading frames for coding sequences (PhyloCSF log-likelihood ratio scores of the two isoforms: -16.8 and -243). In vitro transcription/translation assays failed to detect any peptide products, confirming its noncoding status (fig. S6B). ChIP-seq data in human adipocytes revealed high occupancy of H3K4me3 at the *linc-ADAL* locus and multiple PPAR γ and C/EBP α binding peaks as well as H3K4me and H3K27ac histone enhancer marks upstream of its TSS (Fig. 3A). *Linc-ADAL* expression was reduced in mature adipocytes treated with GW9662, a PPAR γ antagonist (Fig. 3C). Collectively, these data suggest that PPAR γ modulates adipocyte-specific expression of *linc-ADAL*. Interrogation of public RNA-seq data (31) revealed that *linc-ADAL* expression was about threefold higher in white than in brown adipocytes, and our qPCR data showed that its expression is comparable in omental and abdominal subcutaneous adipose depots of obese subjects (Fig. 3E). Consistent with many biologically important adipose protein-coding genes (36, 37), subcutaneous adipose *linc-ADAL* expression was higher in females than in males, as demonstrated by qPCR analysis in a larger independent sample ($n = 107$) (Fig. 3F and table S12).

Although the human genetic locus of *linc-ADAL* is syntenic in mouse, no transcript was present in this intergenic region in published data of steady-state (RNA-seq) and nascent [Global run-on sequencing (GRO-seq)] transcriptomes from mouse 3T3-L1 adipocytes or adipose tissues (fig. S6C) (38, 39). Notably, *linc-ADAL* sequence consists of a high proportion of transposable elements (43% long terminal repeat and 42% short interspersed nuclear element), a characteristic of many recently evolved lincRNAs in primates (40, 41).

***Linc-ADAL* regulates adipocyte differentiation and modulates de novo lipogenesis in mature adipocytes**

Linc-ADAL transcripts were equally distributed in the cytoplasm and nucleus of mature human adipocytes (Fig. 4A). To investigate *linc-ADAL* function, we applied locked nucleic acid antisense oligonucleotides (ASO) to ASC adipocytes and achieved substantial *linc-ADAL* knockdown (~70%) in both the nucleus and cytoplasm (fig. S7A). ASO-mediated knockdown of *linc-ADAL* in ASC preadipocytes markedly impaired subsequent adipocyte differentiation (Fig. 4B) and suppressed mRNA and protein expression of *PPARG* and other lipogenic genes (for example, *CEBPA*, *SREBF1*, and *FASN*), strongly suggesting a regulatory role of *linc-ADAL* in adipocyte differentiation (Fig. 4, C to D). In addition, ASO knockdown of *linc-ADAL* in day 7 differentiated adipocytes resulted in a ~50% decrease in triglyceride accumulation and lipid biosynthesis gene expression (for example, *SREBF1*, *FASN*, and *ELOVL6*) but had no effect on *PPARG* expression in mature adipocytes (Fig. 5,

A to C). These results suggest that *linc-ADAL* might play modulatory roles both in adipocyte differentiation and in lipid storage in mature adipocytes.

To probe the mechanism of *linc-ADAL* effects on lipid storage, we examined lipolysis and de novo lipogenesis in differentiated adipocytes. Neither at baseline nor after forskolin stimulation was lipolysis affected by knockdown of *linc-ADAL*, despite reduced mRNA expression of adipocyte triglyceride lipase (*ATGL*) (Fig. 5D). In contrast, using stable isotope ^{13}C -labeled glucose as substrate followed by mass spectrometry, ASO-mediated *linc-ADAL* knockdown in differentiated adipocytes reduced the amount of newly synthesized, ^{13}C -labeled palmitic, stearic, and oleic acids (Fig. 5E) (42), suggesting that *linc-ADAL* regulates lipid accumulation in mature adipocytes by modulating de novo lipogenesis. RNA-seq analysis of *linc-ADAL* knockdown in mature adipocytes revealed ~1200 differentially expressed genes (fold change > 1.5; FDR-adjusted $P < 0.05$). Ingenuity pathway analyses of down-regulated genes upon *linc-ADAL* knockdown suggest modulation of fatty acid oxidation, liver X receptor/retinoid X receptor signaling, cholesterol biosynthesis, and lipid metabolism gene networks (fig. S8, A to C), including uncoupling protein-1 (*UCPI*), a key gene in adipose browning (fig. S8D). Thus, *linc-ADAL* may regulate a broad network of lipid and metabolic programs in human adipocytes.

***Linc-ADAL* interacts with hnRNPU in the nucleus and insulin-like growth factor 2 mRNA binding protein 2 in the cytoplasm**

Relative to ASO-mediated knockdown, short-hairpin RNA (shRNA) repression of *linc-ADAL* expression elicited a more discrete phenotype (fig. S7). Consistent with the known inefficiency of shRNA in knocking down nuclear RNAs (43), cellular fractionation revealed that cytoplasmic but not nuclear *linc-ADAL* transcripts were depleted by shRNA-mediated knockdown (fig. S7A). Administration of shRNA both before differentiation and after 7 days of differentiation resulted in a similar (~40 to 50%) decrease of triglycerides and lipid biosynthesis genes but did not reduce *PPARG* expression (fig. S7, B to E). The phenotypic pattern of *linc-ADAL* knockdown with shRNA relative to ASO suggests that suppression of cytoplasmic *linc-ADAL* alone is sufficient to modulate triglyceride storage in differentiated adipocytes but has limited impact on adipocyte differentiation per se.

The differential effects of shRNA versus ASO knockdown of *linc-ADAL* on *PPARG* expression and adipocyte differentiation raise the possibility of distinct nuclear and cytoplasmic actions of *lincADAL*. LincRNAs can act in cis or in trans to modulate gene expression. Arguing against a cis regulatory function, *linc-ADAL* knockdown does not affect the expression of its nearest-neighbor genes, *AQPEP* and *AP3SI*, or any of the 13 closest protein-coding genes within 1 Mb upstream or downstream of the transcript (fig. S9). Therefore, we sought to identify *linc-ADAL*-interacting proteins that might mediate in trans actions. We performed RNA pulldown in whole-cell lysates of mature ASC adipocytes using biotinylated full-length *linc-ADAL* and green fluorescent protein transcripts (similar in length to *linc-ADAL*) as a negative control. Three unique bands enriched in *linc-ADAL* pulldown samples were analyzed by mass spectrometry (Fig. 6A). Isolated proteins were ranked on the basis of highest unique peptide counts (table S13), revealing the nuclear protein hnRNPU and the cytoplasmic protein IGF2BP2 as two of the highest ranked putative

linc-ADAL-bound partners. RNA pulldown coupled with Western blotting confirmed *linc-ADAL* coprecipitation with hnRNPU and IGF2BP2 (Fig. 6A). Complementary RNA immunoprecipitation (RIP) of endogenous hnRNPU and IGF2BP2 proteins in differentiated adipocytes revealed *linc-ADAL* enrichment by ~10 and ~40-fold compared to an immunoglobulin G (IgG) control, respectively (Fig. 6B). In addition, we found multiple putative binding regions in the *linc-ADAL* transcript matching hnRNPU and IGF2BP2 binding motifs identified through in vivo CLIP (cross-linking immunoprecipitation) sequencing studies (fig. S10) (44, 45). Collectively, these data strongly suggest that *linc-ADAL* interacts with hnRNPU and IGF2BP2 in human adipocytes to form ribonucleoprotein complexes in nuclear and cytoplasmic locations, respectively.

HnRNPU, a nuclear complex protein, was recently shown to interact with three mouse adipose lincRNAs (*Firre*, *lnc-BATE1*, and *Blncl*) to regulate white and brown adipocyte differentiation by lincRNA-mediated gene regulation in trans (16, 19, 46). In human ASC preadipocytes, ASO knockdown of hnRNPU blocked adipocyte differentiation (Fig. 6C) and adipogenic gene expression, including *PPARG* (Fig. 6D), thus reproducing the effects of nuclear *linc-ADAL* knockdown via ASO. In differentiated human ASC adipocytes, hnRNPU knockdown reduced triglyceride accumulation and lipogenic gene expression with modest and inconsistent effects on *PPARG* mRNA expression (Fig. 6, C and D, and fig. S11, A and B), more closely mimicking the effects of ASO-mediated *linc-ADAL* knockdown in differentiated adipocytes.

***Linc-ADAL* modulates IGF2BP2 posttranscriptional regulation of distinct metabolic genes in adipocytes**

IGF2BP2 is a cytoplasmic mRNA binding protein (mRBP) that mediates the posttranscriptional regulation of many genes, including metabolic genes, by enhancing or inhibiting mRNA translation and by increasing mRNA stability (45, 47–50). Photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP) sequencing in human embryonic kidney–293 cells (45) showed that IGF2BP2 binds a few thousand mRNAs, suggesting a wide range of gene targets. However, only a few IGF2BP2 targets, for example, *IGF2* (insulin-like growth factor 2) and *HMGA1* (high mobility group AT-hook 1), have been validated and well studied (49, 50). Furthermore, IGF2BP2 target genes are poorly characterized in human adipocytes.

Knockdown of IGF2BP2 both in ASC preadipocytes and in fully differentiated ASC adipocytes resulted in similar decreases in triglyceride accumulation (~50%) and lipogenic gene expression with minimal impact on *PPARG* mRNA levels (Fig. 6, E and F, and fig. S11, C and D). This recapitulates the effects of shRNA selective knockdown of cytoplasmic *linc-ADAL* (fig. S6). IGF2BP2 knockdown had no impact on *linc-ADAL* abundance in mature ASC adipocytes (fig. S12A), suggesting that IGF2BP2 does not modulate *linc-ADAL* stability and turnover. Recent studies have revealed that some cytoplasmic lincRNAs bind with mRBPs and act as molecular decoys or sponges to regulate the cellular activity of the mRBPs, in turn altering expression of mRBP targets [for example, lincRNA *NORAD* and its mRBP partner PUMILIO (51)]. We confirmed that IGF2BP2 interacts with *IGF2* mRNA and promotes *IGF2* mRNA translation in adipocytes (Fig. 7, A and B); however,

linc-ADAL knockdown had no impact on *IGF2*-IGF2BP2 binding, IGF2 protein abundance, or IGF2 downstream AKT signaling (Fig. 7, A and C, and fig. S12, C and D), suggesting that *linc-ADAL* had little impact on the posttranscriptional regulation of well-established IGF2BP2 targets. These results do not support the idea that *linc-ADAL* has general decoy functions in the regulation of IGF2BP2 activity or bioavailability in adipocytes.

Therefore, we probed alternative modes of action for the *linc-ADAL*-IGF2BP2 ribonucleoprotein complex. PAR-CLIP data revealed IGF2BP2 binding with *PPARA* mRNA, an important transcription factor for lipid metabolism (45). Our RIP assay in human adipocytes showed similar enrichment levels of *PPARA* mRNA to that of *IGF2* mRNA in IGF2BP2 immunoprecipitation samples (Fig. 7A). IGF2BP2 knockdown had no impact on *PPARA* mRNA abundance but increased PPAR α protein in adipocytes, suggesting that IGF2BP2 might suppress *PPARA* mRNA translation (Fig. 7B and fig. S12E). In contrast to its lack of impact on IGF2BP2-*IGF2* mRNA binding, knockdown of *linc-ADAL* in adipocytes increased *PPARA* mRNA interaction with IGF2BP2 (Fig. 7A) while *PPARA* mRNA expression was unaffected (fig. S12E). PPAR α protein abundance was increased by *linc-ADAL* knockdown despite the increase in IGF2BP2-*PPARA* mRNA interaction (Fig. 7, A and C). Consistent with increased PPAR α protein expression, the mRNA abundance of PPAR α target genes *EHHADH* and *SLC27A1* (52) was increased in both *linc-ADAL* and IGF2BP2 knockdown adipocytes (fig. S12F). These findings suggest that IGF2BP2 negatively regulates PPAR α protein expression in human adipocytes and that *linc-ADAL* may be required for IGF2BP2-mediated posttranscriptional regulation of a subset of IGF2BP2 targets in adipocytes (for example, *PPARA*). Our RIP assays and public PAR-CLIP data demonstrated that additional adipocyte genes might be potential targets of IGF2BP2, for example, lipid metabolism genes *ELOVL6* and *SCD* and adiponectin receptor 1 (Fig. 7A). Notably, both *linc-ADAL* and IGF2BP2 knockdown reduced *ELOVL6* mRNA expression in differentiated adipocytes (Figs. 5C and 6F), suggesting that *linc-ADAL*-IGF2BP2 positively regulates *ELOVL6* mRNA abundance. Collectively, these results suggest that *linc-ADAL* modulates IGF2BP2 posttranscriptional regulation of a specific subset of metabolically active target genes in adipocytes (fig. S13). In summary, our data suggest that *linc-ADAL* interacts with two distinct proteins, hnRNPU in the nucleus and IGF2BP2 in the cytoplasm, to modulate adipocyte differentiation and lipid biosynthesis in human adipocytes.

DISCUSSION

Here, we presented a bioinformatic and clinical profile of human subcutaneous adipose lincRNAs and characterized the biological roles of *linc-ADAL*, an abundant, nonconserved, adipose-enriched, human lincRNA associated with obesity. We identified 1001 putative lincRNAs expressed in all participants, 144 newly annotated, including a subset of lincRNAs that were modulated after bariatric surgery. Given the low conservation of human lincRNAs in nonprimate species, these data provide a unique resource for studying lincRNAs, particularly nonconserved ones, in human adipose biology, obesity, and related metabolic diseases. As proof of principle for the functional effects of nonconserved adipose lincRNAs, we demonstrated that *linc-ADAL* modulated adipocyte differentiation of preadipocytes and

de novo lipid biosynthesis in differentiated adipocytes through distinct subcellular interactions with hnRNP in the nucleus and IGF2BP2 in the cytoplasm.

LincRNAs are increasingly recognized as key noncoding regulatory elements in the genome and as important modulators of human cellular functions and pathophysiology. Initial studies of adipose lincRNAs were based on widely used model organisms, for example, mouse adipose lincRNAs (16, 17) and *lnc-BATE1* (20) in mouse brown adipocytes, or used species conservation to prioritize discoveries and functional examination, for example, *Firre* and *Blnc1* (16, 19). However, most human adipose lincRNAs lack sequence conservation or homologs in mouse, underscoring the need for their identification in humans as well as specific functional and translational studies in humans or humanized model systems. By corollary, discovery and functional interrogation of lincRNAs in mouse alone may fail to reveal the biological and clinical relevance of most human lincRNAs in adipose physiology and disease. Recent genetic studies have linked GWAS signals with lincRNA expression and revealed functional nonconserved human lincRNAs at disease-associated loci. For example, long ncRNA *ANRIL* at the 9p21.3 locus has been implicated in coronary heart disease (41, 53), *linc-NFE2L-3* has been implicated in central obesity (54), and *CCAT2* at 8q24.21 has been implicated in colorectal cancer (55). These findings highlight important physiological and disease roles for recently evolved regulatory elements in humans and suggest that many nonconserved lincRNAs are unique and integral parts of human genetic regulatory networks.

One major challenge in human lincRNA research is the incomplete annotation of lincRNAs due to their low abundance and high tissue/lineage specificity (11, 12). Recently, the MiTranscriptome study identified ~46,000 putative lincRNA genes through >7000 RNA-seq data sets of normal and tumor tissues from 18 human organs, highlighting the importance of comprehensive deep RNA-seq analysis in specific cell lineages/tissues for complete lincRNA annotation (56). Through deep RNA-seq of subcutaneous adipose tissue samples in 25 individuals from both African and European ancestry, we detected 4787 potential adipose lincRNAs in at least one person and 1001 lincRNAs present in all 25 participants. Through de novo assembly and bioinformatic triage of adipose transcriptomes, we identified a stringent set of 144 newly annotated adipose lincRNAs reliably expressed in all subjects. In support of their functional relevance, newly annotated lincRNAs exhibited a higher degree of adipose-enriched expression and similar PPAR γ or C/EBP α binding around lincRNA TSSs compared to known lincRNAs. Our analyses provide a detailed bioinformatic annotation of human subcutaneous adipose lincRNAs for functional and translational investigation, most of which are not conserved in rodents. Our data set includes candidates for regulation of obesity and related pathophysiologies, including 54 adipose-enriched, transcription factor-bound lincRNAs, 53 lincRNAs modulated by bariatric surgery, and 49 lincRNAs differentially expressed between sexes that may contribute to the sex dimorphism in human obesity (27, 28) and to sex-linked differences in obesity-linked genetic loci (57).

Previous studies suggested that conserved lincRNAs exhibit genomic synteny but display very limited sequence similarity across species (7, 8). By integrating published mouse adipose lincRNA transcriptomes with synteny analysis at human adipose lincRNA loci, we demonstrated that as few as 15% of human adipose lincRNA loci have syntenic noncoding transcripts expressed in mouse. LincRNAs not conserved in mouse showed roughly

comparable adipose expression, histone modification, and PPAR γ or C/EBP α binding as conserved lincRNAs. PPAR γ , often with C/EBP α , plays a key role in orchestrating gene expression and adipocyte biology, and PPAR γ binding sites are highly enriched in the vicinity of genes induced during adipogenesis (4). Human adipocyte ChIP-seq has shown that only ~3% of PPAR γ and C/EBP α genome-wide binding sites are located in promoters of protein-coding genes in contrast to ~50% at intergenic regions (4). We found 254 PPAR γ and 321 C/EBP α binding sites located near TSSs of 218 and 262 adipose lincRNAs, respectively. In addition, adipose-enriched lincRNAs exhibited about fourfold more PPAR γ and C/EBP α binding sites near their TSS than non-tissue-specific lincRNAs. Up to 80% of highly expressed and PPAR γ and C/EBP α -bound human adipose lincRNAs appear to have no mouse homolog, underscoring the critical need for human data and humanized models for functional interrogation.

We selected an illustrative, nonconserved human lincRNA, *linc-ADAL*, for functional investigation because it was the most abundant adipose-enriched human lincRNA in our data, it was not expressed in mouse adipose or adipocytes, its expression was regulated by PPAR γ , and it is of potential clinical interest because its adipose expression was increased in obesity. *Linc-ADAL* transcripts comprised ~80% transposable element-derived sequences. It is known that such transposable elements in recently evolved nonconserved lincRNAs contribute to diversification, regulation, and potential lineage-specific functions (58). For example, a class of nonconserved lincRNAs, containing up to 90% transposable element sequences, has been found to modulate nuclear reprogramming in stem cells (5). Our clinical studies in independent samples revealed that *linc-ADAL* expression in gluteal subcutaneous white adipose tissue varies by sex and is increased in obesity. Loss-of-function studies via two distinct RNA knockdown methods showed that knockdown of *linc-ADAL* in preadipocytes markedly impaired adipocyte differentiation, whereas knockdown in differentiated adipocytes reduced adipocyte maturity and triglyceride accumulation via suppression of de novo lipogenesis. These findings suggest that *linc-ADAL* may modulate regulatory networks in both adipogenesis and in mature adipocyte lipid-synthetic functions. Although *linc-ADAL* was more lowly expressed in brown adipocytes (31), its knockdown reduced *UCP-1* mRNA expression in a beige/brite adipocyte model, suggesting possible modulatory roles of *linc-ADAL* in brown adipose biology.

LincRNAs can interact with distinct partners in different subcellular locations, such as transcription factors and histone modifying protein complexes in the nucleus and ubiquitin ligases, mRBPs, and microRNAs in the cytoplasm. *Linc-ADAL* is present in both nuclear and cytoplasmic fractions of adipocytes. We identified two distinct *linc-ADAL* protein partners, the nuclear protein hnRNPU and the cytoplasmic RNA binding protein IGF2BP2. Many hnRNP family members interact with lincRNAs to regulate lincRNA gene regulation in trans, for example, hnRNPd-*lnc13* interactions in macrophages (59). As a nuclear matrix factor, hnRNPU was recently revealed to bind with conserved lincRNAs *Firre* and *Blnc1* as well as mouse lincRNA *lnc-BATE1* to mediate white and brown adipocyte differentiation (16, 19, 22, 46). HnRNPU mediates transchromosomal interactions between *Firre* and adipogenic genes (19) and facilitates formation of a ribonucleoprotein complex between *Blnc1* and transcriptional factor EBF2 (46). It is likely that hnRNPU regulates in trans interactions between *linc-ADAL* and specific *linc-ADAL* target genes or transcriptional

regulators through similar mechanisms. HnRNPU also acts as an important mediator of pre-mRNA splicing and specific lincRNAs (for example, *lincGET*) bind with hnRNPU to modulate alternative splicing (60). Detailed investigations beyond the scope of this work, such as genome-wide analysis of transcription and alternative splicing, are required to determine how *linc-ADAL* acts in trans through hnRNPU as a transcriptional regulator and whether it regulates hnRNPU splicing functions. Furthermore, because hnRNPU has now been shown to interact with at least four human adipocyte lincRNAs (*Firre*, *linc-BATE1*, *Blnc1*, and *linc-ADAL*) (16, 19, 46), further studies are required to clarify the specificity and precise functional mechanisms of each validated lincRNA-hnRNPU interaction, as well as their integrated actions, on adipogenesis and adipocyte metabolism.

We focused on the *linc-ADAL*–IGF2BP2 interaction partly because of its novelty as a cytoplasmic program of posttranscriptional regulation in adipocyte metabolism and also because it reflects a unique adipocyte lincRNA activity distinct from other recently identified, nucleus-enriched adipocyte lincRNAs (for example, *Firre*, *linc-BATE1*, and *Blnc1*) that, like *linc-ADAL*, interact with nuclear hnRNPU. Furthermore, genetic variation in *IGF2BP2* has been associated with risk of type 2 diabetes in large GWAS (61). IGF2BP2 can increase the RNA stability of its target mRNAs by inhibiting RNA decay and can also inhibit or activate mRNA translation (49, 50). One of the best-studied IGF2BP2 targets is IGF2, particularly in the context of IGF2/PI3K/AKT antiapoptotic signaling in cancer biology (62, 63). Through PAR-CLIP, thousands of mRNAs, including multiple metabolic genes, have been identified as potential IGF2BP2 targets (45). IGF2BP2 activates the lipogenic targets SREBF1 and ELOVL6 in hepatocytes (47), represses *UCP1* mRNA translation, and modulates many mitochondrial genes to regulate mouse brown adipose function (48). Recently, lincRNAs have been implicated in IGF2BP2 posttranscriptional gene regulation. For example, IGF2BP2-binding ncRNAs, HIF1A-AS2 and *Airn*, promote translation of IGF2BP2 target mRNAs by enhancing their binding with IGF2BP2 (64, 65). In contrast, *LncMyoD* binds with IGF2BP2 and blocks IGF2BP2-mediated translation of proliferation genes to regulate myoblast differentiation (66). One possibility is that *linc-ADAL* may serve as a molecular decoy or sponge to regulate IGF2BP2 bioavailability and function. Such models have been reported; for example, *NORAD*, a cytoplasmic lincRNA, acts as a molecular sponge to regulate the activity of PUMILIO, an mRBP, thus modulating PUMILIO target gene expression (51). However, our data show that the *linc-ADAL* interaction with IGF2BP2 has no impact on *IGF2* mRNA binding with IGF2BP2, IGF2 protein abundance, or insulin-stimulated AKT phosphorylation in human adipocytes. These data suggest that the *lincADAL*–IGF2BP2 interaction has little impact on the posttranscriptional regulation of well-established IGF2BP2 target genes and thus do not support *linc-ADAL* as a broad nonselective molecular decoy for IGF2BP2. In our search for alternative mechanisms, we identified *PPARA*, an important mediator of lipid oxidation, as an IGF2BP2 target gene in human adipocytes and found that *linc-ADAL* is required for IGF2BP2-mediated negative regulation of PPAR α protein expression. Knockdown of *linc-ADAL* increased PPAR α protein expression in human adipocytes, consistent with the observation that brown adipocytes exhibit lower *linc-ADAL* abundance (31) and enhanced expression of PPAR α (67). Our results suggest several additional lipid-metabolism genes, including *ELOVL6* and *SCD*, as IGF2BP2–*linc-ADAL* targets in adipocytes. Thus, *linc-*

ADAL may act as a targeted coregulator of IGF2BP2-mRNA interactions in adipocytes, modulating posttranscriptional cytoplasmic regulation of subsets of IGF2BP2 target genes in adipocytes, specifically genes involved in lipid synthesis and fatty acid oxidation. Overall, our work suggests that *linc-ADAL* integrates signals in both the nucleus and cytoplasm via key target proteins including hnRNPU and IGF2BP2 to modulate human adipocyte differentiation and lipid metabolism.

Our studies provide human-specific mechanistic insights and may be of clinical and translational relevance in obesity and related metabolic disorders. However, several questions remain to be addressed. Our adipose lincRNA profiling was constructed on gluteal subcutaneous adipose from healthy subjects, but it is well established that adipose tissues differ in morphology, transcriptomic regulation, and metabolic functions among depots, even among subcutaneous adipose (68). However, we show that ~70% (695) of our gluteal lincRNAs were expressed in abdominal subcutaneous adipose tissue of obese patients and more than 66% (35 of 53) of lincRNAs modulated by bariatric surgery in abdominal adipose were present in gluteal adipose tissue. Although *PPARG* expression was not affected specifically by cytoplasmic *linc-ADAL* knockdown in adipocytes, the reduction in de novo lipogenesis might be secondary to suppressed *CEBPA* expression or broader impairment of genetic networks involved in adipocyte maturation. Further in-depth investigations concerning target gene specificity, interaction with other coregulators, and genome-wide analysis of transcription and alternative splicing are needed to advance mechanistic insights. Additional study is also required to fully investigate additional adipocyte metabolic functions such as browning, thermogenesis, and adipocyte hyperplasia or hypertrophy. In vivo mechanistic studies of nonconserved lincRNAs are not technically straightforward because rodent models are not easily applied. This emphasizes both the challenge and opportunity in understanding recently evolved, nonconserved genomic regulatory features including alternative splicing, enhancer elements, and lincRNAs. Molecular insights from human cell systems, for example, gene editing in human-induced pluripotent stem cells (69), human-relevant in vivo models such as human adipocyte transplant in mouse models (70), bacterial artificial chromosomes (71), and nonhuman primate studies, as well as human Mendelian randomization approaches and direct genome-based human therapeutic targeting (72), are all required for a complete physiological understanding and clinical translation of human adipose lincRNAs.

In summary, we found that most lincRNAs in human adipose tissue were not conserved in mouse, yet many of these nonconserved adipose lincRNAs showed evidence of active regulation by PPAR γ and C/EBP α or were modulated by sex, race, or weight loss. We showed that *linc-ADAL*, an abundant, nonconserved human adipose lincRNA, is associated with obesity and modulates adipocyte differentiation and de novo lipogenesis through interaction with distinct nuclear and cytoplasmic protein partners. These findings support the functional and potential clinical significance of recently evolved, noncoding regulatory elements in the human genome.

MATERIALS AND METHODS

Detailed Materials and Methods are provided in the Supplementary Materials.

Study design

The objectives of this study were to characterize lincRNAs expressed in human subcutaneous adipose tissue and to seek evidence of roles for nonconserved lincRNAs in human adipocyte biology and pathophysiology. First, deep RNA-seq and de novo transcriptome assembly of gluteal subcutaneous adipose tissue was performed in 25 lean and healthy Genetics of Evoked responses to Niacin and Endotoxemia (GENE) study (24) participants (see below). We focused on adipose lincRNAs expressed in all 25 individuals and interrogated sex and race-related differences in lincRNA expression. We used a separate study of 22 obese patients with adipose RNA-seq data before and after bariatric surgery (33) to identify adipose lincRNAs modulated by weight loss in humans. Next, we integrated published human tissue RNA-seq and ChIP-seq data sets into our analysis and identified a set of lincRNAs exhibiting adipose tissue enrichment as well as PPAR γ and C/EBP α -regulated expression. Mechanistic studies examined the role of *linc-ADAL*, the most abundant adipose-enriched and nonconserved lincRNA in our data set, in adipocyte metabolism. In vitro adipocyte studies were repeated four to five times with at least triplicate samples per experimental group. Detailed description of bioinformatic analyses and experimental procedures is available in Supplemental Materials and Methods.

LincRNA identification in human adipose

To identify annotated lincRNAs from Human BodyMap (11) and GENCODE v19 present in our human gluteal adipose RNA-seq data, we first merged all the annotated lincRNA transcripts. Briefly, we first filtered single-exonic transcripts and transcripts less than 200 bp from GENCODE and Human BodyMap data sets. We then extended each lincRNA transcript by 1 kb and filtered the ones that overlapped an mRNA. These steps generated 7939 lincRNA transcripts in Human BodyMap and 8685 lincRNA transcripts in GENCODE v19 with 1887 overlapping transcripts. For overlapping lincRNA transcripts, we used their annotation from GENCODE v19 in our adipose lincRNA identification pipeline. Our merged annotated human lincRNA data set consisted of 7578 lincRNA genes (2331 Human BodyMap, 4030 GENCODE, and 1217 overlapped lincRNAs). To filter possible coding transcripts, we evaluated the coding potential of these annotated lincRNAs using bioinformatics tools (HMMER-3, CPAT, and PhyloCSF) and published human adipose proteome mass spectrometry profiles (see details below). Next, lincRNA expression was estimated by Cufflinks v2.1.1 (25). LincRNAs expressed in adipose tissue were defined as those with FPKMs (Fragments Per Kilobase of transcript per Million mapped reads) above the first percentile of all adipose transcripts, including protein-coding mRNAs. We focused primarily on the set of lincRNAs expressed in all 25 subjects. To identify previously unannotated lincRNAs, we merged 25 adipose RNA-seq files to improve the sensitivity of transcriptome reconstruction for low-abundance lincRNA transcripts. After de novo assembly by Cufflinks v2.1.1 (25), we excluded transcripts shorter than 200 nt and with only a single exon because they have a greater probability of being transcriptional noise (26). We further removed transcripts with coding potential or overlapping any known protein-coding genes, small RNAs, and pseudogenes (Supplementary Materials and Methods).

Statistical analysis

For adipocyte experiments, numeric outcomes are summarized as means $\pm$ SEM. Adipocyte experimental groups were compared using unpaired Student's *t* test (two groups) or ANOVA followed by Dunnett's or Tukey's test for pairwise comparisons (more than two groups). Analyses were performed using GraphPad Prism. Statistical significance was defined as $P < 0.05$. Other experimental procedures and bioinformatic analyses are described in the Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Global discovery of lincRNAs in human adipose tissue.

(A) Overview of human lincRNA identification pipeline in gluteal subcutaneous adipose tissue. CPAT, coding potential assessment tool; MS, mass spectrometry. (B and C) Venn diagram of differentially expressed (DE) adipose lincRNAs that differ by sex (B) and race (C). EA, European ancestry; AA, African ancestry. (D) Average abundance of protein-coding genes ($n = 14,642$) and lincRNAs ($n = 1001$) in gluteal subcutaneous adipose tissue. (E) Average expression of adipose lincRNAs in human white ($n = 314$) and brown ($n = 311$) adipocytes. Expression analyses were performed on published RNA-seq data sets of human white and brown adipocytes (31). (F) Venn diagram of lincRNAs identified from gluteal subcutaneous (subQ) adipose tissue of lean subjects ($n = 25$; BMI ~ 24) and abdominal subcutaneous adipose tissue of severely obese subjects ($n = 22$; BMI ~ 46) (33). (G) Heatmap representation of differentially expressed lincRNA examples (fold change > 1.5; FDR-adjusted $P < 0.05$) in abdominal subcutaneous adipose tissue of obese patients before (BMI ~ 46.5) and 3 months after bariatric surgery-induced weight loss (BMI ~ 39.9). †LincRNAs that were previously unannotated. (H) Enriched gene pathways for mRNAs identified from differentially expressed lincRNAs-mRNA coexpression analysis in abdominal subcutaneous adipose after bariatric surgery-induced weight loss. The dot and bar plots denote the P values and fold enrichment of the indicated gene ontology terms and

pathways, respectively. Wilcoxon rank sum tests were performed on lincRNA and mRNA expression data (D and E).

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Fig. 2. Transcription of a subset of human adipose lincRNAs is actively regulated despite their poor conservation.

(A) Expression of adipose lincRNAs and mRNAs across 15 tissues from Human BodyMap RNA-seq data sets. Color intensity represents the fractional expression levels in each tissue, calculated as previously described (10). (B) Expression of adipose-enriched lincRNAs with PPAR γ or C/EBP α transcription factor (TF) occupancy near TSSs (± 1.5 kb) and other adipose lincRNAs. (C) Categorization of human adipose lincRNAs and subgroups of adipose-enriched lincRNAs based on locus synteny and mouse adipose lincRNA expression in mouse syntenic regions. Human lincRNAs with mouse lincRNAs annotated in syntenic regions defined as “conserved.” (D) The average adipose abundance and (E) the average H3K4me3, H3K4me1, and H3K27ac ChIP-seq enrichment per 100 base pair (bp) bins within ± 3 kb of TSSs for transcription factor-bound, adipose-enriched lincRNAs based on conservation in mouse. (F) Examples of nonconserved adipose lincRNAs with transcription factor binding near TSSs. 5' CAGE tags near TSSs from FANTOM5 data demonstrate induction of these lincRNAs during adipocyte differentiation. *PLIN1*, a PPAR γ target gene, is shown for comparison. Top tracks depict known genes/lincRNAs and de novo noncoding

transcripts assembled by Cufflinks. Average expression is compared among different lincRNA subsets using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test (B and D).

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Fig. 3. Inducible, adipose-enriched *linc-ADAL* expression is regulated by PPAR γ .

(A) *Linc-ADAL* locus on human chromosome 5 (chr5) exhibits strong transcription by RNA-seq coverage, transcription factor binding, and active histone modification markers in human adipose tissue and adipocytes. (B) *Linc-ADAL*, *PPARG*, and *CEBPA* expression during in vitro differentiation of human ASC adipocytes. ** $P < 0.01$ compared to differentiation day 0. (C) Suppression of *linc-ADAL* expression by short-term PPAR γ antagonist GW9662 treatment (10 μ M, 12 hours) in mature adipocytes. (D) *Linc-ADAL* expression in abdominal subcutaneous adipose tissue of obese ($n = 40$; BMI ~ 48) humans compared to gluteal subcutaneous adipose from lean ($n = 60$; BMI ~ 24) humans. (E) *Linc-ADAL* in paired abdominal subcutaneous and visceral (Vis) omental adipose of obese subjects ($n = 40$; BMI ~ 48). (F) Sex-differential expression of *linc-ADAL* in gluteal subcutaneous adipose of healthy lean subjects ($n = 51$ to 56 subjects per group). qPCR data are normalized by reference gene *ACTB* expression. Unpaired *t* tests were used to compare

variables between two groups; statistical differences among more than two groups were assessed by one-way ANOVA and Dunnett's multiple comparison test.

Fig. 4. Depletion of *linc-ADAL* markedly impairs adipocyte differentiation.

(A) qPCR quantification of *linc-ADAL* in cytoplasmic and nuclear fractions of mature ASC adipocytes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Oil red O staining and triglyceride (TG) content in control and ASO-mediated *linc-ADAL* knockdown ASCs after 14-day adipogenic induction. (C and D) Lipogenic gene (C) protein and (D) mRNA expression in control and ASO-mediated *linc-ADAL* knockdown ASCs. qPCR data are normalized using reference gene *ACTB* for relative gene expression. Data are expressed as the mean $\pm$ SEM from at least three independent experiments and assessed by one-way ANOVA and Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$ compared to control group.

Fig. 5. *Linc-ADAL* modulates de novo lipogenesis in differentiated human adipocytes.

(A) Triglyceride content in mature ASC adipocytes after ASO-mediated *linc-ADAL* knockdown. (B and C) Lipid metabolism gene (B) protein and (C) mRNA expression in control and ASO-mediated *linc-ADAL* knockdown ASC adipocytes. (D) Lipolysis in control and ASO-mediated *linc-ADAL* knockdown differentiated adipocytes with or without forskolin. (E) De novo lipogenesis in control and ASO-mediated *linc-ADAL* knockdown differentiated adipocytes. Newly synthesized adipocyte fatty acids were extracted and quantified by mass spectrometry after 24-hour incubation with ^{13}C -labeled glucose. qPCR results are normalized using reference gene *ACTB* for relative gene expression. Data are expressed as the mean $\pm$ SEM from at least three independent experiments and assessed by one-way ANOVA and Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$ compared to control group.

Fig. 6. *Linc-ADAL* regulates adipocyte differentiation and lipid synthesis by interacting with hnRNPU and IGF2BP2.

(A) RNA pulldown assay with adipocyte whole-cell lysate identifying hnRNPU and IGF2BP2 as *linc-ADAL* binding proteins. Silver-stained protein bands specific to *linc-ADAL* pulldown were used for protein identification by mass spectrometry (top). IGF2BP2 and hnRNPU were confirmed by immunoblot (bottom). (B) qPCR quantification of *Linc-ADAL* from retrieved RNAs in RIP assays using whole adipocyte lysates and IGF2BP2- or hnRNPU-specific antibodies. (C and D) Triglyceride accumulation and lipogenic gene expression in ASC adipocytes with knockdown (KD) of hnRNPU before or 7 days (D7) after adipogenic induction. (E and F) Triglyceride accumulation and lipogenic gene expression in ASC adipocytes with KD of IGF2BP2 before or 7 days after adipogenic induction. qPCR results were normalized using reference gene *ACTB* for relative gene expression. Data are expressed as the mean $\pm$ SEM from at least three independent experiments and assessed by one-way ANOVA and Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$ compared to control group.

Fig. 7. *Linc-ADAL* modulates IGF2BP2-mediated posttranscriptional regulation of specific metabolism genes in adipocytes.

(A) Enrichment of known IGF2BP2 targets and other IGF2BP2-binding mRNAs in IGF2BP2 RIP samples from adipocyte lysates with or without *linc-ADAL* KD. (B and C) Protein expression of IGF2 (a known IGF2BP2 target gene) and PPAR α in adipocytes with IGF2BP2 KD (B) and with *linc-ADAL* KD (C). Data are expressed as the mean $\pm$ SEM from at least two independent experiments and assessed by two-way ANOVA followed by Tukey's multiple comparison test (A) and one-way ANOVA followed by Dunnett's multiple comparison test (B and C). * P < 0.05, ** P < 0.01 compared to IgG or control group; $^{\dagger}P$ < 0.05 control versus *linc-ADAL* KD IGF2BP2 RIP.