

# Long Noncoding RNA *ADINR* Regulates Adipogenesis by Transcriptionally Activating *C/EBP $\alpha$*

Tengfei Xiao,<sup>1,2,3,7</sup> Lihui Liu,<sup>1,2,5,7</sup> Hongling Li,<sup>4,7</sup> Yu Sun,<sup>1,2,5,7</sup> Huaxia Luo,<sup>1,2,5</sup> Tangping Li,<sup>4</sup> Shihua Wang,<sup>4</sup> Stephen Dalton,<sup>6</sup> Robert Chunhua Zhao,<sup>4,\*</sup> and Runsheng Chen<sup>1,2,\*</sup>

<sup>1</sup>Key Laboratory of RNA Biology, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

<sup>2</sup>Key Laboratory of Non-coding RNA, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

<sup>3</sup>Center for Functional Cancer Epigenetics, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, MA 02215, USA

<sup>4</sup>Center of Excellence in Tissue Engineering, Institute of Basic Medical Sciences, School of Basic Medicine, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100005, China

<sup>5</sup>Graduate School of the Chinese Academy of Sciences, Beijing 100080, China

<sup>6</sup>Paul D. Coverdell Center for Biomedical and Health Sciences, Department of Biochemistry and Molecular Biology, The University of Georgia, 500 D.W. Brooks Drive, Athens, GA 30602, USA

<sup>7</sup>Co-first author

\*Correspondence: [zhaochunhua@vip.163.com](mailto:zhaochunhua@vip.163.com) (R.C.Z.), [rschen@ibp.ac.cn](mailto:rschen@ibp.ac.cn) (R.C.)

<http://dx.doi.org/10.1016/j.stemcr.2015.09.007>

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## SUMMARY

*C/EBP $\alpha$*  is a critical transcriptional regulator of adipogenesis. How *C/EBP $\alpha$*  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/EBP $\alpha$*  gene, that orchestrates *C/EBP $\alpha$*  transcription in vivo. Depletion of *ADINR* leads to a severe adipogenic defect that is rescued by overexpression of *C/EBP $\alpha$* . 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/EBP $\alpha$*  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/EBP $\alpha$*  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 (Klattehoff 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 tri-

methylation (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/EBP $\alpha$*  gene, interacts with DNMT-1 to block *C/EBP $\alpha$*  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 (Jandrová 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). *PPAR $\gamma$*  and *C/EBP $\alpha$*  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 *PPAR* $\gamma$  and *C/EBP* $\alpha$  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/EBP* $\alpha$  gene in *cis* by impacting H3K4me3 and H3K27me3 histone modification of the *C/EBP* $\alpha$  locus. These findings have major implications for obesity and human disease.

## RESULTS

### *ADINR* Is a lncRNA Upregulated during Adipogenesis

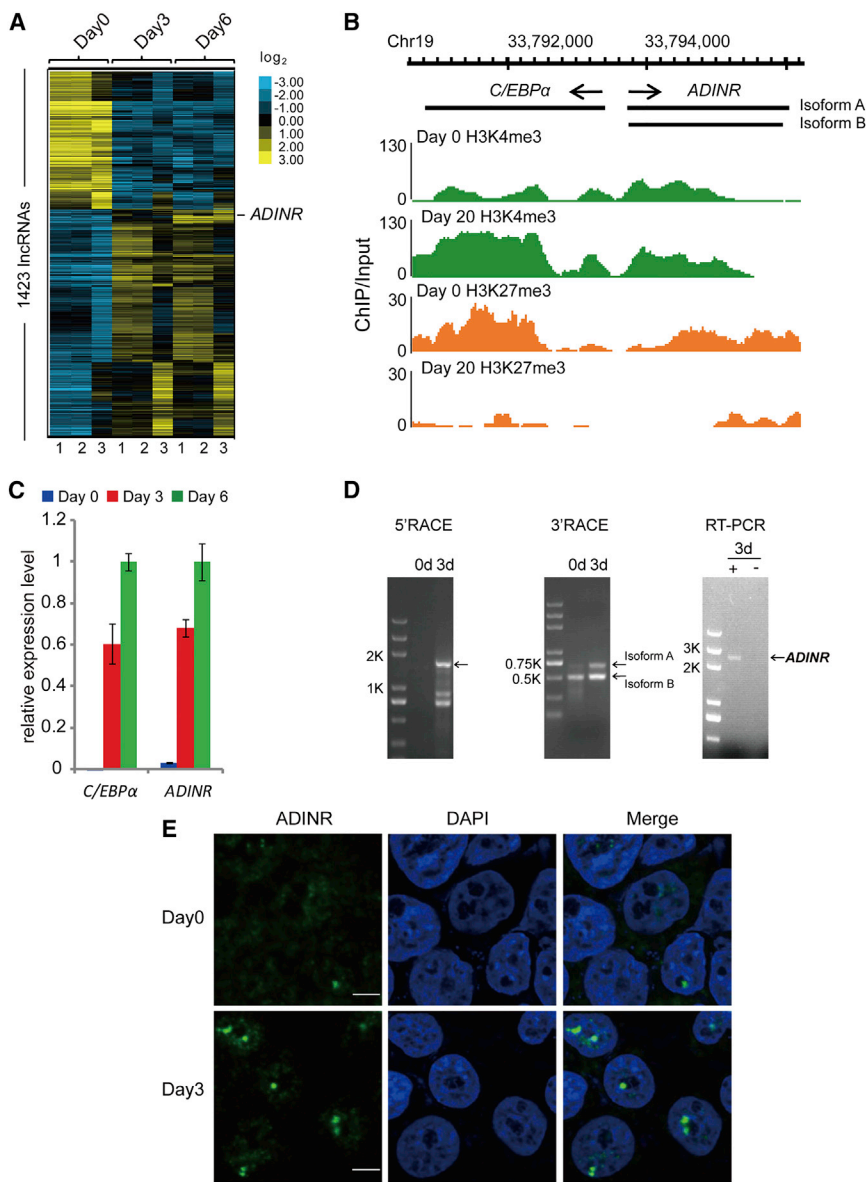
To explore whether lncRNAs are involved in the adipogenic differentiation of hMSCs, we designed a customized microarray to probe the expression profiles of 39,303 human transcripts that have been annotated as potential noncoding RNAs (see [Experimental Procedures](#)). The expression profiles were probed 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/EBP* $\alpha$  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/EBP* $\alpha$  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 hu-

man 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/EBP* $\alpha$  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/EBP* $\alpha$  and *PPAR* $\gamma$ , 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/EBP* $\alpha$  and *PPAR* $\gamma$  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<sup>+</sup> cells and reduced adipogenic transcripts *C/EBP* $\alpha$ , *PPAR* $\gamma$ , 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/EBP* $\alpha$ , *PPAR* $\gamma$ , *FABP4*, and LPL ([Figure 2F](#)) but also decrease the number of oil red O<sup>+</sup> 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/EBP* $\alpha$  mRNA as well as other adipogenic markers. However, the adipogenic deficiency was readily rescued by lentivirus-mediated ectopic expression of *C/EBP* $\alpha$  ([Figures 2I and 2J](#); [Figure S2C](#)). In addition, ectopic



### Figure 1. IncRNA *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  $\pm$  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*.

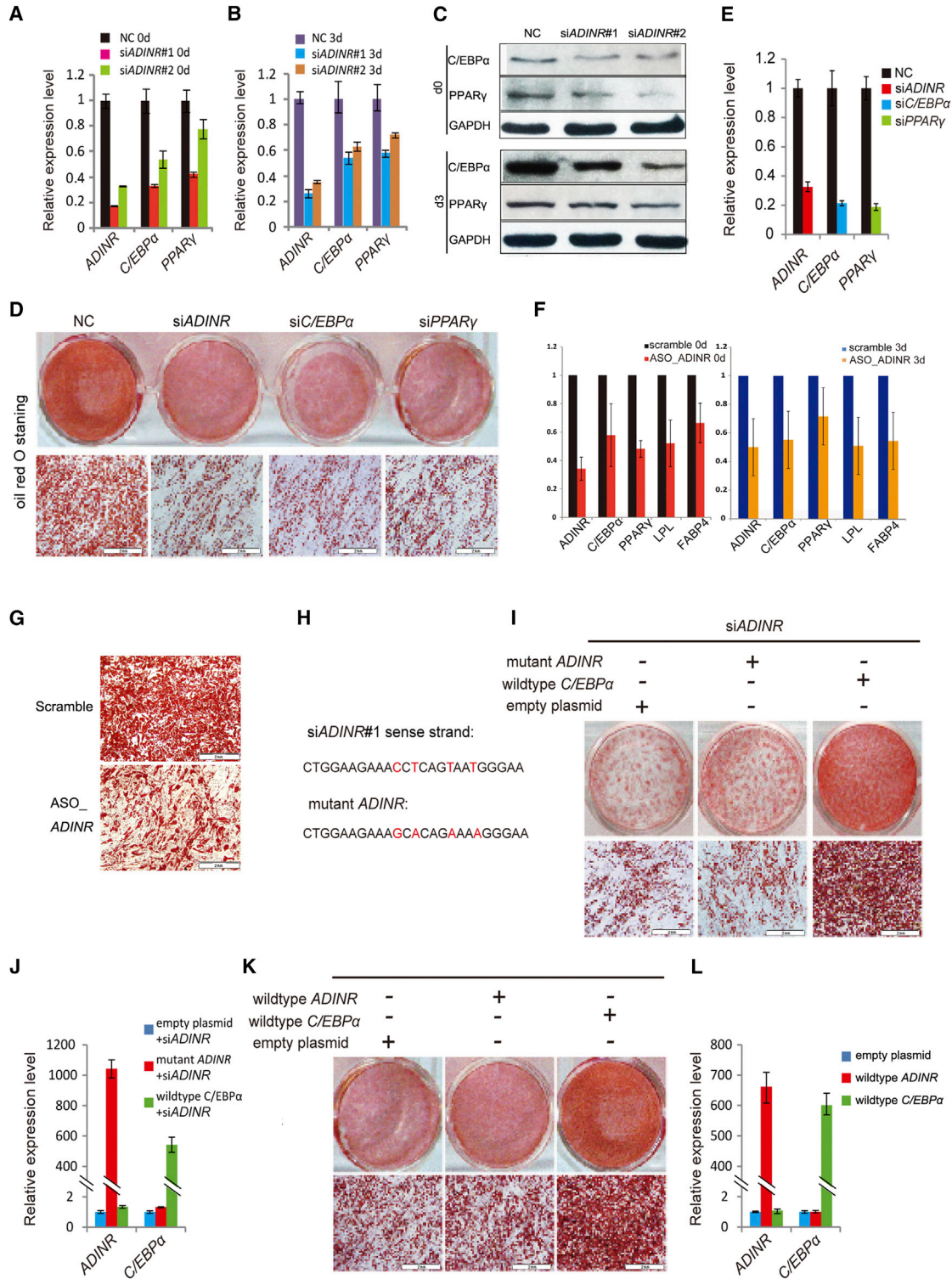
### *ADINR* Interacts with MLL3/4 Complexes to Regulate *C/EBPα* Expression

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 (siADINR#1 and siADINR#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.

(legend continued on next page)



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/EBP $\alpha$*  (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/EBP $\alpha$*  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/EBP $\alpha$*  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/EBP $\alpha$*  and *ADINR* on days 0 and 3 (Figure S3E), while knockdown of Menin and WDR82 had no detectable effect on *C/EBP $\alpha$*  or *ADINR* expression (Figures S3F and S3G). Knockdown of MLL3 and MLL4 indicated that both proteins are required for *C/EBP $\alpha$*  expression on day 3, whereas only the knockdown of MLL4 markedly altered *C/EBP $\alpha$*  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/EBP $\alpha$*  and *ADINR* expression. This finding is consistent with previous reports in mouse adipocytes that PTIP and MLL4 are recruited to the *C/EBP $\alpha$*  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 immu-

noprecipitation 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/EBP $\alpha$*  expression.

### PA1 Is Recruited to the *C/EBP $\alpha$* 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/EBP $\alpha$*  gene in *cis*. To confirm that *ADINR* regulates the *C/EBP $\alpha$*  gene by recruiting the MLL3/4 complexes to the *C/EBP $\alpha$*  promoter, we performed ChIP assays on PA1, in the presence or absence of *ADINR*. The results showed that PA1 is recruited to the *C/EBP $\alpha$*  promoter on days 0 and 3, and the knockdown of *ADINR* significantly inhibits the binding of PA1 to the *C/EBP $\alpha$*  promoter (Figure 4B). These findings indicate that the MLL3/4 complexes are recruited to the *C/EBP $\alpha$*  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* (siADINR#1), *C/EBP $\alpha$* , and *PPAR $\gamma$*  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/EBP $\alpha$* , and *PPAR $\gamma$*  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/EBP $\alpha$*  mRNA.

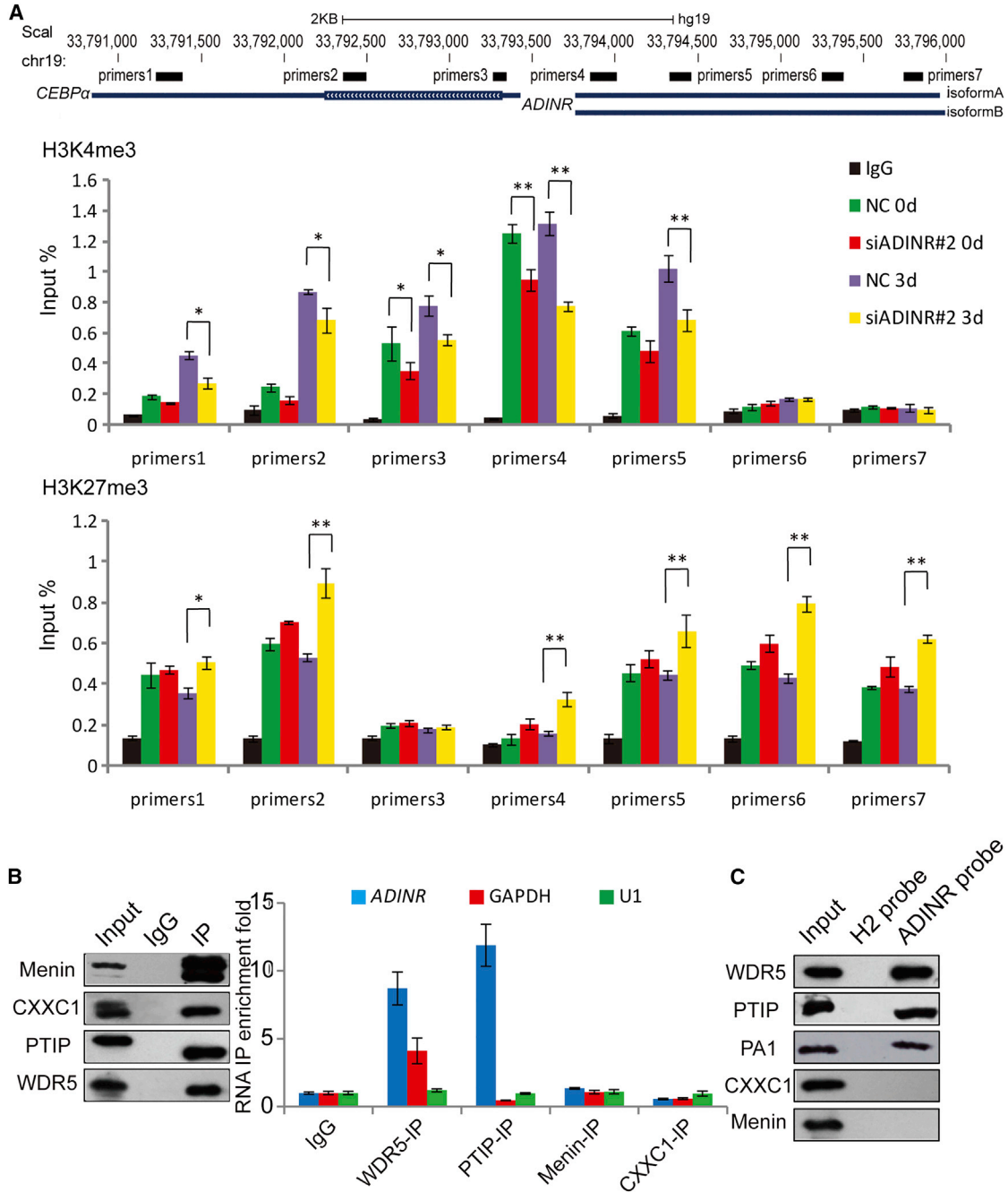
(J) qRT-PCR analysis of *ADINR* and *C/EBP $\alpha$*  expression after adipogenesis.

(K) Overexpression assay for *ADINR* and *C/EBP $\alpha$*  adipocytes on day 6.

(L) qRT-PCR analysis of *ADINR* and *C/EBP $\alpha$*  expression after adipogenesis.

All of the expression levels derived via qPCR were normalized to *GAPDH* and are presented as the means  $\pm$  SD in three independent experiments. Scale bars, 2 mm.

See also Figure S2.



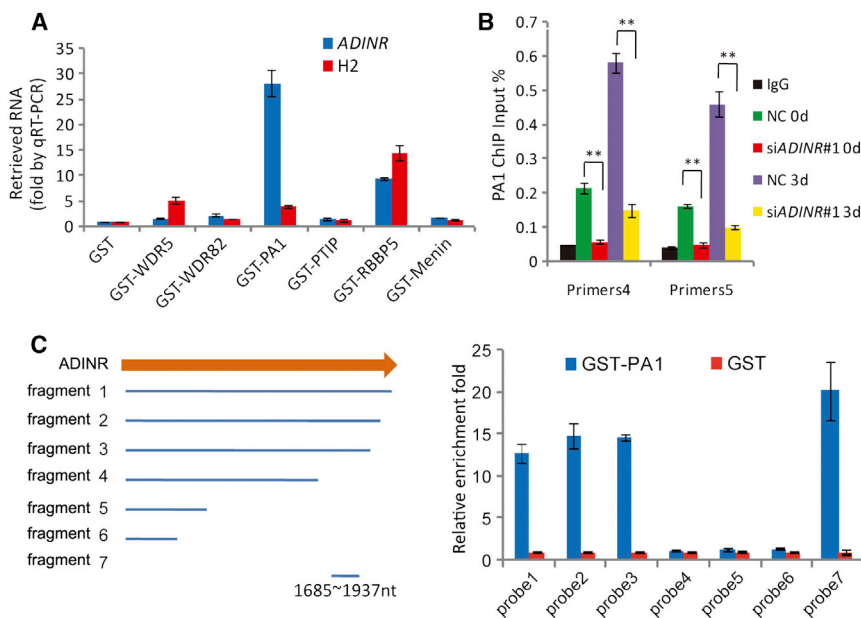
**Figure 3. *ADINR* RNA Is Required for the Active Chromatin State of the *C/EBPα* and *ADINR* Loci**

(A) ChIP assay of H3K4me3 and H3K27me3 at the *C/EBPα* and *ADINR* loci. The qPCR data are presented as the mean  $\pm$  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  $\mu$ g of cell lysate was loaded as the input control. Error bars represent the mean  $\pm$  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 func-

tions 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/EBP $\alpha$*  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 *PPAR $\gamma$*  and *C/EBP $\alpha$*  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/EBP $\alpha$*  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/EBP $\alpha$*  transcriptional activity in *cis* by affecting the level of H3K4me3 in the promoter region of *C/EBP $\alpha$* , 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 $\times$  insulin transferrin selenium, 10<sup>-8</sup> M dexamethasone, 10<sup>-4</sup> 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.

### ACKNOWLEDGMENTS

We thank Myles Brown and Shirley Liu for helpful discussions and Jianqing Zhao for assistance with microarray data. This work was supported by the Chinese Academy of Sciences Strategic Project of Leading Science and Technology (XDA01020402), the National High Technology Research and Development Program ("863" Program) (2012AA020402), and the National Natural Science Foundation of China (No. 81370466). R.Z. was supported by a National Collaborative Innovation Program grant (for Biotherapy).

Received: April 16, 2015

Revised: September 9, 2015

Accepted: September 10, 2015

Published: October 15, 2015

### REFERENCES

- Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315–326.
- Cabili, M.N., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., and Rinn, J.L. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* **25**, 1915–1927.





- Cao, Y., Sun, Z., Liao, L., Meng, Y., Han, Q., and Zhao, R.C. (2005). Human adipose tissue-derived stem cells differentiate into endothelial cells in vitro and improve postnatal neovascularization in vivo. *Biochem. Biophys. Res. Commun.* *332*, 370–379.
- Cheng, J., Kapranov, P., Drenkow, J., Dike, S., Brubaker, S., Patel, S., Long, J., Stern, D., Tammana, H., Helt, G., et al. (2005). Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. *Science* *308*, 1149–1154.
- Cho, Y.W., Hong, S., Jin, Q., Wang, L., Lee, J.E., Gavrilo, O., and Ge, K. (2009). Histone methylation regulator PTIP is required for PPAR $\gamma$  and C/EBP $\alpha$  expression and adipogenesis. *Cell Metab.* *10*, 27–39.
- Di Ruscio, A., Ebraldiz, A.K., Benoukraf, T., Amabile, G., Goff, L.A., Terragni, J., Figueroa, M.E., De Figueiredo Pontes, L.L., Alberich-Jorda, M., Zhang, P., et al. (2013). DNMT1-interacting RNAs block gene-specific DNA methylation. *Nature* *503*, 371–376.
- Farmer, S.R. (2006). Transcriptional control of adipocyte formation. *Cell Metab.* *4*, 263–273.
- Friedman, J.M. (2000). Obesity in the new millennium. *Nature* *404*, 632–634.
- Grote, P., Wittler, L., Hendrix, D., Koch, F., Währisch, S., Beisaw, A., Macura, K., Bläss, G., Kellis, M., Werber, M., and Herrmann, B.G. (2013). The tissue-specific lincRNA Fendrr is an essential regulator of heart and body wall development in the mouse. *Dev. Cell* *24*, 206–214.
- Guttman, M., and Rinn, J.L. (2012). Modular regulatory principles of large non-coding RNAs. *Nature* *482*, 339–346.
- Guttman, M., Amit, I., Garber, M., French, C., Lin, M.F., Feldser, D., Huarte, M., Zuk, O., Carey, B.W., Cassady, J.P., et al. (2009). Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* *458*, 223–227.
- Guttman, M., Donaghey, J., Carey, B.W., Garber, M., Grenier, J.K., Munson, G., Young, G., Lucas, A.B., Ach, R., Bruhn, L., et al. (2011). lincRNAs act in the circuitry controlling pluripotency and differentiation. *Nature* *477*, 295–300.
- Hsieh, C.L., Fei, T., Chen, Y., Li, T., Gao, Y., Wang, X., Sun, T., Sweeney, C.J., Lee, G.S., Chen, S., et al. (2014). Enhancer RNAs participate in androgen receptor-driven looping that selectively enhances gene activation. *Proc. Natl. Acad. Sci. USA* *111*, 7319–7324.
- Hung, T., Wang, Y., Lin, M.F., Koegel, A.K., Kotake, Y., Grant, G.D., Horlings, H.M., Shah, N., Umbrecht, C., Wang, P., et al. (2011). Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. *Nat. Genet.* *43*, 621–629.
- Janderová, L., McNeil, M., Murrell, A.N., Mynatt, R.L., and Smith, S.R. (2003). Human mesenchymal stem cells as an in vitro model for human adipogenesis. *Obes. Res.* *11*, 65–74.
- Janowski, B.A., Huffman, K.E., Schwartz, J.C., Ram, R., Hardy, D., Shames, D.S., Minna, J.D., and Corey, D.R. (2005). Inhibiting gene expression at transcription start sites in chromosomal DNA with antigene RNAs. *Nat. Chem. Biol.* *1*, 216–222.
- Kapranov, P., Cheng, J., Dike, S., Nix, D.A., Duttagupta, R., Willingham, A.T., Stadler, P.F., Hertel, J., Hackermüller, J., Hofacker, I.L., et al. (2007). RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* *316*, 1484–1488.
- Kikuchi, K., Fukuda, M., Ito, T., Inoue, M., Yokoi, T., Chiku, S., Mitsuyama, T., Asai, K., Hirose, T., and Aizawa, Y. (2009). Transcripts of unknown function in multiple-signaling pathways involved in human stem cell differentiation. *Nucleic Acids Res.* *37*, 4987–5000.
- Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S., et al. (2010). Widespread transcription at neuronal activity-regulated enhancers. *Nature* *465*, 182–187.
- Klattenhoff, C.A., Scheuermann, J.C., Surface, L.E., Bradley, R.K., Fields, P.A., Steinhauser, M.L., Ding, H., Butty, V.L., Torrey, L., Haas, S., et al. (2013). Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. *Cell* *152*, 570–583.
- Kretz, M., Webster, D.E., Flockhart, R.J., Lee, C.S., Zehnder, A., Lopez-Pajares, V., Qu, K., Zheng, G.X., Chow, J., Kim, G.E., et al. (2012). Suppression of progenitor differentiation requires the long noncoding RNA ANCR. *Genes Dev.* *26*, 338–343.
- Lee, J.E., Wang, C., Xu, S., Cho, Y.W., Wang, L., Feng, X., Baldrige, A., Sartorelli, V., Zhuang, L., Peng, W., and Ge, K. (2013). H3K4 mono- and di-methyltransferase MLL4 is required for enhancer activation during cell differentiation. *eLife* *2*, e01503.
- Loewer, S., Cabili, M.N., Guttman, M., Loh, Y.H., Thomas, K., Park, I.H., Garber, M., Curran, M., Onder, T., Agarwal, S., et al. (2010). Large intergenic non-coding RNA-RoR modulates reprogramming of human induced pluripotent stem cells. *Nat. Genet.* *42*, 1113–1117.
- Mohan, M., Herz, H.M., and Shilatifard, A. (2012). SnapShot: Histone lysine methylase complexes. *Cell* *149*, 498–498.e1.
- Musahl, A.S., Huang, X., Rusakiewicz, S., Ntini, E., Marsico, A., Kroemer, G., Kepp, O., and Ørom, U.A. (2015). A long non-coding RNA links calreticulin-mediated immunogenic cell removal to RB1 transcription. *Oncogene*. Published online January 12, 2015. <http://dx.doi.org/10.1038/onc.2014.424>.
- Ørom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytznicki, M., Notredame, C., Huang, Q., et al. (2010). Long noncoding RNAs with enhancer-like function in human cells. *Cell* *143*, 46–58.
- Pandey, R.R., Mondal, T., Mohammad, F., Enroth, S., Redrup, L., Komorowski, J., Nagano, T., Mancini-Dinardo, D., and Kanduri, C. (2008). Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol. Cell* *32*, 232–246.
- Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* *284*, 143–147.
- Ponjavic, J., Ponting, C.P., and Lunter, G. (2007). Functionality or transcriptional noise? Evidence for selection within long noncoding RNAs. *Genome Res.* *17*, 556–565.
- Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Bruggmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., and Chang, H.Y. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* *129*, 1311–1323.
- Rosen, E.D., Hsu, C.H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., and Spiegelman, B.M. (2002). C/EBP $\alpha$  induces



adipogenesis through PPARgamma: a unified pathway. *Genes Dev.* *16*, 22–26.

Sigova, A.A., Mullen, A.C., Molinie, B., Gupta, S., Orlando, D.A., Guenther, M.G., Almada, A.E., Lin, C., Sharp, P.A., Giallourakis, C.C., and Young, R.A. (2013). Divergent transcription of long noncoding RNA/mRNA gene pairs in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* *110*, 2876–2881.

Struhl, K. (2007). Transcriptional noise and the fidelity of initiation by RNA polymerase II. *Nat. Struct. Mol. Biol.* *14*, 103–105.

Sun, L., Goff, L.A., Trapnell, C., Alexander, R., Lo, K.A., Haciosuleyman, E., Sauvageau, M., Tazon-Vega, B., Kelley, D.R., Hendrickson, D.G., et al. (2013). Long noncoding RNAs regulate adipogenesis. *Proc. Natl. Acad. Sci. USA* *110*, 3387–3392.

Tsai, M.C., Manor, O., Wan, Y., Mosammamaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. *Science* *329*, 689–693.

Wang, K.C., Yang, Y.W., Liu, B., Sanyal, A., Corces-Zimmerman, R., Chen, Y., Lajoie, B.R., Protacio, A., Flynn, R.A., Gupta, R.A., et al. (2011). A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. *Nature* *472*, 120–124.

Wapinski, O., and Chang, H.Y. (2011). Long noncoding RNAs and human disease. *Trends Cell Biol.* *21*, 354–361.

Zhao, J., Sun, B.K., Erwin, J.A., Song, J.J., and Lee, J.T. (2008). Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* *322*, 750–756.