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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*.

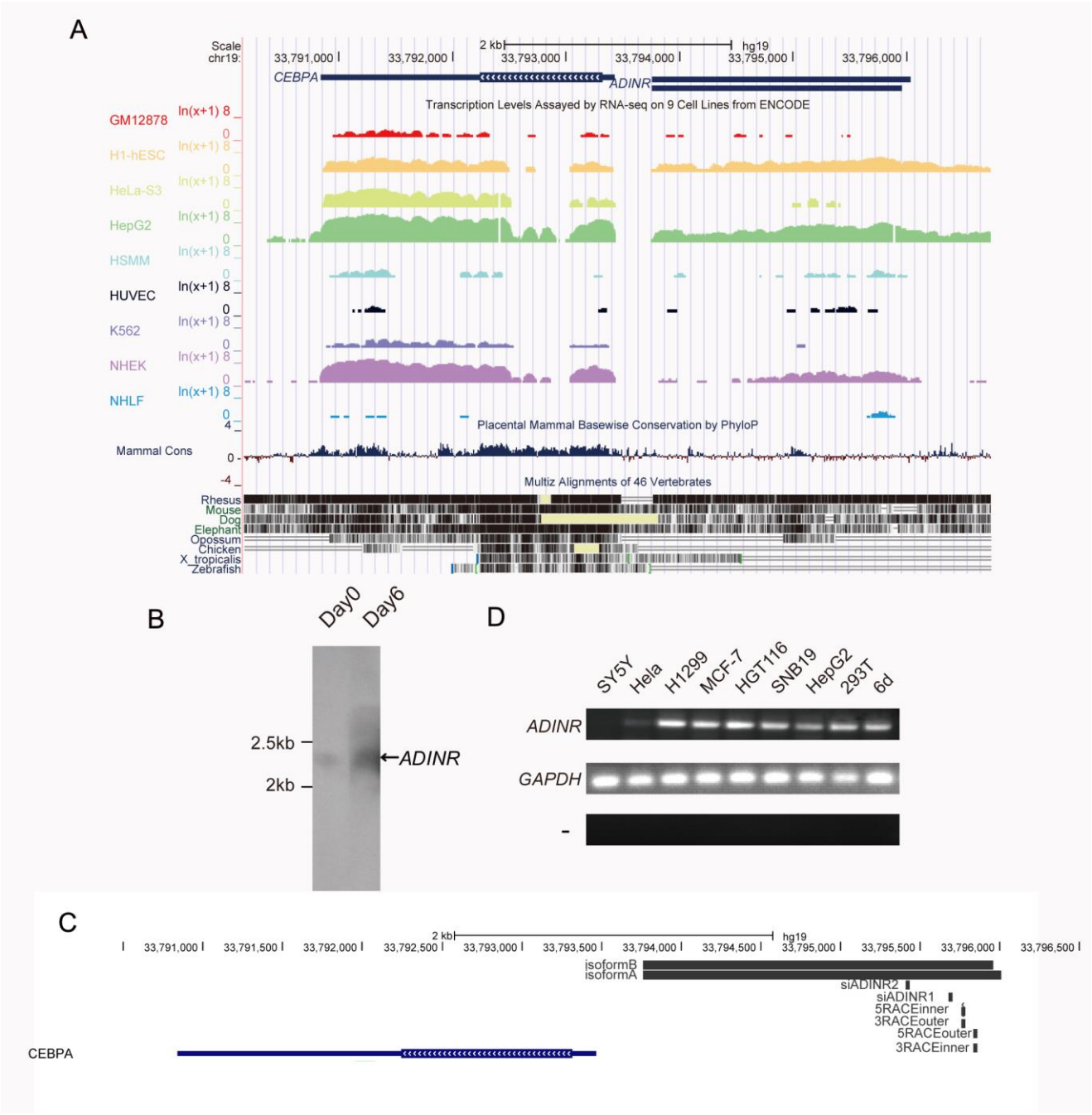

Figure S1, related to Figure 1. Expression and conservation analysis of *ADINR*.

(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*.

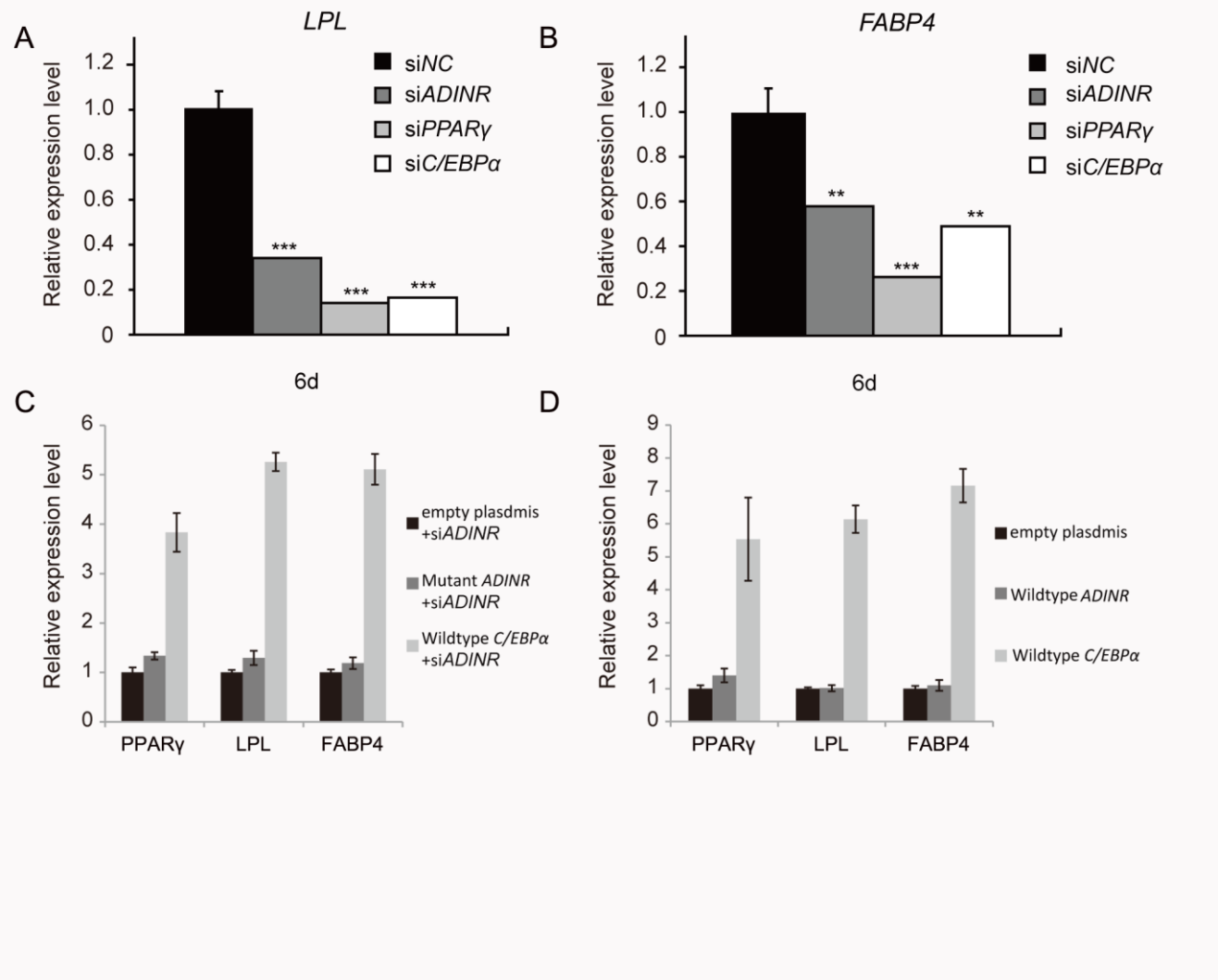

Figure S2, related to Figure 2. The expression of adipogenic marker genes.

(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.

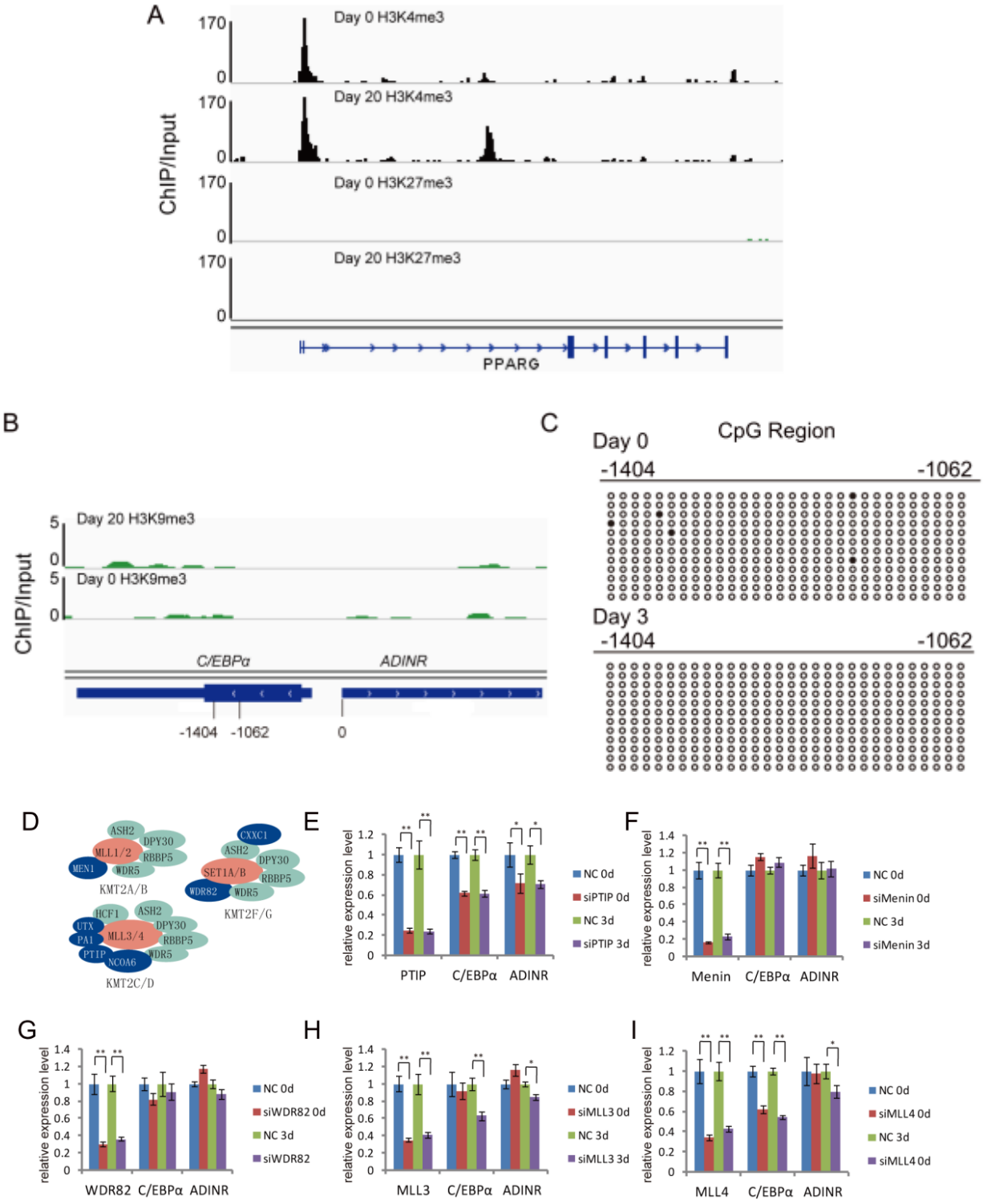

Figure S3, related to Figure 3. Epigenetic analysis of the *PPARγ*, *C/EBPα* and *ADINR* loci.

(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*.

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(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

Xiao T, Wang Y, Luo H, Liu L, Wei G, Chen X, Sun Y, Chen X, Skogerbø G, Chen R (2012) A differential sequencing-based analysis of the C. elegans noncoding transcriptome. RNA *18*, 626-639.

Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E, Chang HY (2010) Long noncoding RNA as modular scaffold of histone modification complexes .Science *329*, 689-693.

Nelson JD, Denisenko O, Bomsztyk K (2006) Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nature Protocols *1*, 179-185.