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Long Noncoding RNA *ADINR* Regulates Adipogenesis by Transcriptionally Activating *C/EBPα*

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SUMMARY

C/EBPα is a critical transcriptional regulator of adipogenesis. How *C/EBPα* transcription is itself regulated is poorly understood, however, and remains a key question that needs to be addressed for a complete understanding of adipogenic development. Here, we identify a lncRNA, *ADINR* (adipogenic differentiation induced noncoding RNA), transcribed from a position ~450 bp upstream of the *C/EBPα* gene, that orchestrates *C/EBPα* transcription in vivo. Depletion of *ADINR* leads to a severe adipogenic defect that is rescued by overexpression of *C/EBPα*. Moreover, we reveal that *ADINR* RNA specifically binds to PA1 and recruits MLL3/4 histone methyl-transferase complexes so as to increase H3K4me3 and decrease H3K27me3 histone modification in the *C/EBPα* locus during adipogenesis. These results show that *ADINR* plays important roles in regulating the differentiation of human mesenchymal stem cells into adipocytes by modulating *C/EBPα* in *cis*.

INTRODUCTION

Analyses of the human transcriptome using tiling arrays (Cheng et al., 2005; Kapranov et al., 2007) and RNA-sequencing (RNA-seq) technology (Cabili et al., 2011; Guttman et al., 2009) have revealed that the entire genome is extensively transcribed as RNA. Substantial fractions of these transcripts are relatively long, apparently do not encode proteins, and are commonly termed "long noncoding RNAs" (lncRNAs). However, the functions of the lncRNAs are not yet well understood. One point of view, in fact, suggests that they mostly represent "transcriptional noise" (Ponjavic et al., 2007; Struhl, 2007). Nonetheless, an increasing body of recent evidence suggests that specific lncRNAs play a regulatory role in numerous cellular processes, including stem cell pluripotency (Guttman et al., 2011; Loewer et al., 2010), cell-cycle regulation (Hung et al., 2011), cell development and differentiation (Klattenhoff et al., 2013; Kretz et al., 2012), and human disease pathogenesis (Wapinski and Chang, 2011). Other studies have documented that lncRNAs recruit chromatin modification complexes involved in gene silencing or gene activation. For example, the lncRNAs *Xist* (Zhao et al., 2008) and *HOTAIR* (Rinn et al., 2007; Tsai et al., 2010) physically associate with Polycomb repressive complex 2 (PRC2) in the *cis* or *trans* form (Guttman and Rinn, 2012) and specifically repress target genes by modulating histone H3 lysine 27 trimethylation (H3K27me3) at various sites along the genome. Similarly, the lncRNAs *HOTTIP* (Wang et al., 2011) activate *HOXA* genes by recruiting the TrxG: MLL1 (Trithorax group activator:Mixed lineage leukemia 1) complex to chromatin, which leads to increased H3K4 trimethylation (H3K4me3) at the *HOXA* locus. A very recent study reported that the lncRNA *ecCEBPA*, which is transcribed in the same direction as the *C/EBPα* gene, interacts with DNMT-1 to block *C/EBPα* gene methylation in leukemic cell lines (Di Ruscio et al., 2013).

Obesity is an important risk factor for various diseases, particularly heart disease, diabetes, hypertension, and cancer (Friedman, 2000). Thus, understanding the molecular mechanism that regulates adipogenesis would be expected to facilitate the development of methods for the treatment of obesity and other adipogenic-differentiation-related disorders. In recent years, human mesenchymal stem cells (hMSCs) have been widely used as an alternative model for the study of human adipogenesis. In contrast to human pre-adipocytes, hMSCs are multipotent, and there is less individual variability between donor-derived hMSCs (Janderová et al., 2003; Pittenger et al., 1999). Over the past 2 decades, transcriptional regulation of adipogenesis has been shown to involve an elaborate network of transcription factors that coordinate the expression of several hundred proteins (Farmer, 2006). *PPARγ* and *C/EBPα* are considered to be the two principal adipogenic transcription

factors in this network, positively regulating each other's expression and cooperating in the control of adipogenesis (Rosen et al., 2002). Nevertheless, the detailed molecular mechanism that activates the expression of *PPARγ* and *C/EBPα* during adipogenesis is not yet known. Although several lncRNAs are differentially regulated during adipogenesis (Kikuchi et al., 2009; Sun et al., 2013), none have been identified to directly participate in the genetic control of adipogenic differentiation. Here, we reveal that, during adipogenic differentiation, an uncharacterized noncoding RNA (*ADINR*) activates the *C/EBPα* gene in *cis* by impacting H3K4me3 and H3K27me3 histone modification of the *C/EBPα* locus. These findings have major implications for obesity and human disease.

RESULTS

ADINR Is a lncRNA Upregulated during Adipogenesis

To explore whether lncRNAs are involved in the adipogenic differentiation of hMSCs, we designed a customized microarray to probe the expression profiles of 39,303 human transcripts that have been annotated as potential noncoding RNAs (see Experimental Procedures). The expression profiles were probed on days 0, 3, and 6 of adipogenic differentiation. Analysis showed that 1,423 annotated or potential lncRNAs were up- or downregulated by >2-fold during adipogenic differentiation (Student's t test, false discovery rate [FDR] < 0.2; Figure 1A; Table S1). Of these, 37.7% (536) showed reduced expression during differentiation relative to uninduced cells, while the remaining lncRNAs (887) were highly induced at an early (day 3) and/or a late (day 6) stage of adipogenic differentiation. We focused on an uncharacterized RNA transcript, *ADINR*, whose genomic location suggested that it was divergently transcribed from a position ~450 bp upstream of the *C/EBPα* gene (Figure 1B). *ADINR* exhibited significantly increased expression on days 3 and 6 ($p < 0.02$) compared to undifferentiated hMSCs. Sequence analysis of the *ADINR* locus revealed it to be highly conserved in rhesus macaque, mouse, dog, and elephant (Figure S1A). Furthermore, qRT-PCR showed that *ADINR* expression increased 20- to 30-fold on days 3 and 6 relative to day 0 (Figure 1C), confirming the microarray data. The qRT-PCR results further showed that *ADINR* and *C/EBPα* are co-expressed during adipogenic differentiation (Figure 1C). Analysis performed by the 5′ and 3′ rapid amplification of cDNA ends (RACE) suggested that the *ADINR* locus produces two polyadenylated, unspliced transcripts (Figure 1D; Figure S1B). Isoform A has an ~190-nt-long extension at the 3′ end relative to isoform B, the latter being the major form as determined by RACE (Figure 1D), and the primers used are listed in Figure S1C. Additional RT-PCR analyses of multiple human cell lines suggested that *ADINR* is expressed in a number of tissues (lung, liver, colon, etc.) and not restricted to hMSCs and adipocytes (Figure S1D). RNA-seq data from ENCODE indicates that expression of the *ADINR* and *C/EBPα* genes is correlated in all nine cell lines tested (Figure S1A). Single-molecule RNA fluorescence in situ hybridization of *ADINR* showed that the transcript is exclusively localized in the nucleus of hMSCs and day-3 differentiated cells (Figure 1E).

ADINR Regulates the Adipogenesis Program In Vitro and In Vivo

To study the functional role of *ADINR* during adipogenesis, we used small interfering RNAs (siRNAs) to knock down *ADINR* transcripts on day 0, followed by culture in hMSC medium (adipogenesis d0) or adipogenic medium for 3 days (adipogenesis d3). Knockdown of *ADINR* with two independent siRNAs markedly reduced the expression of *C/EBPα* and *PPARγ*, both at mRNA and protein levels on day 0 and day 3 (Figures 2A–2C), suggesting that *ADINR* RNA plays a key role in the coordinated activation of *C/EBPα* and *PPARγ* during adipogenic differentiation. We further transfected the siRNAs (#1) and depleted *ADINR* in hMSCs on day 0, followed by 8 days of adipogenic differentiation. Notably, knockdown of *ADINR* resulted in a dramatic adipogenic defect, as shown by the decreased number of oil red O^+ cells and reduced adipogenic transcripts *C/EBPα*, *PPARγ*, fatty acid binding protein 4 (Fabp4) and lipoprotein lipase (LPL; Figures 2D and 2E; Figures S2A and S2B). To exclude the off-target effects of the siRNAs for *ADINR* knockdown, we also performed more knockdown assays using RNase-H-based antisense oligonucleotides (ASO). Likewise, the ASO-targeted *ADINR* could not only downregulate the expression of *C/EBPα*, *PPARγ*, FABP4, and LPL (Figure 2F) but also decrease the number of oil red O^+ cells (Figure 2G).

Subsequently, we tested whether the adipogenic defect could be rescued by ectopic expression of the *ADINR* RNA. To eliminate the possibility that "adipogenic rescue" was caused by the ectopic *ADINR* RNA annealing to the siRNA (#1) and titrating it away, hMSCs were sequentially transfected with the siRNA (#1) on day 0 and then infected with a lentivirus expressing a mutant *ADINR* RNA with four mutations in the siRNA target site (Figure 2H). After dexamethasone treatment for adipogenic differentiation, we found that the ectopic mutant *ADINR* exhibited scant rescue of the severe adipogenic defects caused by the depletion of endogenous *ADINR* (Figure 2I). This rescue assay only resulted in an ~1.2- to ~1.3-fold increase in the expression of *C/EBPα* mRNA as well as other adipogenic markers. However, the adipogenic deficiency was readily rescued by lentivirus-mediated ectopic expression of *C/EBPα* (Figures 2I and 2J; Figure S2C). In addition, ectopic

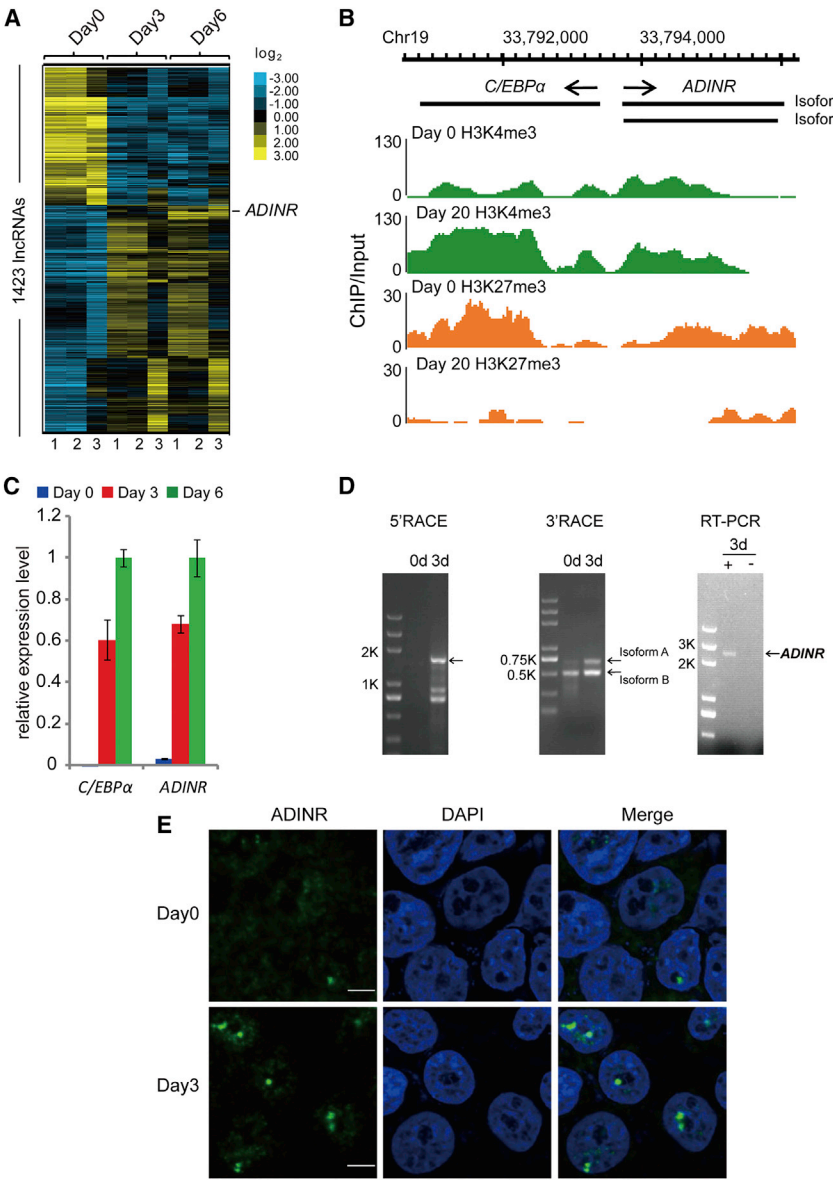

Figure 1. lncRNA *ADINR* Is Upregulated during Adipogenic Differentiation

(A) Mean-centered, hierarchical clustering of 1,423 differentially ($\geq$2-fold) expressed (two-tailed, paired Student's t test, FDR < 0.2), previously annotated noncoding RNAs on days 0, 3, and 6 of adipogenic differentiation. The microarray data are from three independent biological replicates. NC, negative control.

(B) ChIP-seq analysis of H3K4me3 and H3K27me3 at the *C/EBPα* and *ADINR* loci in adipose-derived hMSCs on day 20 of adipogenic differentiation relative to the undifferentiated cells (day 0). The data were obtained from the Roadmap Epigenomics Project.

(C) qRT-PCR analysis of *C/EBPα* and *ADINR* expression across three time points (days 0, 3, and 6) of adipogenic differentiation. The relative expression levels after normalizing to the amount of *GAPDH* signal in each sample are shown. qPCR data are presented as the mean ± SD in three independent experiments.

(D) 5′ and 3′ RACE and RT-PCR assays detecting full-length *ADINR* RNA in undifferentiated (0d) and 3-day adipogenic-differentiated (3d) hMSCs. The longest bands (arrows) for *ADINR* RNA in the RACE assays were indicated. Through sequencing the PCR product of 5′ RACE, we found that the two shorter bands are non-specific PCR products. +, RT-PCR using DNase-treated 3d total RNA; -, PCR using DNase-treated 3d total RNA (no RT; negative control).

(E) Single-molecule RNA fluorescence in situ hybridization shows greatly increased abundance of *ADINR* molecules during adipogenic differentiation, and *ADINR* RNA is exclusively localized in the nucleus of hMSCs and day-3 differentiated cells. Scale bars, 50 μm.

See also Figure S1.

overexpression of wild-type *ADINR* barely stimulated *C/EBPα* expression and also failed to facilitate the adipogenic differentiation of hMSCs as efficiently as did overexpression of *C/EBPα* (Figures 2K and 2L; Figure S2D). Together, these data suggest that *ADINR* RNA is inactive in *trans* but that it promotes adipogenesis by activating *C/EBPα* transcription in *cis*.

ADINR Interacts with MLL3/4 Complexes to Regulate *C/EBPα* Expression

In hMSCs, the *PPARγ* promoter is enriched in H3K4me3 and depleted of H3K27me3 (Figure S3A). In contrast, the *C/EBPα* promoter is enriched for overlapping domains of H3K4me3 and H3K27me3, a histone modification pattern associated with "poised/bivalent" regulatory sequences (Bernstein et al., 2006). During adipogenic differentiation, H3K4me3 is increased markedly on the *C/EBPα* promoter, while H3K27me3 is decreased, suggesting that the *C/EBPα* gene is transcriptionally activated (Figure 1B). In addition, two other epigenetic markers, histone H3K9me3 and CpG DNA methylation, which are generally associated with gene repression, were present at only low levels and exhibited almost no change during differentiation (Figures S3B and S3C).

Next, we addressed whether *ADINR* regulates *C/EBPα* by impacting H3K4me3 and H3K27me3 marks at the *C/EBPα*

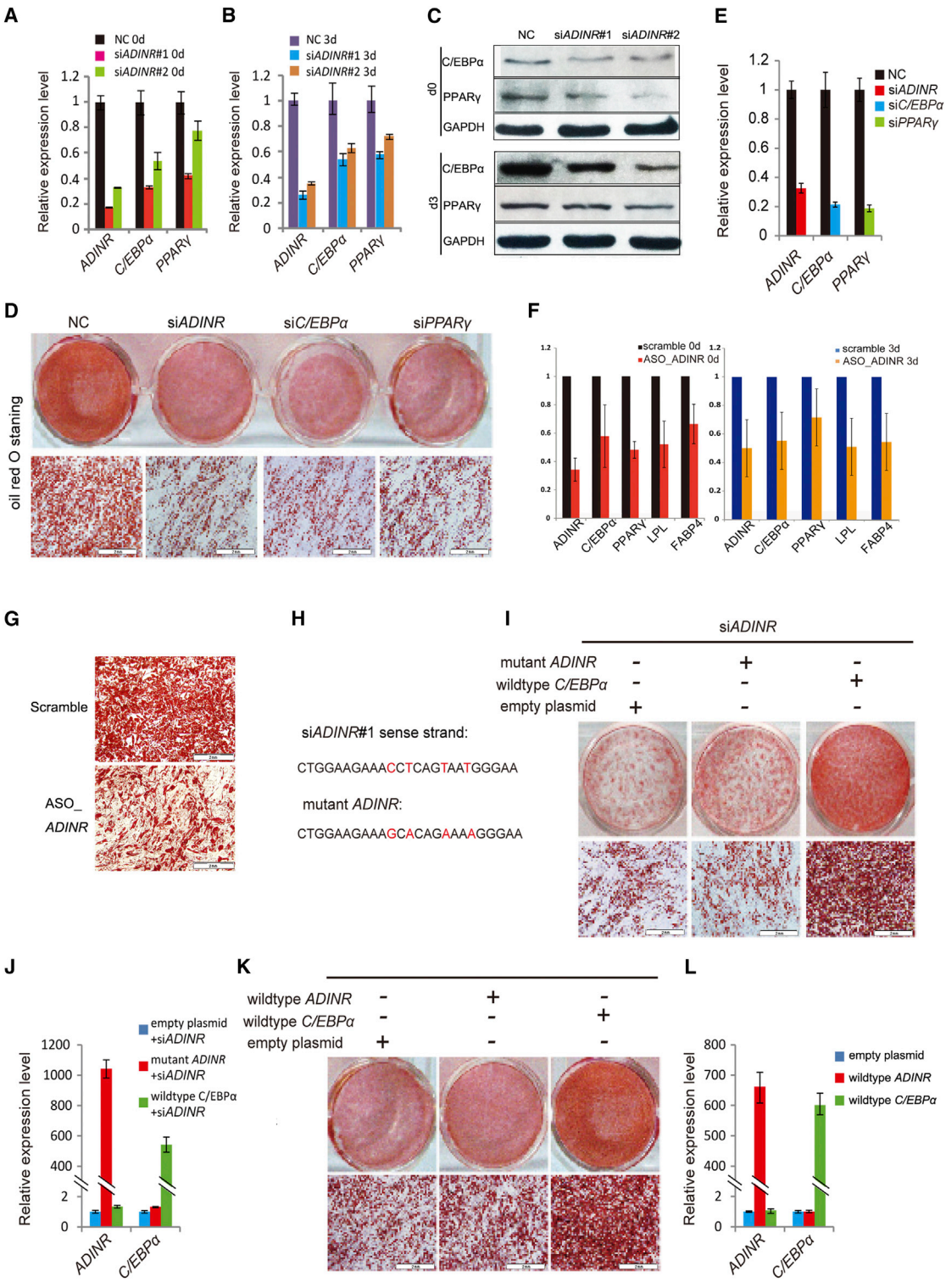

Figure 2. *ADINR* RNA Depletion Represses Adipogenic Differentiation

(A and B) qRT-PCR analysis of *ADINR* RNA, *C/EBPα*, and *PPARγ* after knockdown of *ADINR* RNA using two independent siRNAs (si*ADINR*#1 and si*ADINR*#2) on day 0 (A) and day 3 (B) of adipogenic differentiation.

(C) Western blot of *C/EBPα* and *PPARγ* after knockdown of *ADINR* RNA using two independent siRNAs (siADINR#1 and siADINR#2) on day 0 and day 3 of adipogenic differentiation.

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locus. Chromatin immunoprecipitation (ChIP) assays for days 0 and 3 showed that knockdown of *ADINR* significantly reduced H3K4me3 and increased H3K27me3 in the promoter and coding regions of *C/EBPα* (Figure 3A). This indicates that *ADINR* is involved in the maintenance of H3K4me3 and the removal of H3K27me3 in these regions during adipogenic differentiation. Previous studies in mammals have documented the existence of at least six types of SET-domain-containing lysine methyltransferases that are responsible for H3K4me3 (Mohan et al., 2012). These histone methyltransferases interact with a core complex including WDR5 and RBBP5 in order to form a set of three complexes abbreviated as MLL1/2, MLL3/4, and SET1A/B (Figure S3D). To identify which of the complexes is essential for the expression of *C/EBPα* and *ADINR* RNA, we used siRNAs to knock down the specific protein in each of the three complexes (i.e., Menin, PTIP, and WDR82, respectively). Changes in levels of C/EBPα and *ADINR* were then used as readouts for the function of these complexes in regulation of adipogenic transcription. Knockdown of PTIP, a component of the MLL3/4 complexes, significantly reduced the expression of *C/EBPα* and *ADINR* on days 0 and 3 (Figure S3E), while knockdown of Menin and WDR82 had no detectable effect on C/EBPα or *ADINR* expression (Figures S3F and S3G). Knockdown of MLL3 and MLL4 indicated that both proteins are required for *C/EBPα* expression on day 3, whereas only the knockdown of MLL4 markedly altered *C/EBPα* expression on day 0 (Figures S3H and S3I). Collectively, these results point to an association between the MLL3/4 complexes and the regulation of *C/EBPα* and *ADINR* expression. This finding is consistent with previous reports in mouse adipocytes that PTIP and MLL4 are recruited to the *C/EBPα* promoter during preadipocyte differentiation (Cho et al., 2009).

To identify whether there is a molecular relationship between *ADINR* and MLL3/4 complexes, we demonstrated that we could retrieve endogenous *ADINR* RNA by immunoprecipitation of endogenous PTIP and WDR5 from hMSCs on day 3 (Figure 3B). Subunits of MLL1/2 (Menin) and SET1A/B (CXXC1), however, did not precipitate with *ADINR* RNA. These observations indicate that *ADINR* transcript binds to one or more subunits of the MLL3/4 complex but not MLL1/2 and SET1A/B. To further investigate this interaction of *ADINR* with MLL3/4, we used an RNA pull-down assay that uses in-vitro-transcribed biotin-labeled full-length *ADINR* isoform A and histone H2A mRNA (control) probes. The *ADINR* probe specifically retrieved MLL3/4 subunits (i.e., PTIP, PA1, and WDR5), but not Menin or CXXC1 (Figure 3C). Together, these results confirm that the *ADINR* RNA associates with the MLL3/4 complexes to regulate *C/EBPα* expression.

PA1 Is Recruited to the *C/EBPα* Promoter by Specifically Binding to *ADINR*

To identify subunits of MLL3/4 complexes that directly interact with *ADINR*, six forms of in-vitro-purified glutathione S-transferase (GST) fusion protein were used in binding assays with total RNA from adipogenic-induced hMSCs on day 3. In contrast to recombinant GST-RBBP5, GST-WDR5, GST-PTIP, GST-WDR82, and GST-Menin, GST-PA1 was able to specifically bind to the endogenous *ADINR* RNA (Figure 4A). The functional studies had indicated that *ADINR* RNA regulates the *C/EBPα* gene in *cis*. To confirm that *ADINR* regulates the *C/EBPα* gene by recruiting the MLL3/4 complexes to the *C/EBPα* promoter, we performed ChIP assays on PA1, in the presence or absence of *ADINR*. The results showed that PA1 is recruited to the *C/EBPα* promoter on days 0 and 3, and the knockdown of *ADINR* significantly inhibits the binding of PA1 to the *C/EBPα* promoter (Figure 4B). These findings indicate that the MLL3/4 complexes are recruited to the *C/EBPα* promoter by the binding of PA1 to *ADINR* RNA during adipogenic differentiation. To localize the PA1 binding site on *ADINR* RNA, seven truncated probes from *ADINR* isoform A were used for GST-PA1 in-vitro-binding assays, ultimately

(D) Adipogenesis assay of hMSCs (oil red O staining) after knockdown of the *ADINR* (si*ADINR*#1), *C/EBPα*, and *PPARγ* genes, respectively, on day 6 of adipogenic differentiation (control: scrambled siRNAs).
(E) qRT-PCR analysis of gene knockdown effects after adipogenesis.
(F) qRT-PCR analysis of *ADINR* RNA, *C/EBPα*, and *PPARγ* after knockdown of *ADINR* RNA using ASO on day 0 and day 3 of adipogenic differentiation.
(G) Oil red O staining after knockdown of the *ADINR* using ASO on day 6 of adipogenic differentiation.
(H) Sequences of the *ADINR* mutant.
(I) Rescue assay for *ADINR* RNA-deficient adipocytes on day 6 of adipogenic differentiation. The cells were first transfected with the siRNA#1 for *ADINR* RNA and then infected with lentivirus-expressing mutant *ADINR* RNA and *C/EBPα* mRNA.
(J) qRT-PCR analysis of *ADINR* and *C/EBPα* expression after adipogenesis.
(K) Overexpression assay for *ADINR* and *C/EBPα* adipocytes on day 6.
(L) qRT-PCR analysis of *ADINR* and *C/EBPα* expression after adipogenesis.
All of the expression levels derived via qPCR were normalized to *GAPDH* and are presented as the means ± SD in three independent experiments. Scale bars, 2 mm.
See also Figure S2.

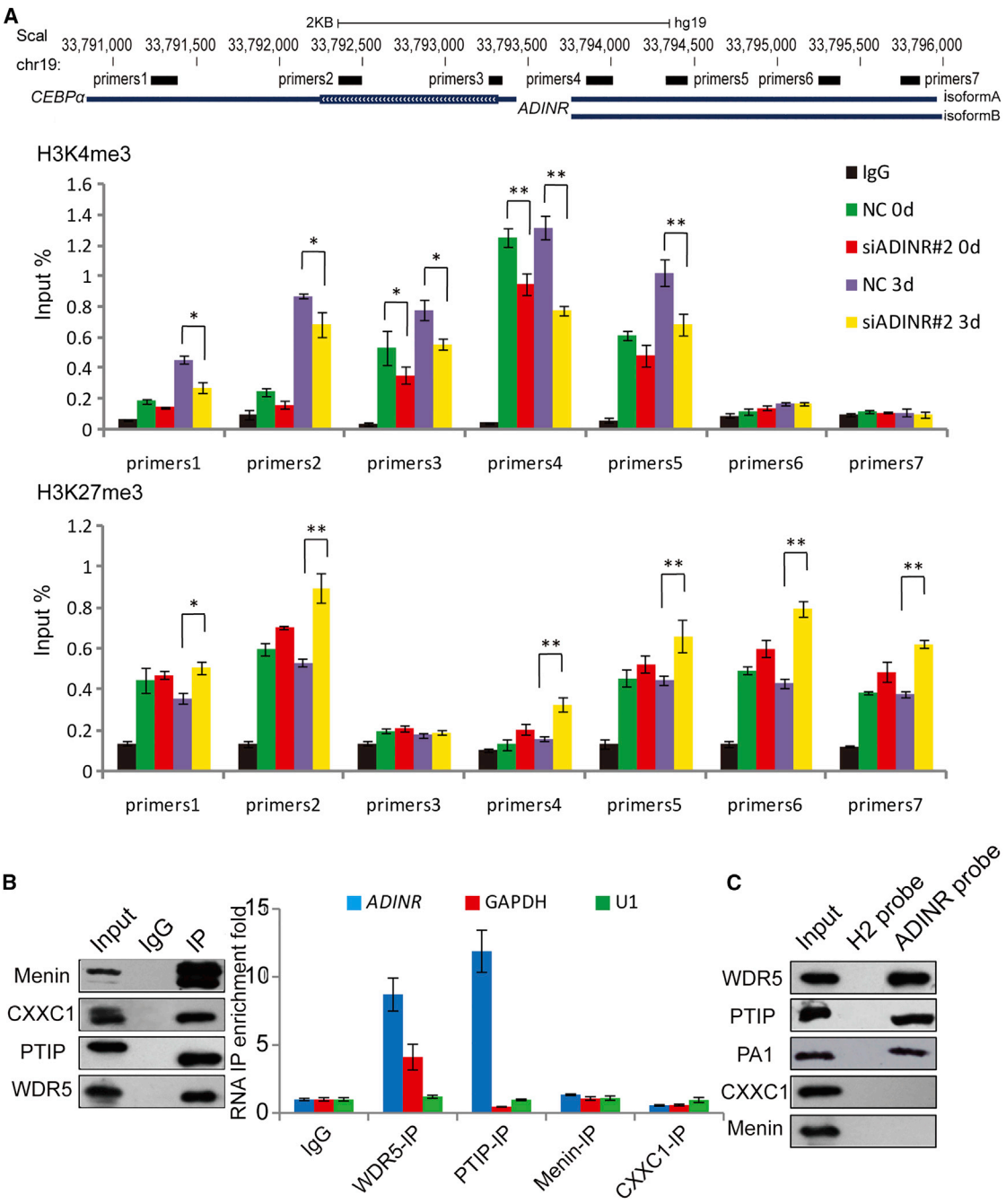

Figure 3. *ADINR* RNA Is Required for the Active Chromatin State of the *C/EBPα* and *ADINR* Loci

(A) ChIP assay of H3K4me3 and H3K27me3 at the *C/EBPα* and *ADINR* loci. The qPCR data are presented as the mean ± SD in six independent experiments. *p < 0.05; **p < 0.01. NC, negative control.

(B) RNA immunoprecipitation (IP) of endogenous WDR5, PTIP, Menin, and CXXC1 in human adipocytes after 3 days of differentiation. The retrieved RNA was quantified by qRT-PCR (negative controls: U1 and GAPDH RNAs). Approximately 2 μg of cell lysate was loaded as the input control. Error bars represent the mean ± SD in three independent experiments.

(C) Immunoblot analysis after RNA pull-down assay of in-vitro-transcribed *ADINR* RNA and histone2 mRNA.

See also Figure S3.

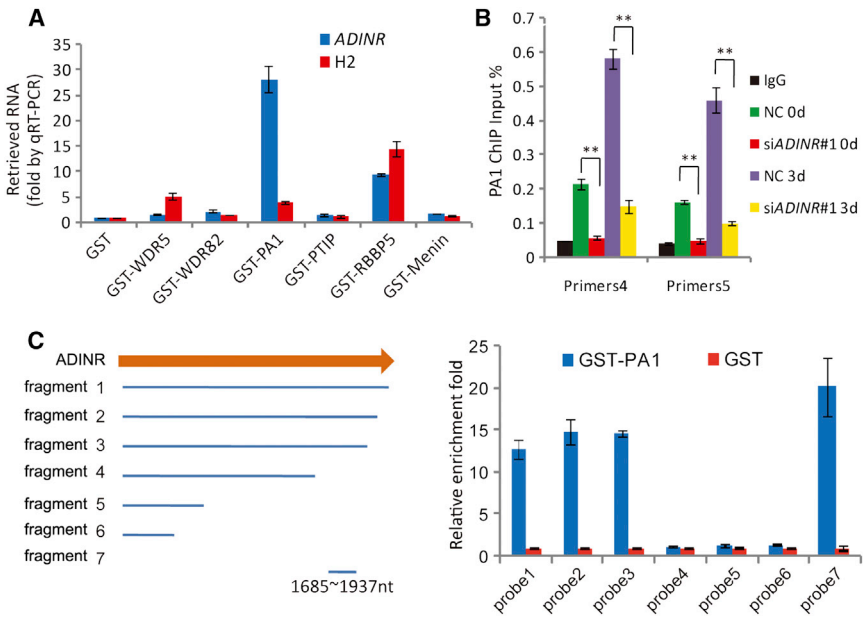

Figure 4. *ADINR* RNA Regulates Active Chromatin via Binding to PA1

(A) RNA pull-down with six different GST-conjugated proteins (WDR5, WDR82, PA1, PTIP, RBBP5, and Menin) from the adipocyte total RNA on day 3. Only GST-PA1 specifically retrieves *ADINR* RNA.

(B) ChIP assay of PA1 in the *C/EBPα* promoter region. Knockdown of *ADINR* RNA significantly abrogates the peaks of PA1 occupancy in the region of primers4 and primers5 (see Figure 3A). **p < 0.01, Student's t test.

(C) Affinity of seven truncated ADINR RNA fragments for GST-PA1 (in vitro binding assay). All the panels show means ± SD in three independent experiments.

See also Figure S4.

indicating that the PA1-binding activity mapped between nucleotides 1685 and 1937 (Figure 4C), a region that includes a LINE repeat element conserved in mammals (Figure S4A).

DISCUSSION

In this report, the long intergenic noncoding RNA *ADINR* was found to regulate the adipogenesis of hMSCs by in *cis* recruitment of the trithorax complex (MLL3/MLL4) to the *C/EBPα* promoter region. We show that the abundance of *ADINR* was increased during the differentiation from hMSC to adipocyte. This phenomenon indicates that *ADINR* may play a key role in the complicated process.

It was reported that *C/EBPα* is a critical transcriptional modulator of adipocyte differentiation and adipogenesis (Farmer, 2006). In this study, downregulation of *ADINR* can downregulate the level of *C/EBPα* via acting on the latter's promoter to impact transcription. Thereby, gene expression cascades involved in adipocytes differentiating into hMSCs are activated and result in adipogenesis. Our findings suggest that *ADINR* is an uncharacterized transcriptional regulator of *C/EBPα*, which shed light on how lncRNA coordinates with key transcriptional factors to regulate downstream gene expression and hMSCs differentiation.

In the human genome, more than 2,000 lncRNAs are bidirectionally transcribed within 2 kb of the transcription start sites of protein-coding genes (Sigova et al., 2013). Many of them are co-expressed with the protein-coding genes (Hung et al., 2011). However, the regulatory functions of these lncRNAs that are associated with the promoters or enhancers of protein-coding genes could be different. Several studies have addressed the roles of these promoter-associated antisense transcripts that could directly regulate the expression of nearby genes by various mechanisms (Hsieh et al., 2014; Pandey et al., 2008), while other studies have shown that some divergent lncRNAs and protein-coding gene pairs may share the same upstream transcriptional network but contribute to the same cellular response independently (Grote et al., 2013; Musahl et al., 2015).

Previous mapping of histone marks in embryonic stem (ES) cells has frequently found overlapping domains of H3K4me3 and H3K27me3 at the promoter regions of developmental regulators (Bernstein et al., 2006). These "bivalent" domains poise the activity of genes encoding developmental transcription factors in pluripotent cells and, during early differentiation, resolve into an H3K4me3-activated state or an H3K27me3-repressed state. During adipogenic differentiation, knockdown of the ADINR dramatically affects the histone marks of both H3K4me3 and H3K27me3 at the promoter of C/EBPα, indicating that the ADINR exerts its functions by modulating this bivalent region. A previous study (Janowski et al., 2005) showed that transfection of siRNA may directly regulate chromatin changes by hybridizing to chromatin DNA, which might be an alternative mechanism through which the siRNAs (#1 and #2) can directly affect the C/EBPα expression. Although we cannot absolutely exclude this possibility, the consistent results that have been shown by three types of knockdown approaches (siRNA, short hairpin RNA [shRNA], and ASO) against ADINR could

make it more convincing that this lncRNA plays essential roles in regulating adipogenesis. The precise molecular mechanism that underlies regulation of these specific histone variations is not fully understood, but information from this study clearly implicates the role for this lncRNA.

The MLL3/4 complexes contain a histone H3K27 demethylase (i.e., UTX) that might be responsible for the removal of H3K27me3 at the *C/EBPα* and *ADINR* loci during adipogenic differentiation. Moreover, MLL4 is required for H3K4 mono- and di-methylation and partially redundant with MLL3 during cell differentiation (Lee et al., 2013); at the same time, MLL3/4 plays a critical part in adipogenesis by enriching H3K4me3 on *PPARγ* and *C/EBPα* promoters (Cho et al., 2009). Our findings suggest that the *ADINR* RNA exerts its effect by acting in concert with the functionally uncharacterized PA1 protein so as to serve as a link between the MLL3/4 complexes and the chromatin, thereby activating *C/EBPα* expression (Figure S4B). Recently, the lncRNAs *HOTTIP* (Wang et al., 2011), plus other RNAs with an enhancer-like function (Kim et al., 2010; Ørom et al., 2010), have been shown to regulate nearby genes in *cis*. Investigation of these RNAs should provide further clues as to whether there is widespread chromatin remodeling associated with RNA recruitment of enzymes that regulate gene expression.

In summary, we identified a lncRNA, named *ADINR*, that functions as a significant positive modulator in hMSC differentiation. Grounded on our findings, we proposed an action model for *ADINR* (Figure S4B). In this model, *ADINR* governs *C/EBPα* transcriptional activity in *cis* by affecting the level of H3K4me3 in the promoter region of *C/EBPα*, and this government occurs through the association of *ADINR* with PA1, which is a member of the histone methylation complex MLL3/4.

EXPERIMENTAL PROCEDURES

RNA Extraction and Microarray Hybridization

The cells on days 0, 3, and 6 during differentiation were harvested, and total RNA was extracted with TRIzol (Invitrogen). Each time point has three replicates. Total RNAs were hybridized using mRNA-lncRNA-combined microarray (CapitalBio).

Plasmids and Antibodies

The following plasmids have been used in this study: pCDH-EF1-MSCV-GFP-Puro (SBI, CD711B-1) and pGEX-6P-1 (Addgene). Antibodies: mouse and rabbit immunoglobulin G (IgG) (sc-69786 and sc-66931) were from Santa Cruz Biotechnology. anti-PTIP (A300-369A and A300-370A), anti-PA1 (A301-978A and A301-979A), anti-Menin (A300-105A), and anti-CXXC1 (A303-161A) were ordered from Bethyl Laboratories. anti-WDR5 (ab56919), anti-H3K4me3 (ab8580), and anti-H3K27me3 (ab6002) were from Abcam.

Cell Isolation and Culture

Human adipose tissue was obtained from patients undergoing liposuction according to procedures approved by the Ethics Committee at the Chinese Academy of Medical Sciences and Peking Union Medical College. Fresh liposuction tissue was collected, digested, and isolated according to an established method (Cao et al., 2005). The cells were then cultured with hADSC culture medium containing DMEM/F-12, MCDB-201, 2% fetal bovine serum, 1× insulin transferrin selenium, 10^{-8} M dexamethasone, 10^{-4} M ascorbic acid 2-phosphate, 10 ng/ml EGF, 10 ng/ml PDGF-BB, and 1 ng/ml Activin A. See also the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO GSE57593 (http://www.ncbi.nlm.nih.gov/geo/).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.09.007.

AUTHOR CONTRIBUTIONS

T.X., L.L., Y.S., H.L., R.C.Z., S.D., and R.C. designed experiments, T.X. analyzed microarray data. T.X., L.L., Y.S., H.L., T.L., and S.W. performed experiments. T.X. drafted the manuscript. L.L., H.L., and S.D. revised the article, which all authors edited and approved. R.C.Z., S.D., and R.C. directed and supervised the research.

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Stem Cell Reports, Volume *5*

Supplemental Information

Long Noncoding RNA *ADINR* Regulates Adipogenesis by Transcriptionally Activating *C/EBP*α

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Figure S1, related to Figure 1. Expression and conservation analysis of *ADINR*.

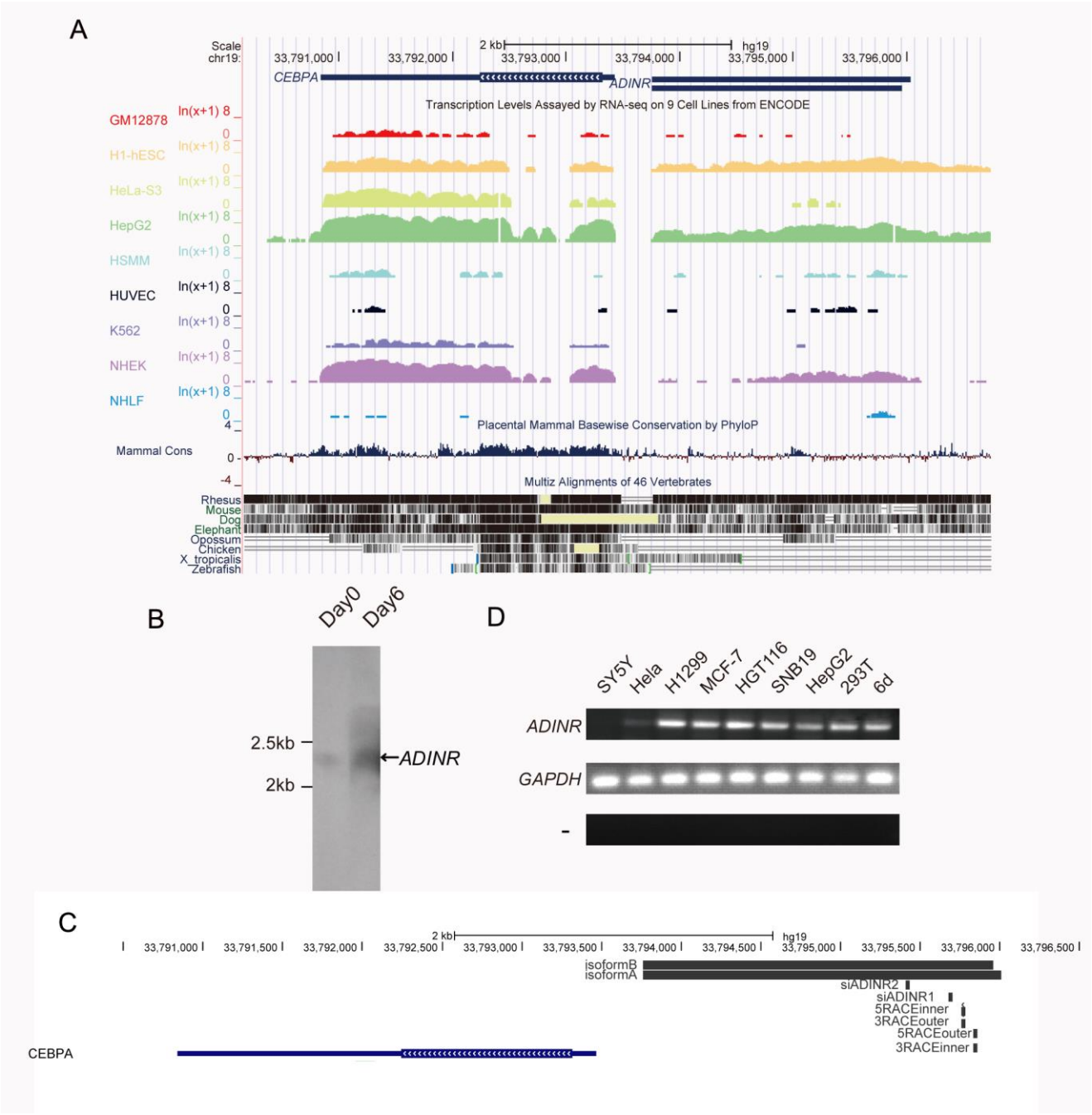

Figure S1, related to Figure 1. Expression and conservation analysis of *ADINR*.

(A) Public data for expression and conservation analysis of *C/EBPα* and *ADINR* genes are shown. (B) Northern blot analysis suggests that *ADINR* RNA ranges from ~2.3-kb to ~2.4-kb and is up-regulated in day 6 adipogenic differentiated cells. (C) RACE primers and siRNA target region in the genomic locus. (D) RT-PCR assays detecting *ADINR* RNA in 9 different cell lines. “6d” denotes 6 day adipogenic differentiated hMSCs. *GAPDH* mRNA was used as positive control, and (-) indicates PCR using DNase-treated total RNAs (no RT; negative control).

Figure S2, related to Figure 2. ADINR Regulates the Adipogenesis *in vitro* and *in vivo*.

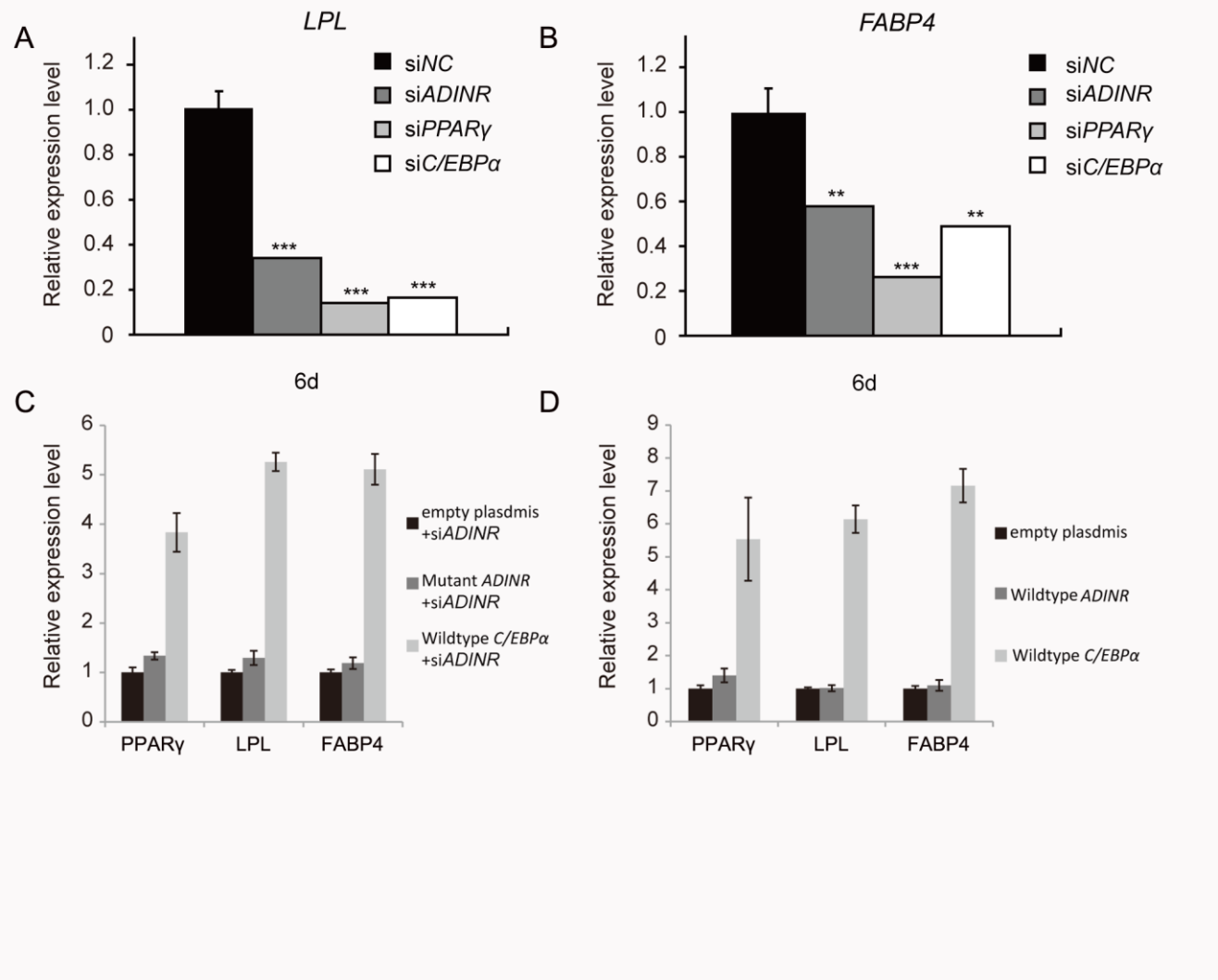

Figure S2, related to Figure 2. The expression of adipogenic marker genes.

(A and B) The expression of two adipogenic marker genes was repressed after knockdown of *ADINR*, *C/EBPα* and *PPARγ*. (C) qRT-PCR analysis of *PPARγ*, *LPL* and *FABP4* expression after transfected with the siRNAs for *ADINR* RNA, and subsequently infected with lentivirus expressing mutant *ADINR* RNA and *C/EBPα* mRNA. (D) qRT-PCR analysis of *PPARγ*, *LPL* and *FABP4* expression after overexpression of wildtype *ADINR* RNA and *C/EBPα* mRNA. The qPCR data are presented as mean ± s.d. in 3 independent experiments.

Figure S3, related to Figure 3. Epigenetic analysis of the *PPARγ*, *C/EBPα* and *ADINR* loci.

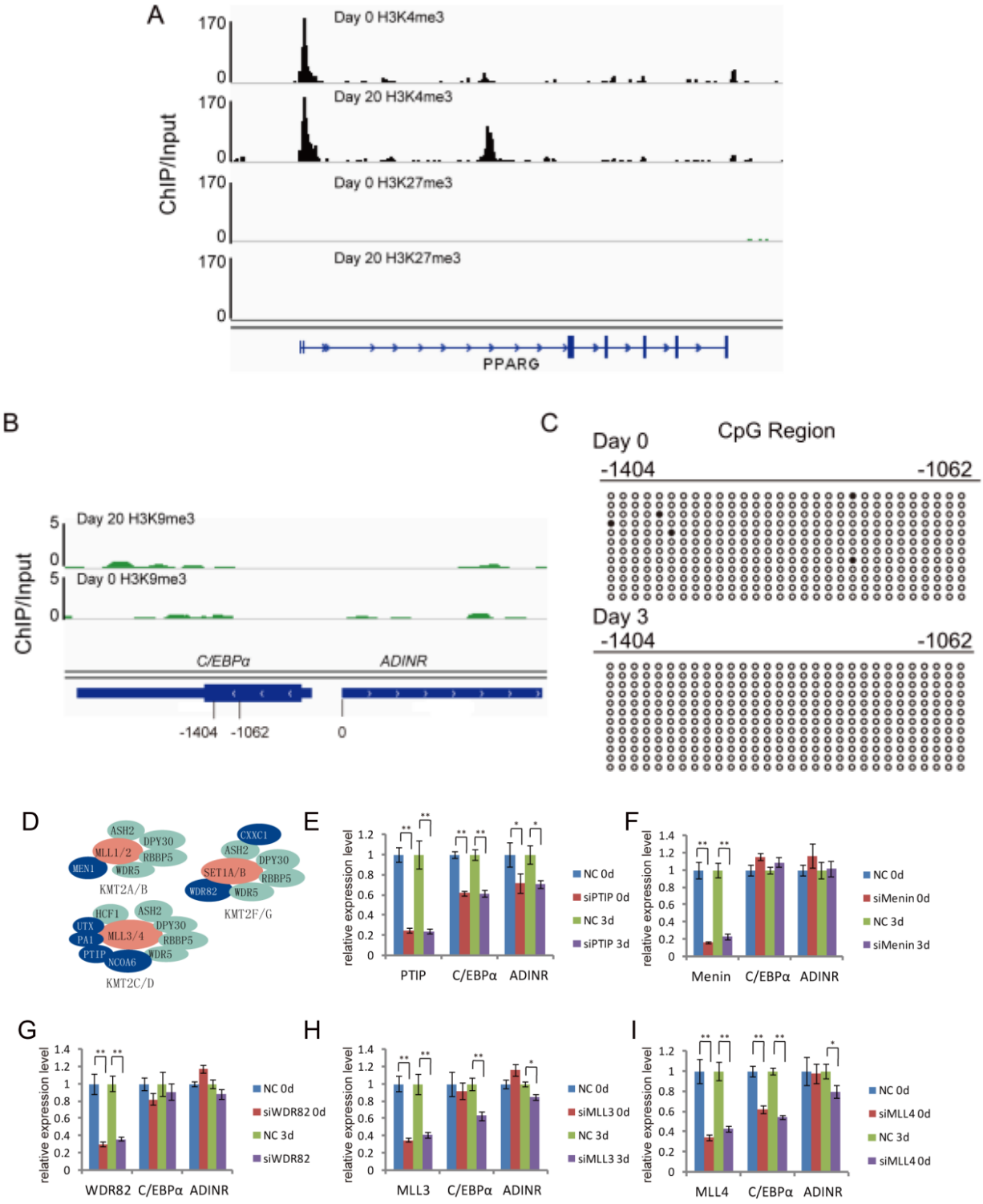

Figure S3, related to Figure 3. Epigenetic analysis of the *PPARγ*, *C/EBPα* and *ADINR* loci.

(A) ChIP-Seq analysis of H3K4me3 and H3K27me3 at the *PPARγ* locus in human adipose-derived mesenchymal stem cells at day 20 of adipogenic differentiation relative to undifferentiated cells (day 0). (B) ChIP-Seq analysis of H3K9me3 in human adipose-derived mesenchymal stem cells and at day 20 of differentiated human adipocytes. (C) Bisulfite sequencing of the *ADINR* promoter region in human

adipose-derived mesenchymal stem cells and at day 20 differentiated human adipocytes. Each row represents one bacterial clone with one circle symbolizing one CpG. Top numbers indicate the position of the CpGs relative to the *ADINR* TSS. (D) Six MLL/Trithorax (Trx) family members of SET-domain-containing lysine methytransferases are shown. (E-G) Knockdown of each the unique protein of these three complexes (Menin, unique to MLL1/MLL2 complexes; PTIP, unique to MLL3/MLL4 complexes; and WDR82 , unique to the Set1A/Set1B) at day 0 and day 3 of adipogenic differentiation, showed that only PTIP significantly regulated *C/EBPα* gene induction during adipogenesis. (H-I) Both MLL3 and MLL4 genes affect *C/EBPα* gene expression during adipogenesis. The relative expression levels after normalizing to the amount of *GAPDH* signal in each sample are shown. The qPCR data are presented as mean ± s.d. in 3 independent experiments.

Figure S4, Related to Figure 4. *PA1* interacts with *ADINR* to regulate *CEBPA*.

Figure S4, Related to Figure 4. *PA1* interacts with *ADINR* to regulate *CEBPA*.

(A) Genomic locus of PA1 protein binding site for *ADINR*. (B) Model of *ADINR* RNA inducing *C/EBPα* gene expression during adipogenesis.

Table S2. List of primers, Related to Figures 1-4.

Primer	Sequence	Application
ADINR Probe_F	GGCTGGAAAACTTTCTTTATAATTACTT	northern
ADINR Probe_R	TAATACGACTCACTATAGGGGCGTCCCTCGCATTCTTTACC	northern
Menin_qF	AGCTGGCTGTACCTGAAAGGATCA	qPCR
Menin_qR	AATGGAAGGGTTGATGGCACACAC	qPCR
WDR82_qF	TTTCCACCAACGGCAGCTTCATTC	qPCR
WDR82_qR	TGGATCTTGCCATCCTCTGAACCA	qPCR
PTIP_qF	TTTCACTGGATTCGAGCCTGTCCA	qPCR
PTIP_qR	CACTTTGCTGGCAATGAGGTGTGT	qPCR
MLL3_qF	TGCATCCTACAGCTGCTGAGAACA	qPCR
MLL3_qR	AAACCCATGGATGGGACATCTGGA	qPCR
MLL4_qF	ACACACTGATCTCCTGGATGGCAA	qPCR
MLL4_qR	GAACCAATGAGCACGTTGATGGCA	qPCR
ADINR_qF	AGGGTGGATGTGCTGTGATGAAGA	qPCR
ADINR_qR	AGTCCATAACACCTCCGCAGACAA	qPCR
CEBPA_qF	ATTGCCTAGGAACACGAAGCACGA	qPCR
CEBPA_qR	TTTAGCAGAGACGCGCACATTCAC	qPCR
PPARG_qF	GGAGCCCAAGTTTGAGTTTGCTGTG	qPCR
PPARG_qR	CAGGGCTTGTAGCAGGTTGTCTTG	qPCR
FABP4_qF	AGCACCATAACCTTAGATGGGG	qPCR
FABP4_qR	CGTGGAAGTGACGCCTTTCA	qPCR
LPL_qF	ACAAGAGAGAACCAGACTCCAA	qPCR
LPL_qR	AGGGTAGTTAAACTCCTCCTCC	qPCR
U1_qF	ATACTTACCTGGCAGGGGAG	qPCR
U1_qR	CAGGGGAAAGCGCGAACGCA	qPCR
GAPDH_qF	AATCCCATCACCATCTTCCA	qPCR
GAPDH_qR	TGGACTCCACGACGTACTCA	qPCR
siADINR#1 sense	GGAGGAAGGAGGAGACAGAAGGAdGdT	siRNA
siADINR#1 antisense	ACUCCUUCUGUCUCCUCCUUCCUCCCA	siRNA
siADINR#2 sense	CUGGAAGAAACCUCAGUAAUGGGdAdA	siRNA
siADINR#2 antisense	UUCCCAUUACUGAGGUUUCUUCCAGUC	siRNA
siMenin sense	CUUCAGUCCUGCUCCAGAAUUUGdGdA	siRNA
siMenin antisense	UCCAAAUUCUGGAGCAGGACUGAAGUU	siRNA
siWDR82 sense	GCACAAUAGGGUUAUGACUUGCUdCdA	siRNA
siWDR82 antisense	UGAGCAAGUCAUAACCCUAUUGUGCCU	siRNA
siPTIP sense	GCAGGAAGACAGAUAUAAUAAAUdAdT	siRNA
siPTIP antisense	AUAUUUAUUAUAUCUGUCUUCCUGCUU	siRNA
siMLL3 sense	GCACCAAGAGGGUAGAGAAGGACdAdT	siRNA
siMLL3 antisense	AUGUCCUUCUCUACCCUCUUGGUGCUU	siRNA
siMLL4 sense	GCACCACCUUCCUGAAGAAUAUCdCdG	siRNA
siMLL4 antisense	CGGAUAUUCUUCAGGAAGGUGGUGCUU	siRNA
siCEBPA sense	GGAACACGAAGCACGAUCAGUCCdAdT	siRNA
siCEBPA antisense	AUGGACUGAUCGUGCUUCGUGUUCCUA	siRNA
siPPARG sense	CCACUGCCAACAUUUCCCUUCUUdCdC	siRNA
siPPARG antisense	GGAAGAAGGGAAAUGUUGGCAGUGGCU	siRNA

Primer1_F	ATTGCCTAGGAACACGAAGCACGA	ChIP qPCR
Primer1_R	TTTAGCAGAGACGCGCACATTCAC	ChIP qPCR
Primer2_F	ATTGTCACTGGTCAGCTCCAGCA	ChIP qPCR
Primer2_R	AGAAGTCGGTGGACAAGAACAGCA	ChIP qPCR
Primer3_F	CCATGCCGGGAGAACTCTAACT	ChIP qPCR
Primer3_R	CTCTGCAGGTGGCTGCTCAT	ChIP qPCR
Primer4_F	CGGATTCTCTTTCAAAGCCAGA	ChIP qPCR
Primer4_R	CTGGAGATCAGAGCTAGGAGACG	ChIP qPCR
Primer5_F	CACCGAGGGAGGAGACAAACTT	ChIP qPCR
Primer5_R	ACACCCTCGCTCCCGCCGTT	ChIP qPCR
Primer6_F	TGCGGTGCTAAAACTAATGGCT	ChIP qPCR
Primer6_R	AGGCAACAGCAGCTGTGCAA	ChIP qPCR
Primer7_F	AGGCACAAATGGGAATCAAGGGTG	ChIP qPCR
Primer7_R	TCAGTCCATAACACCTCCGCAGAC	ChIP qPCR
WDR5-EcoR1_F	TATCCGGAATTCGGTGGCGGAGGGAGTATGGCGACGGAGGAGAAGAA	GST vector
WDR5-Xho1_R	TATCCGCTCGAGTTAGCAGTCACTCTTCCACAGTT	GST vector
RBBP5-BamH1_F	TATCGCGGATCCGGTGGCGGAGGGAGTATGAACCTCGAGTTGC	GST vector
RBBP5-EcoR1_R	ATACCGGAATTCTCATAACAGTTCTGAGATTGCTC	GST vector
MEN1-EcoR1_F	ATGTAGAATTCGAGGAGGAGGAGTCGATGGGGCTGAAGGCCGCCCAGAAGA	GST vector
MEN1-Not1_R	AACTAGCGGCCGCTCAGAGGCCTTTGCGCTGCCGCTT	GST vector
PA1-EcoRI_F	GAATTCAATGTCCCTTGCTCGGGGCCAT	GST vector
PA1-BamH1_R	GGATCCTCAGTATTTCCGCTGCCGAGGGAAG;	GST vector
PTIP-EcoR1_F	ATGTAGAATTCGAGGAGGAGGAGTCGATGTCGGACCAGGCGCCCA	GST vector
PTIP-Not1_R	AACTAGCGGCCGCTCAGTTAAACTTATATGATTCATAGTCCAGCGTT	GST vector
WDR82-EcoR1_F	ATGTAGAATTCGAGGAGGAGGAGTCGATGAAGCTGACCGACAGCGTGTTGC	GST vector
WDR82-NotI_R	AACTAGCGGCCGCTCAGTCATCAATGGTGGGCAACCA	GST vector

Supplemental Experimental Procedures

RNA extraction and microarray hybridization

The cells on day 0, 3, 6 during differentiation were harvested and total RNA was extracted with Trizol (invitrogen). Each time-point has three replicates. Total RNAs were hybridized using mRNA-lncRNA-combined microarray (CapitalBio).

Plasmids and antibodies

The following plasmids have been used in this study: pCDH-EF1-MSCV-GFP-Puro (SBI, CD711B-1), pGEX-6P-1 (Addgene). Antibodies: Mouse and Rabbit IgG (sc-69786, sc-66931) were from Santa Cruz Biotechnology. anti-PTIP (A300-369A, A300-370A), anti-PA1 (A301-978A, A301-979A), anti-Menin

(A300-105A), anti-CXXC1 (A303-161A) were ordered from Bethyl Laboratories. anti-WDR5 (ab56919), anti-H3K4me3 (ab8580), anti-H3K27me3 (ab6002) were from Abcam.

Cell isolation and culture

Human adipose tissue was obtained from patients undergoing liposuction according to procedures approved by the Ethics Committee at the Chinese Academy of Medical Sciences and Peking Union Medical College. Fresh liposuction tissue was collected, digested and isolated according to an established method[37]. The cells were then cultured with hADSC culture medium containing DMEM/F-12, MCDB-201, 2% fetal bovine serum, 1×insulin transferrin selenium, 10^{-8}M dexamethasone, 10^{-4}M ascorbic acid 2-phosphate, 10ng/ml EGF, 10 ng/ml PDGF-BB and 1 ng/ml Activin A.

Adipogenic differentiation

The culture-expanded cells of 3^{th} passage at 100% confluence were induced in the following adipogenic medium for 8 days: H-DMEM supplemented with 10% FCS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 1 mM ascorbic acid. Adipogenesis was determined by oil red O staining. All reagents used in osteogenic and adipogenic differentiation were from Sigma Aldrich.

Oil red O staining

Cells were washed twice with PBS and fixed with 10% formalin for 10 min at room temperature. After fixation, cells were stained with filtered oil red O solution (stock solution: 3 mg/ml in isopropanol; working solution: 60% oil red O stock solution and 40% distilled water) for 1h at room temperature. After staining, cells were washed with water to remove unbound dye, visualized by light microscopy, and photographed. In order to quantify the degree of adipogenic differentiation, after visualized by light microscopy, the dye in cells was extracted with isopropanol and OD value was measured at 510 nm wavelength.

Lentivirus production and purification

T-225 flasks of 293T cells were cultured at 40%~50% confluence the day before transfection. Transfection was performed using Lipofectamine 2000 (Life Technologies). For each flask, 20 μg of lentivectors, 5 μg of pMD2.G, and 15 μg of psPAX2 (Addgene) were added into 4 ml OptiMEM (Life Technologies). 100 μl of Lipofectamine 2000 was diluted in 4 ml OptiMEM and, after 5 min, it was added to the plasmid mixture. The complete mixture was incubated for 20 min before being added to cells. After 6 hr, the media was changed to 30 ml DMEM + 10% FBS. After 60 hr, the media was removed and centrifuged at 3,000 rpm at 4 ℃ for 10 min to pellet cell debris. The supernatant was filtered through a 0.45 um low protein binding membrane. The virus was ultracentrifuged at 24,000 rpm for 2 hr at 4 ℃ and then resuspended overnight at 4 ℃ in DMEM + 10% FBS. Aliquots were stored at –80 ℃.

Northern Blot

RNA probes were synthesized and labeled by *in vitro* transcription of plasmids with T7 RNA polymerase (Fermentas) and Dig-11-UTP (Roche). Northern blot were done as done by NorthernMax kit (Ambion) according to manufacturer's instructions. For Northern blotting, 30 μg of total RNA isolated with Trizol was loaded into each lane. and the primers used in the experiments are listed in Supplementary Table S2.

Western Blot

Protein concentrations were measured by BCA method (Pierce), followed by loaded to SDS PAGE gel for electrophoresis. Then the proteins were transferred from gel to PVDF membrane. The membranes were blocked for 1 hr at room temperature with 5% milk TBS buffer, and incubated overnight at 4 ℃ with 1:1000 diluted primary antibody in blocking buffer supplemented with 0.1% Tween-20. Subsequently, the membranes were washed three times with TBST buffer and incubated with secondary antibody 1:10000

diluted in Blocking Buffer with 0.1% Tween-20 in dark for 1 hr at room temperature. The membranes were washed two times with Tween-TBS for 10 min each and a final wash with PBS for 10 min. Results were obtained using the Odyssey Infrared Imager (Licor).

RNA interference

Human mesenchymal stem cells were transfected with siRNA targeting *ADINR*, *PPARγ*, *C/EBPα*, *PTIP*, *Menin* and *WDR82* using HiPerFect Transfection Reagent (Qiagen) according to manufacturer's instructions. After 72 hr, the total RNA of the cells was isolated by TRIzol (Invitrogen) as previously described (T. Xiao et al, 2012). A shRNA delivered by lentivirus was used to stably deplete *ADINR* RNA in *de novo* adipose formation model. The siRNA and shRNA sequences used in the experiments are listed in Supplementary Table S2.

Quantitative RT-PCR (qRT-PCR) for analysis of RNA expression

qRT-PCR analysis was performed as previously described (T. Xiao et al, 2012). The primers used in the experiments are listed in Supplementary Table S2.

RNA Immunoprecipitation

RIP assay was performed as described (M. C. Tsai et al, 2010) with minor modifications. 4×10^6 cells were treated with 1% formaldehyde in medium for 10 min at room temperature. The crosslinking was stopped by the addition of 0.125 M glycine for 5 min. Cells were then washed twice in ice cold PBS and resuspended in 2 ml PBS, 2 ml nuclear isolation buffer (15 mM Tris pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.5 mM spermidine, 1% NP40, 1× protease inhibitor cocktail (Pierce)) for 10 min with frequent mixing. After pelleting nuclei by centrifugation at 2500 rpm for 5 min, the nuclei were resuspended in 500 μl RIP buffer [150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM DTT, 100 U/ml RNase inhibitor (Promega), 1× protease inhibitor cocktail (Pierce)],

incubated on ice with frequent vortex for 10 min and pass through 20 up and down strokes in a Dounce homogenizer, followed by brief sonication, and then the nuclear debris were pelleted by centrifugation at 16000 g for 10min. About 2 μg antibodies were added and samples were incubated for overnight at 4℃, with gentle rotation and then 40 μl protein A/G beads (Invitrogen) were added and incubated for 1 hr at 4℃. Subsequently the beads were washed five times with RIP buffer. 20% of the beads were boiled in SDS loading buffer for western blot analysis, while RNAs from the rest of beads were extracted with 1ml Trizol (Invitrogen). Then, qRT-PCR analysis was performed by using three pairs of primers listed in Supplementary Table S2.

Chromatin Immunoprecipitation

ChIP was performed as described (J. D. Nelson et al, 2006) with some modifications. 4×10^6 cells were treated with 1% formaldehyde in medium for 10 min at room temperature. The crosslinking was stopped by the addition of 0.125 M glycine for 5 min. Cells were then washed twice in ice cold PBS and resuspended in lysis buffer (50 mM Tris-HCl pH=8.0, 10 mM EDTA, 1% SDS). Then, the sample was sonicated to DNA fragments from 400-600 bp length on average. After pelleting debris at 16000 g for 10 min, the supernatant was diluted to 10 folds with dilution buffer (16.7 mM Tris-HCl pH=8.0, 167 mM NaCl, 1.1% Triton X-100, 1.2 mM EDTA, protease inhibitor cocktail tablet). Then the sample was pre-cleared by 50 μl Salmon Sperm DNA blocked protein A beads (Millipore) for 1 hr. Keep 1% volume of the sample as Input, and subsequently 2~4 μg antibodies were added to the samples for overnight incubation at 4℃. The immunocomplex was pulled down by 50 μl protein A beads for 1.5 hr co-incubation and then washed sequentially with low salt buffer (0.1% SDS, 20 mM Tris pH=8.0, 2 mM EDTA, 1% Trion X-100, 150 mM NaCl, proteinase inhibitors), high salt buffer (0.1% SDS, 20 mM Tris pH=8.0, 2 mM EDTA, 1% Trion X-100, 500 mM NaCl, proteinase inhibitors), LiCl buffer (10 mM Tris pH=8.0, 1 mM EDTA, 0.25 mM LiCl, 0.1% NP-40, 1% deoxycholate sodium) and TE (10 mM Tris pH 8.0, 1 mM EDTA pH=8.0). Subsequently,

the bound immunocomplex was eluted with 250 μl fresh elution buffer (0.1 M $NaHCO_3$, 1% SDS) by heating at 65℃ with briefly vortex for 15 min and crosslinking was reversed by overnight incubation at 65℃. Whole cell extract DNA (input fraction reserved from the sonication step) was also treated for crosslinking reversal. Immunoprecipitated DNA and WCE DNA were then purified by treatment with RNaseA, proteinase K and multiple phenol: chloroform: isoamyl alcohol extraction. Then, qRT-PCR was performed by using seven pairs of primers listed in Supplementary Table S2.

RNA pull-down assay

Biotin-labeled RNAs were *in vitro* transcribed with Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Promega), treated with RNase-free DNase I (Roche) and purified with Trizol (Invitrogen). About 1 μg of biotinylated RNA was denatured at 65℃ for 2 min in RNA structure buffer (10 mM Tris pH=7.0, 0.1 M KCl, 10 mM MgCl2), and then shifted to room temperature (RT) for 20 min to form proper secondary structure. Nuclei of 5×10^7 293T cells were isolated with 2 ml nuclear isolation buffer, 2 ml PBS, 6 ml water, and then the nuclei were lysed in 1ml RIP buffer (150 mM KCl, 25 mM Tris pH 7.4, 0.5 mM DTT, 0.5% NP40, 1 mM PMSF, 100 U/ml RNase inhibitor (Promega) and protease Inhibitor cocktail (Pierce)). After pelleting the nuclear debris by centrifugation at 16000 g for 10 min, the biotinylated RNA was then added to the nuclear extract and incubated at 4℃ for three hours with gentle rotation. Then fifty microliters washed Streptavidin T1 beads (Invitrogen) were added to the binding reaction for another three hours at 4℃. After five times washes with wash buffer (10 mM Tris pH=8.0, 1mM EDTA, 0.5M NaCl), the beads were boiled for 5 min in 0.1% SDS loading buffer. The eluted proteins can then be detected by Western bolt analysis.

GST pull-down assay

Probes for full-length and truncated *ADINR* RNA were *in vitro* transcribed from plasmids or PCR templates

containing a T7 promoter by T7 RNA polymerase (Promega), followed by treated with RNase-free DNase I (Roche) and purified with Trizol (Invitrogen). The mRNAs for WDR5, WDR82, PTIP, PA1, RBBP5, Menin were amplified by RT-PCR from adipocyte total RNAs, and cloned in the pGEX-6P-1 vector (GE Healthcare), and expressed in *Escherichia coli*. The primers for these vectors are listed in Supplementary Table S2. Each GST-conjugated protein was bound to Glutathione-Sepharose beads (GE Healthcare) in binding buffer (20 mM HEPES pH=7.6, 150 mM KCl, 0.05% NP40, 1 mM DTT, 0.5 mM PMSF at room temperature for one hour incubation. The beads were then incubated with either adipocyte total RNAs or pre-denatured *CAR* truncated RNA probes together with yeast total RNAs for one hour incubation at room temperature. After five times washes with wash buffer (20 mM HEPES pH=7.6, 300 mM KCl, 0.05% NP40, 1 mM DTT, 0.5 mM PMSF), RNAs from the beads were extracted with 1 ml Trizol (Invitrogen) and analysed by qRT-PCR as previously described.

Supplemental Reference

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