

# Long Noncoding RNAs with Enhancer-like Function in Human Cells

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## SUMMARY

While the long noncoding RNAs (ncRNAs) constitute a large portion of the mammalian transcriptome, their biological functions has remained elusive. A few long ncRNAs that have been studied in any detail silence gene expression in processes such as X-inactivation and imprinting. We used a GENCODE annotation of the human genome to characterize over a thousand long ncRNAs that are expressed in multiple cell lines. Unexpectedly, we found an enhancer-like function for a set of these long ncRNAs in human cell lines. Depletion of a number of ncRNAs led to decreased expression of their neighboring protein-coding genes, including the master regulator of hematopoiesis, *SCL* (also called *TAL1*), *Snai1* and *Snai2*. Using heterologous transcription assays we demonstrated a requirement for the ncRNAs in activation of gene expression. These results reveal an unanticipated role for a class of long ncRNAs in activation of critical regulators of development and differentiation.

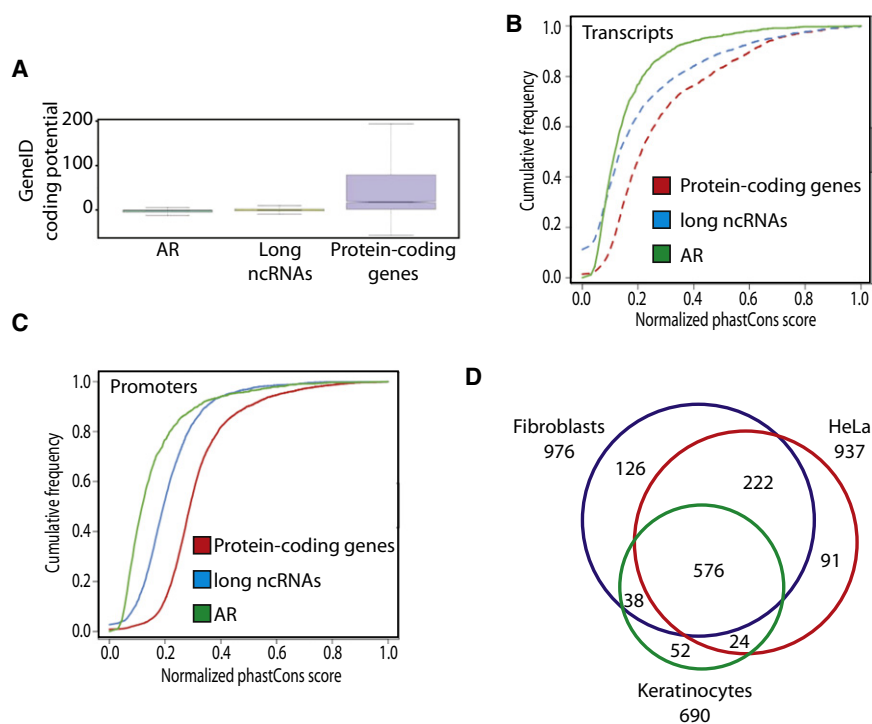
## INTRODUCTION

Recent technological advances have allowed the analysis of the human and mouse transcriptomes with an unprecedented resolution. These experiments indicate that a major portion of the genome is being transcribed and that protein-coding sequences only account for a minority of cellular transcriptional output (Bertone et al., 2004; Birney et al., 2007; Cheng et al., 2005; Kapranov et al., 2007). Discovery of RNA interference (RNAi) (Fire et al., 1998) in *C. elegans* and the identification of a new class of small RNAs known as microRNAs (Lee et al., 1993; Wightman et al., 1993) led to a greater appreciation of RNA's role in regulation of gene expression. MicroRNAs are endogenously expressed noncoding transcripts that silence gene expression by targeting specific mRNAs on the basis of sequence recognition (Carthew and Sontheimer, 2009). Over 1000 microRNA loci are estimated

to be functional in humans, modulating roughly 30% of protein-coding genes (Berezikov and Plasterk, 2005).

While microRNAs represent a minority of the noncoding transcriptome, the tangle of long and short noncoding transcripts is much more intricate, and is likely to contain as yet unidentified classes of molecules forming transcriptional regulatory networks (Efroni et al., 2008; Kapranov et al., 2007). Long ncRNAs are transcripts longer than 100 nts which in most cases mirror the features of protein-coding genes without containing a functional open reading frame (ORF). Long ncRNAs have been implicated as principal players in imprinting and X-inactivation. The imprinting phenomenon dictates the repression of a particular allele, depending on its paternal or maternal origin. Many clusters of imprinted genes contain ncRNAs, and some of them have been implicated in the transcriptional silencing (Yang and Kuroda, 2007). Similarly, the X chromosome inactivation relies on the expression of a long ncRNA named *Xist*, which is thought to recruit, in a *cis*-specific manner, protein complexes establishing repressive epigenetic marks that encompass the chromosome (Heard and Distèche, 2006). There is also a report indicating that a long ncRNA expressed from the *HOXC* locus may affect the expression of genes in the *HOXD* locus which is located on a different chromosome (Rinn et al., 2007). More recently, a set of long ncRNAs has been identified in mouse, through the analysis of the chromatin signatures (Guttman et al., 2009). There has also been reports of divergent transcription of short RNAs flanking transcriptional start sites of the active promoters (Core et al., 2008; Preker et al., 2008; Seila et al., 2008).

In search of a function for long ncRNAs, we used the GENCODE annotation (Harrow et al., 2006) of the human genome. To simplify our search we subtracted transcripts overlapping the protein-coding genes. Moreover, we filtered out the transcripts that may correspond to promoters of protein-coding genes and the transcripts that belong to known classes of ncRNAs. We identified 3019 putative long ncRNAs that display differential patterns of expression. Functional knockdown of multiple ncRNAs revealed their positive influence on the neighboring protein-coding genes. Furthermore, detailed functional analysis of a long ncRNA adjacent to the *Snai1* locus using reporter assays demonstrated a role for this ncRNA in an RNA-dependent potentiation of gene expression. Our studies suggest



**Figure 1. Identification of Novel Long ncRNAs in Human Annotated by GENCODE**

(A) Analysis of coding potential using Gene ID for ancestral repeats (AR), long ncRNAs annotated by GENCODE and protein-coding genes.

(B) Conservation of the genomic transcript sequences for AR, long ncRNAs, protein-coding genes, and (C) of their promoters.

(D) Expression analysis of 3,019 long ncRNA in human fibroblasts, HeLa cells and primary human keratinocytes, showing numbers for transcripts detected in each cell line and the overlaps between cell lines. All microarray experiments have been done in four replicates. See also Figure S1 and Table S1 and Table S2.

a role for a class of long ncRNAs in positive regulation of protein-coding genes.

## RESULTS

### Noncoding RNAs Are Expressed and Respond to Cellular Differentiating Signals

To assign a function to uncharacterized human long ncRNAs, we identified unique long noncoding transcripts using the annotation of the human genome provided by the GENCODE (Harrow et al., 2006) and performed by human and vertebrate analysis and annotation (HAVANA) group at Sanger Institute. Such genomic annotation is being produced in the framework of the ENCODE project (Birney et al., 2007). At the time of our analysis, the GENCODE annotation encompassed about one third of the human genome. Such an annotation relies on the human expert curation of all available experimental data on transcriptional evidence, such as cloned cDNA sequences, spliced RNAs and ESTs mapped on to the human genome.

We focused on ncRNAs that do not overlap the protein-coding genes in order to simplify the interpretation of our functional analysis of ncRNAs. This included the subtraction of all transcripts mapping to exons, introns and the antisense transcripts overlapping the protein-coding genes. We also excluded transcripts within 1 kb of the first and the last exons as to avoid promoter and 3'-associated transcripts (Fejes-Toth et al., 2009; Kapranov et al., 2007), that display a complicated pattern of short transcripts (Core et al., 2008; Preker et al., 2008; Seila et al., 2008). Furthermore, we excluded all known noncoding transcripts from our list of putative long ncRNAs. This analysis resulted in 3019 ncRNAs, which are annotated by HAVANA to have no

coding potential, expressed from 2286 unique loci (some loci display multiple alternative spliced transcripts) of the human genome (Experimental Procedures, Table S1 available online). The average size of the noncoding transcripts is about 800 nts with a range from 100 nts to 9100 nts. Interestingly, the long ncRNAs display a simpler transcription unit than that of protein-coding genes (Figure S1A). Nearly 50% of our long

ncRNAs contain a single intron in their primary transcript (Figure S1A). Moreover, analysis of their chromatin signatures indicated similarities with protein-coding genes. Transcriptionally active ncRNAs display histone H3K4 trimethylation at their 5'-end (Figure S1B) and histone H3K36 trimethylation in the body of the gene (Figure S1C). Analysis of protein coding potential of the ncRNAs using GeneID (Blanco et al., 2007; Parra et al., 2000) shows ncRNAs coding potential comparable to that of ancestral repeats (Lunter et al., 2006), supporting the HAVANA annotation of these transcripts as noncoding (Figure 1A). Moreover, comparison of ncRNAs with protein-coding genes and control sequences corresponding to ancestral repeats (Lunter et al., 2006) reveals that ncRNA sequence conservation is lower than that of protein-coding genes, but higher than that of ancestral repeats (Figure 1B). A similar case is seen with the promoter regions (Figure 1C). These results are in concordance with previous observations in the mouse genome (Guttman et al., 2009; Ponting et al., 2009).

Next we used custom-made microarrays (Experimental Procedures) which were designed to include an average of six probes (nonrepetitive sequences) against each ncRNA transcript to detect their expression. We analyzed the expression pattern of ncRNAs using three different human cell lines (Figure 1D). Overall, we detected 1167 ncRNAs expressed in at least one of the three cell types and 576 transcripts common among the three cell types (Figure 1D). We validated the expression of 16 ncRNAs that mapped to the 1% of the human genome investigated by the original ENCODE study (Birney et al., 2007) using quantitative polymerase chain reaction (qPCR) in three different cell lines (Table S2). Furthermore, we could find evidence for expression of 80% of our noncoding transcripts in at least one

human tissue in a recent high throughput sequencing of the human transcriptome (Wang et al., 2008).

To assess whether ncRNAs respond to cellular differentiating signals, we induced the differentiation of human primary keratinocytes using 12-O-tetradecanoylphorbol 13-acetate (TPA). We monitored the expression of ncRNAs using custom microarrays. Expression of protein-coding genes was monitored using conventional Agilent arrays containing nearly all human mRNAs. We prepared RNA from human primary keratinocytes before and following treatment with TPA. As shown in Figure 2A and Table S3, we could detect 687 ncRNAs in keratinocytes, where 104 (or 15.1%) respond to TPA treatment by over 1.5-fold. Similarly, 21.3% of protein coding-genes display a change in expression of over 1.5-fold (Figure 2B). While around half of the TPA-regulated protein-coding genes increase and a similar proportion decrease their expression following differentiation, 70% of the TPA-regulated ncRNAs increase their expression whereas only 30% show a decrease (Figures 2A and 2B). Furthermore, analysis of the protein-coding genes in the 500 kb window surrounding the TPA-regulated ncRNAs indicates a significant enrichment in genes involved in differentiation and morphogenesis (Figure 2C). An example of such change in expression of an important gene involved in extra-cellular matrix is shown in Figure 2D. Extracellular Matrix Protein 1 (ECM1) gene and an ncRNA adjacent to it displayed a 5 and 1.7 fold induction following TPA treatment, respectively. (Figure 2D, upper panel). qPCR analysis shows the TPA-mediated induction of ECM1 and the ncRNA as 14 and 4 fold, respectively (Figure 2D, bottom panel). Taken together, we found that many of the GENCODE annotated transcripts are expressed in multiple cell lines and that they display gene expression responsiveness to differentiation signals.

### Noncoding RNAs Display a Transcriptional Activator Function

To assess the function of our set of long ncRNAs, we reasoned that similar to long ncRNAs function at the imprinting loci, our collection of ncRNAs may act to regulate their neighboring genes. To test this hypothesis, we used RNA interference to deplete a set of ncRNAs. We initially chose ncRNAs that showed a differential expression following keratinocyte differentiation. However, to obtain a reproducible knockdown we had to use cell lines that are permissive to transfection by siRNAs. We used five different cell lines for our analyses in which the candidate ncRNAs display a detectable expression (Figure 3).

We validated the expression of our experimental set of ncRNAs and the absence of protein-coding potential using rapid amplification of 5' and 3' complementary DNA ends (5' and 3' RACE), PCR and in vitro translation (Figure S3). These experiments confirmed the expression of ncRNAs and showed that they do not yield a product in an in vitro translation assay (Figures S3A and S3B), supporting the noncoding annotation of our set of ncRNAs. In two cases, the ncRNAs adjacent to *Snai2* and *TAL1* loci, we found evidence of a longer ncRNA transcript than that annotated by HAVANA (Figure S3).

We began by examining small interfering RNAs (siRNAs) against the ncRNA next to *ECM1* in order to assess its functional role following its depletion (for reasons that will follow, this class of RNA is designated as noncoding RNA-activating1 through 7,

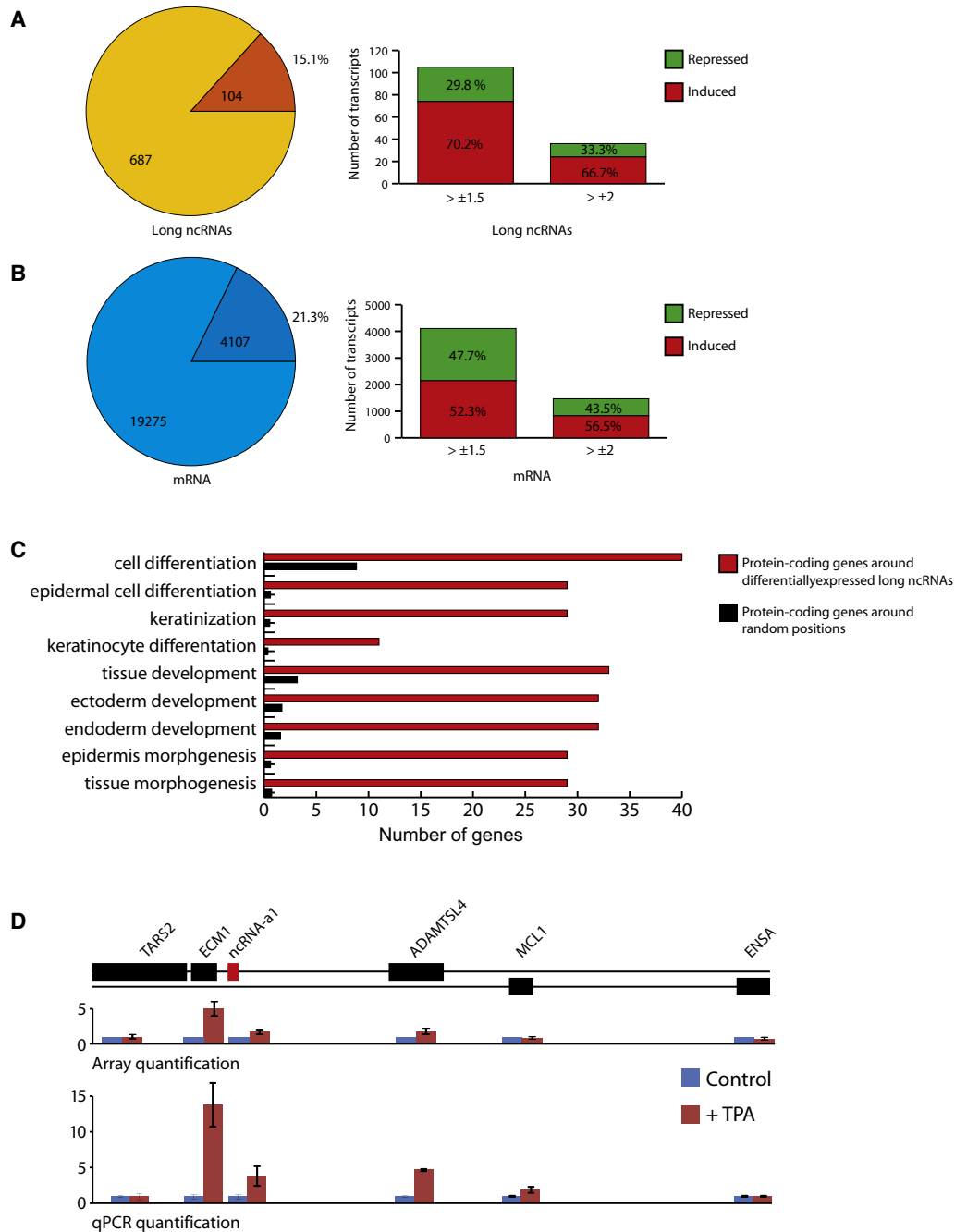
ncRNA-a1-7). HEK293 cells were used for these experiments because of the ease of functional knockdown and the detectable amounts of ncRNA-a1 and *ECM1* in this cell line. We compared the results obtained using two siRNAs against ncRNA-a1 to data obtained following the transfection of two control siRNAs (for the visual simplicity only one siRNA is shown (Figure 3A), the values for both siRNAs can be seen in Table S4). The two siRNAs produced comparable results. We interrogated a 300 kb window around the ncRNA-a1 containing six protein-coding genes using qPCR.

Surprisingly, unlike the silencing action of long ncRNAs in imprinting and X-inactivation, depletion of ncRNA-a1 adjacent to *ECM1* resulted in a concomitant decrease in expression of the neighboring *ECM1* gene (Figure 3A). This effect was specific, as we did not detect any change in the other protein-coding genes surrounding ncRNA-a1 (Figure 3A). To ascertain that ncRNA-a1 is not a component of the *ECM1* 3' untranslated region, we used primer pairs spanning the *ECM1* and ncRNA-a1 genes. We were not able to detect a transcript comprised of the two genes in HEK293 cells, supporting the contention that the two transcripts are independent transcriptional units (Figure S2A). Furthermore, published ChIP experiments (Euskirchen et al., 2007) show the presence of RNA polymerase II and trimethyl H3K4 peaks at the transcription start site of ncRNA-a1 in several cell lines, further attesting to an independent transcriptional start site for ncRNA-a1. Moreover, knocking down the *ECM1* gene did not affect the expression level of ncRNA-a1 or any of the other protein-coding genes analyzed in the locus, further supporting the independence of *ECM1* transcript from that of ncRNA-a1 (Figure S2B).

Next we analyzed ncRNA-a2 flanking the histone demethylase *JARID1B/KDAM 5B* which also shows increased expression following keratinocyte differentiation. These experiments were performed in HeLa cells as they showed detectable expression of ncRNA-a2. Interestingly, while depletion of ncRNA-a2 did not change *JARID1B/KDAM 5B* levels, the *KLHL12*, a gene known for its negative regulation of the Wnt-beta catenin pathway, on the opposite strand displayed a significant reduction (Figure 3B). Although the decrease in *KLHL12* was small (about 20%), no other protein-coding gene in the locus displayed a difference in expression (Figure 3B).

To extend our findings and to determine whether regulation of neighboring protein-coding genes is a common function of ncRNAs, we interrogated the ncRNA-a3 flanking the stem cell leukemia gene (*SCL*, also called *TAL1*). *TAL1* is a basic helix-loop-helix protein which serves as the master regulator of hematopoiesis (Lecuyer and Hoang, 2004). This locus contains two ncRNAs on different strands of DNA. We used MCF-7 cells to assess the depletion of ncRNA-a3, since the expression of ncRNA-a3 and *TAL1* could be readily detected in these cells. However, neither *PDZK1IP1* nor ncRNA-a4 could be detected by qPCR in MCF-7 cells. Depletion of ncRNA-a3 resulted in a specific and potent reduction of *TAL1* expression (Figure 3C). While depletion of ncRNA-a3 did not affect either *STIL* or *CMPK1* genes, a significant reduction in *CYP4A11* gene on the opposite strand of the DNA was detected (Figure 3C).

We next turned our attention to ncRNA-a4 which was not expressed at a detectable level in MCF7 cells. We could reliably



**Figure 2. Long ncRNAs Display Responsiveness to Differentiation Signals in Human Primary Keratinocytes**

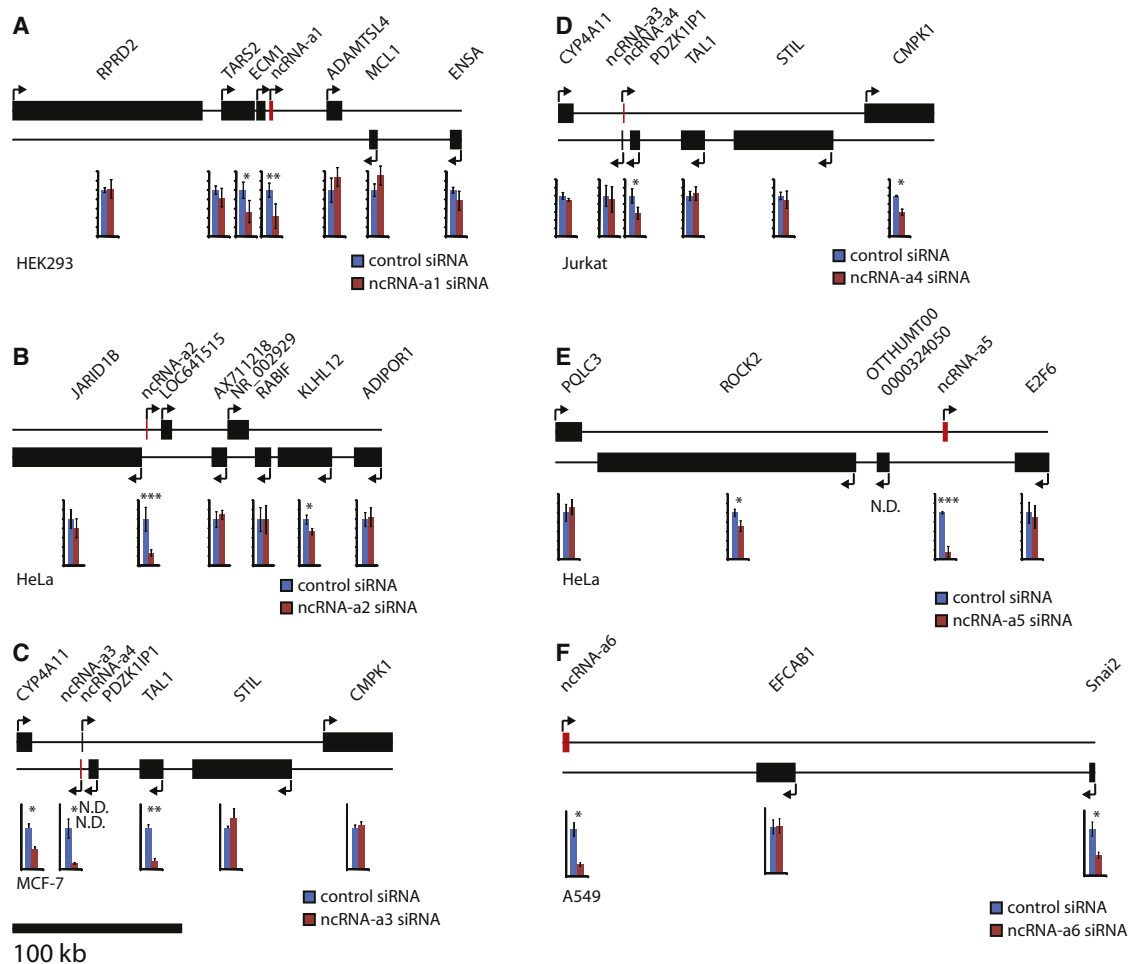
(A and B) Distribution of differentially expressed transcripts (dark colors) following TPA treatment for long ncRNAs (A), and mRNAs (B). Lighter colors show total number of transcripts, darker colors and percentage show number of differentially expressed transcripts. Bar-plots show number and fractions of transcripts induced (red) or repressed (green) at different fold-change cut-offs.

(C) Gene ontology analysis of genes flanking the differentially expressed long ncRNAs (red) compared to genes flanking random positions (black).

(D) Graphic representation of a locus with induction of the long ncRNA ncRNA-a1 and the adjacent ECM1 gene, with expression values from microarrays (upper panel) and qPCR quantification of transcripts (lower panel). Microarray experiments and qPCR validation are done in four replicates. Data shown are mean  $\pm$  SD. See also Figure S2 and Table S3.

detect ncRNA-a4 in Jurkat cells. While we could not efficiently knockdown ncRNA-a3 in Jurkat cells, siRNAs specific to ncRNA-a4 reproducibly reduced its levels by about 50%

(Figure 3D). Importantly, reduced levels of ncRNA-a4 resulted in a consistent and significant decrease in the level of the gene *CMPK1* which is over 150 kb downstream of ncRNA-a4



### Figure 3. Stimulation of Gene Expression by Activating RNAs

The thick black line representing each gene shows the span of the genomic region including exons and introns. The targeted activating RNAs are shown in red. Bar-plots show RNA levels as determined by qPCR.

(A) ncRNA-a1 locus in HEK293 cells.

(B) ncRNA-a2 locus in HeLa cells.

(C) ncRNA-a3 locus in MCF-7 cells.

(D) ncRNA-a4 locus in Jurkat cells.

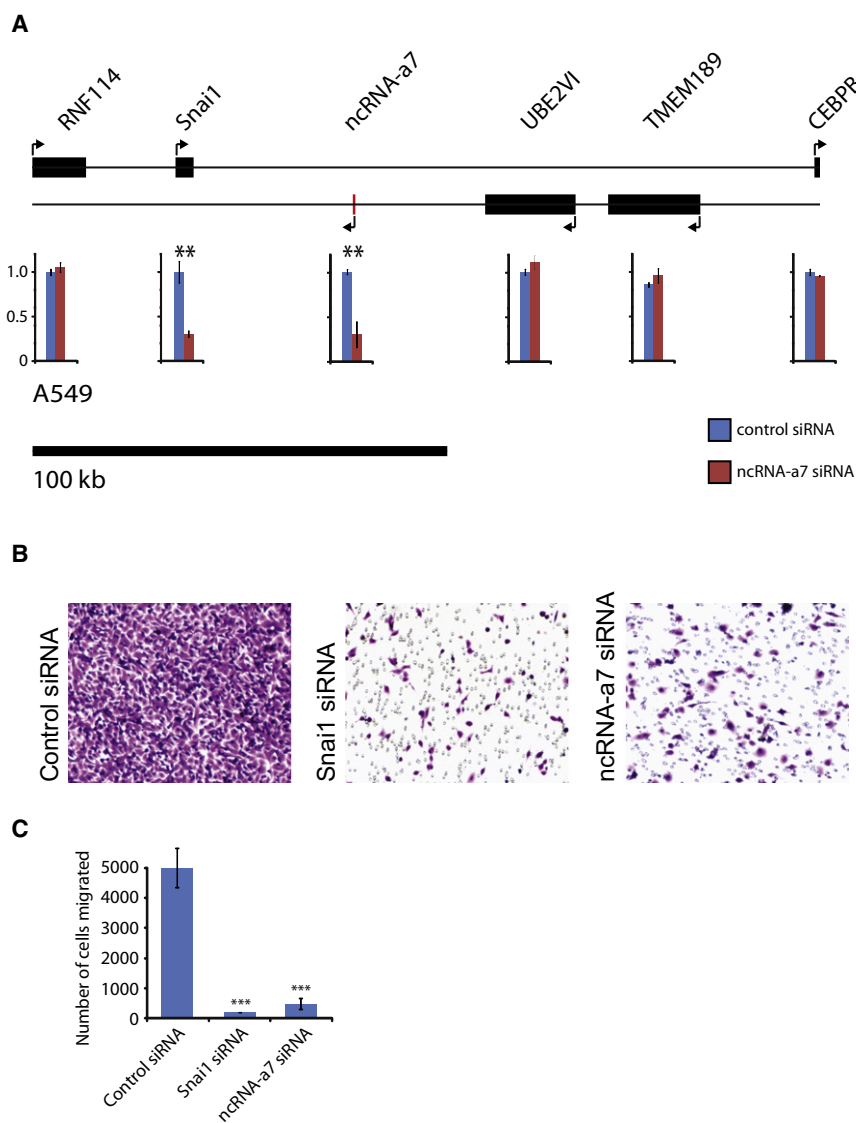
(E) ncRNA-a5 locus in HeLa cells.

(F) ncRNA-a6 locus in A549 cells. All values are relative to GAPDH expression and relative to control siRNA transfected cells set to an average value of 1. The scale bar represents 100 kb and applies to all figure panels. Error bars show mean  $\pm$  SEM of at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by two-tailed Student's *t* test. See also Figure S3 and Table S4. The results represent at least six independent experiments. See also Figure S3 and Table S4.

(Figure 3D). We do not detect any changes in the other protein-coding genes surrounding ncRNA-a4. Next we depleted ncRNA-a5 which is adjacent to the E2F6 gene, an important component of a polycomb-like complex (Ogawa et al., 2002). Knockdown of ncRNA-a5 did not affect the E2F6 gene. However, depletion of ncRNA-a5 resulted in a specific reduction in ROCK2 expression levels in HeLa cells, which is located upstream of ncRNA-a5 (Figure 3E).

Finally, we examined the Snai1 and Snai2 loci in A549 cells (Figure 3F and Figure 4). The Snail family of transcription factors are implicated in the differentiation of epithelia cells into mesenchymal cells (epithelial-mesenchymal transition) during embry-

onic development (Barrallo-Gimeno and Nieto, 2005; Savagner, 2001). Snai2 shows a significant reduction in expression when the adjacent ncRNA-a6 is depleted, an effect that is not seen on EFCAB1, the only other protein-coding gene within 300 kb of the ncRNA-a6 (Figure 3F). In total, we have examined 12 loci where we were able to efficiently knockdown the ncRNAs using siRNAs (Table S5). We were able to show that in 7 cases, the ncRNA acts to potentiate the expression of a protein-coding gene within 300 kb of the ncRNA. It is possible that the remaining ncRNAs which did not display a positive effect on the neighboring genes within the 300 kb window, exert their action over longer distances which was not assessed in our analysis. Taken



**Figure 4. Knockdown of ncRNA-a7 Specifically Targets Snai1 Expression**

(A) As in Figure 3, the ncRNA-a7 locus is depicted showing effects on RNA levels for the surrounding genes with and without knockdown of ncRNA-a7. The results represent mean  $\pm$  SEM of at least six independent experiments. \*\* $p < 0.01$  by one-tailed Student's *t* test.

(B) Migration assay of A549 cells with control (right panel) or ncRNA-a7 (left panel) siRNA transfections.

(C) Quantification of the data shown in (B). Experiments in (B) and (C) are done in three replicates and are shown as mean  $\pm$  SEM. \*\*\* $p < 0.001$  by two-tailed Student's *t* test. See also Figure S4 and Table S5.

in a specific reduction in Snai1 levels (Figure 4A). The expression of the four other protein-coding genes in this locus does not change following the depletion of ncRNA-a7. Concomitantly, knockdown of ncRNA-a7 has a significant phenotypic effect in cell migration assays, reducing the number of migrating cells to about 10% of that of the control (Figures 4B and 4C), consistent with the phenotypic changes following the depletion of Snai1 (Figures 4B and 4C).

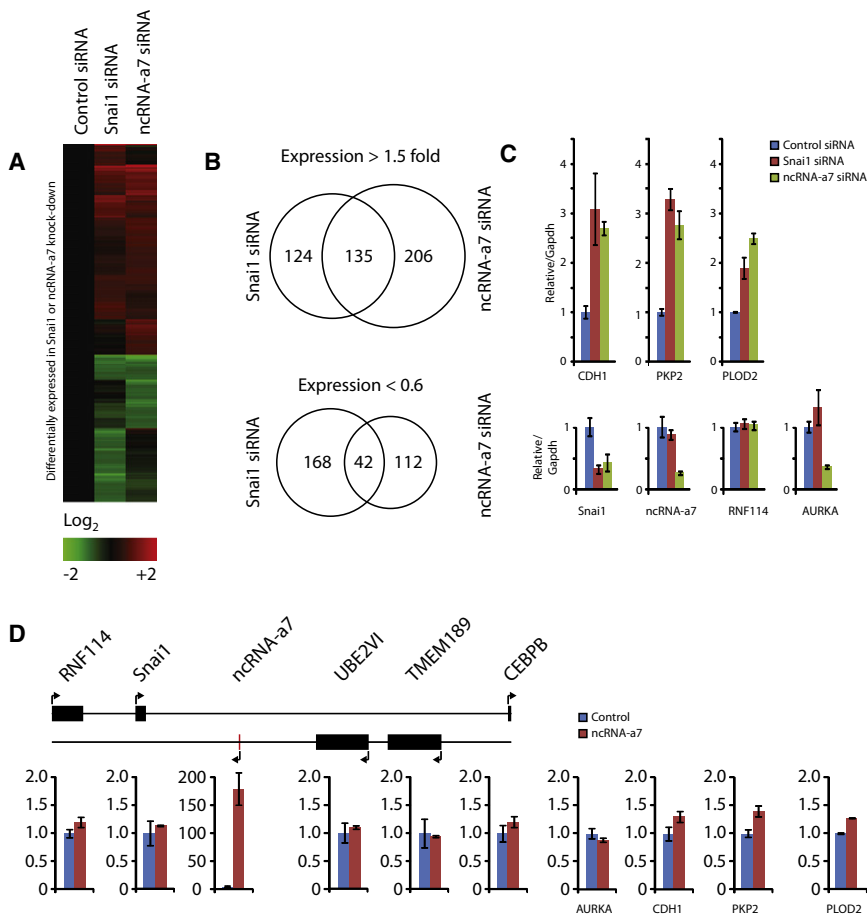
Since the knockdown of ncRNA-a7 or Snai1 had similar consequences on cellular migration, we assessed their depletion on gene expression in A549 cells using Agilent arrays. We could not detect the basal level of Snai1 on the array, while Snai1 was readily detectable using quantitative PCR. Interestingly, depletion of Snai1 or ncRNA-a7 resulted in similar changes in gene expression profiles (Figure 5A and Table S6). Not only did we observe a similar trend in

together, our results indicate that a subset of ncRNAs has activating functions and therefore we have named them ncRNA-activator (ncRNA-a) followed by a number to distinguish each activating long ncRNA.

### ncRNA-a7 Is a Regulator of Snai1

As mentioned above, Snai1 is a member of the Snail zinc-finger family, which comprises transcription factors with diverse functions in development and disease (Barrallo-Gimeno and Nieto, 2005; Nieto, 2002). The Snail gene family is conserved among species from *Drosophila* to human and has been shown to function as mesodermal determinant genes (Barrallo-Gimeno and Nieto, 2005; Nieto, 2002). Snail genes are the regulators of cell adhesion, migration and epithelial-mesenchymal transition (EMT) (Barrallo-Gimeno and Nieto, 2005; Nieto, 2002). Analysis of the ncRNA close to the Snai1 gene provided us with an opportunity to combine our gene expression analysis with analysis of changes in cellular migration. Knockdown of ncRNA-a7 resulted

genes that were affected upon the knockdown of either gene but also a significant number of genes that were upregulated were in common in both treatments (Figures 5A and 5B). Since Snai1 is a known transcriptional repressor, depletion of Snai1 or ncRNA-a7 should result in an upregulation of Snai1 target genes. Indeed, a number of genes that were commonly upregulated were direct targets of Snai1 (Figure 5C, upper panel) (De Craene et al., 2005). Depletion of either ncRNA-a7 or Snai1 also resulted in downregulation of a set of genes with a partial overlap between the genes downregulated following the two treatments (Figure 5B). Interestingly, Aurora-kinase A a gene that is 6 MB down-stream of ncRNA-a7 was specifically downregulated following the depletion of ncRNA-a7, suggesting a long range effect for ncRNA-a7 (Figure 5C). Taken together, these results indicate that while the depletion of ncRNA-a7 partially mimic the gene expression profile observed following Snai1 depletion, there are a number of gene expression changes resulting from the ncRNA-a7 depletion that occur independently



**Figure 5. Microarray Analysis of Snai1 and ncRNA-a7 Knockdown**

Snai1 or ncRNA-a7 were knocked down using siRNA in A549 cells and the isolated RNA analyzed on microarrays in duplicate experiments.

(A) All genes differentially expressed (>1.5-fold or <0.6-fold compared to control) in either Snai1 or ncRNA-a7 knockdown, or both, are shown clustered in a heat map according to expression profile. Numbers are log<sub>2</sub> transformed and color scale is shown below the heat map.

(B) Analysis of genes showing upregulation (>1.5 fold) or downregulation (<0.6 fold) in both Snai1 and ncRNA-a7 knockdown. Numbers represent number of genes regulated in the indicated condition.

(C and D) (C) Validation of microarray data by qPCR and (D) analysis of the Snai1 locus and targets of Snai1 upon overexpression of ncRNA-a7. ncRNA-a7 was overexpressed from a vector in A549 cells and expression of select genes were measured by qPCR. Y-axes show expression value relative to GAPDH of the indicated gene. Values are normalized to those of control siRNA transfected cells, set to 1. \*\*p < 0.01, \*\*\*p < 0.001 by one-tailed Student's t test. See also Table S6.

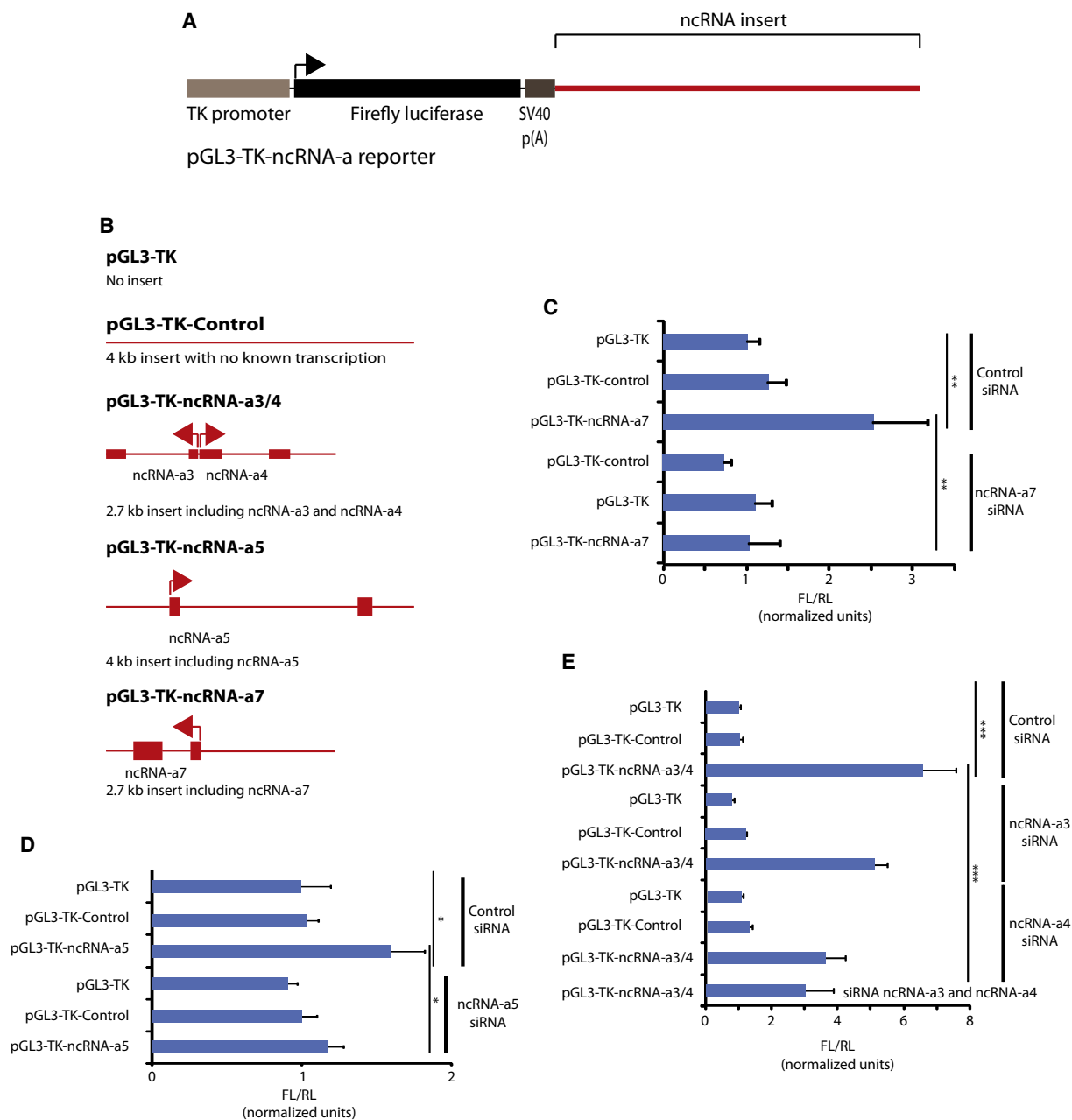
of changes in Snai1. Therefore, it is likely that depletion of ncRNA-a7 may have other effects on gene expression which may be mediated through other targets in *trans*.

To specifically address whether ncRNA-a7 may exert its effects in *trans*, we assessed the gene expression changes in Snai1 locus as well as some of the targets that were changed by depletion of ncRNA-a7 or Snai1 following the overexpression of ncRNA-a7 (Figure 5D). Overall, we did not observe changes in gene expression for any of the ncRNA-a7 targets following its overexpression (Figure 5D, ncRNA-a7 was overexpressed 150 fold). While these results suggest that ncRNA-a7 exerts its local gene expression changes in *cis*, it is likely that other targets may be influenced in *trans*. Taken together, these experiments reveal a role for ncRNA-a in positive regulation of expression of neighboring protein-coding genes and show that this effect is not specific to any one locus and may represent a general function for ncRNAs in mammalian cells.

**ncRNA Activation of Gene Expression of a Heterologous Reporter**

Previous studies have shown that distal activating sequences/enhancers can stimulate transcription when placed adjacent to a heterologous promoter, a methodology widely used to validate potential enhancers (Banerji et al., 1983, 1981; Gillies et al., 1983; Heintzman et al., 2009; Kong et al., 1997). To func-

tionally dissect the influence of the ncRNA activation on the expression of an adjacent gene, we constructed vectors with inserts containing either ncRNA-a3 and -a4 from a bidirectional promoter, ncRNA-a5 or ncRNA-a7, and placed them downstream of Firefly luciferase driven by a thymidine kinase (TK) promoter in a reporter vector (pGL3-TK-ncRNA-a), (Figure 6A). We included 1–1.5 kb upstream of the ncRNA-as to contain their endogenous promoters and 500 bps downstream in the reporter vector. We also produced a control vector (pGL3-TK-control) in which 4 kb of DNA without transcriptional potential was cloned down-stream of Firefly luciferase similar to the ncRNA activation reporters (Figure 6B). A vector containing Renilla luciferase was used to control for transfection efficiency. Importantly, inclusion of either of the three ncRNA-a inserts result in an enhancement of transcription ranging from 2- to 7-fold (Figures 6C–6E). This effect is specific as pGL3-TK-control vector do not enhance the basal TK promoter activity (Figures 6C–6E). To demonstrate that the observed potentiation of gene expression is mediated through the action of ncRNA-a, we knocked down the ncRNA-a in question for each reporter construct using specific siRNAs (Figures 6C–6E). Interestingly while depletion of ncRNA-a7 and ncRNA-a5 completely abolished the increased transcription, depletion of ncRNA-a3 and/or ncRNA-a4 resulted in a partial decrease in transcriptional enhancement (Figures 6C–6E). These results suggest that while ncRNA-a play a major role in transcriptional activation, other DNA elements in the cloned ncRNAa-3/4 region may also contribute to increased transcription.



### Figure 6. ncRNA-Activators Potentiate Transcription of a Reporter Gene

(A) ncRNA-a 3/4, 5 and 7 were cloned and inserted downstream of luciferase driven by a TK-promoter in a reporter plasmid as shown.

(B) Graphical representation of the inserts in the various vectors used. The pGL3-TK-Control vector contains an insert of approximately 4 kb containing no annotated evidence of transcription. The depicted inserts show exons and transcriptional direction of the ncRNA-a.

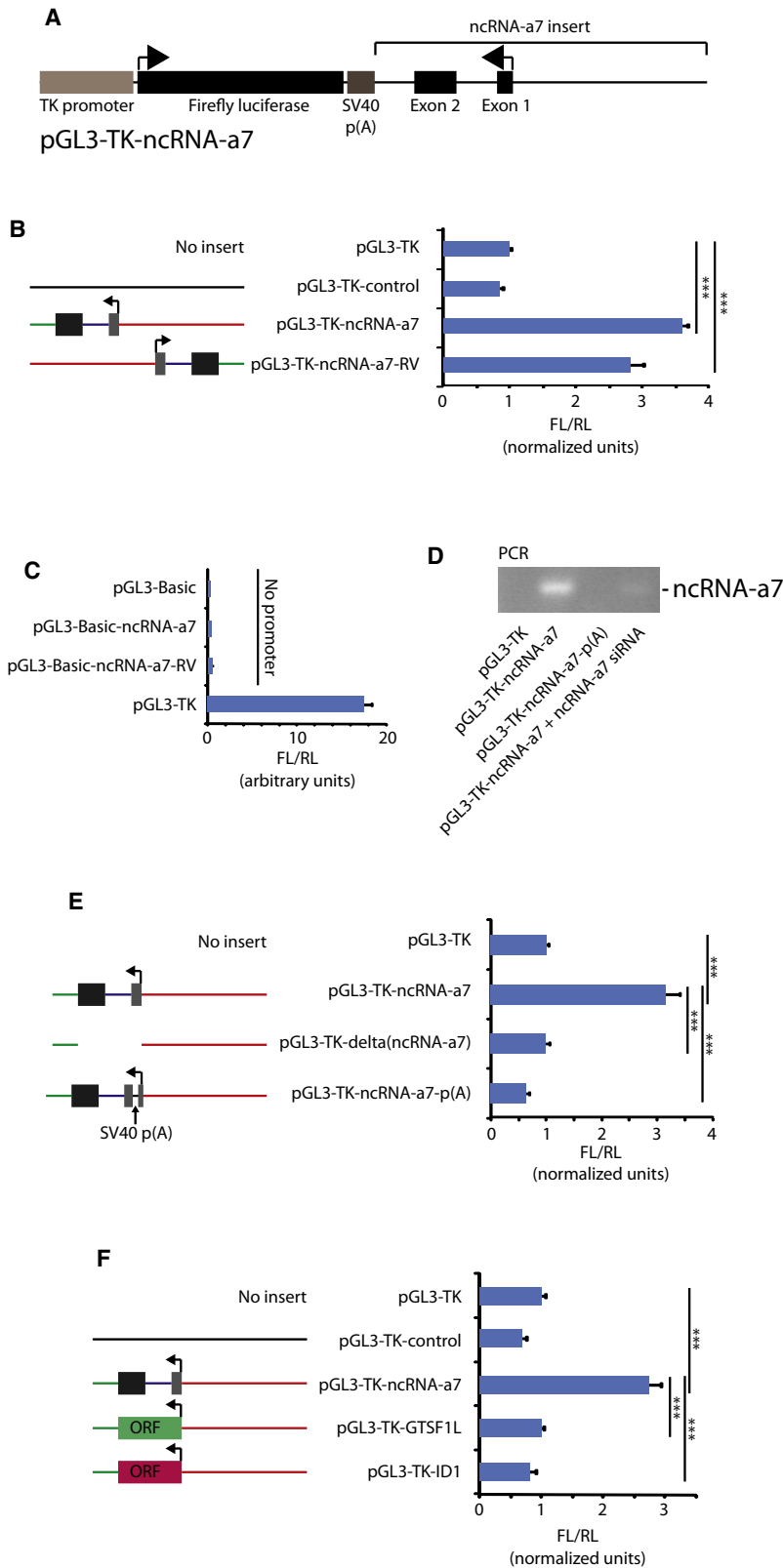
(C–E) Luciferase reporter assays. The Firefly luciferase vectors were cotransfected with a Renilla luciferase vector (pRL-TK) for transfection control. (C) The vector containing ncRNA-a3 and ncRNA-a4 from a bidirectional promoter, with control siRNA or siRNAs toward either of the two ncRNA-a, or both. (D) Reporter with ncRNA-5, and (E) the reporter with the ncRNA-a7 inserted downstream of luciferase. X axes show relative Firefly (FL) to Renilla (RL) luciferase activity. Cotransfected siRNAs are indicated to the right of the bars. All data shown are mean  $\pm$  SE from six independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by one-tailed Student's *t* test.

### Dissection of the ncRNA-a7 in a Reporter Construct

An important property of enhancing sequences is their orientation independence (Imperiale and Nevins, 1984; Khoury and Gruss, 1983; Kong et al., 1997). We designed reporter constructs

(Figure 7A) in which the ncRNA-a7 sequence is reversed (pGL3-TK-ncRNA-a7-RV) in order to assess its orientation independence. The ncRNA-a7-RV construct displayed a similar transcriptional enhancing activity as the construct containing the





**Figure 7. RNA-Dependent Activation of a Reporter Gene by ncRNA-a7**

(A) Properties of the ncRNA-a7 containing luciferase reporter vector. (B, C, E, and F) Luciferase reporter assays. The Firefly luciferase vectors were cotransfected with a Renilla luciferase vector (pRL-TK) for transfection control. (D) Semiquantitative PCR of ncRNA-a7. (B) Reporter experiments with the ncRNA-a7 insert reversed as indicated in the left panel. (C) The TK-promoter driving luciferase expression was deleted from the construct and expression values are shown relative to the pGL3-TK control plasmid as a reference. (E) Truncated reporter constructs containing the ncRNA-a7 promoter and downstream sequences, but not the ncRNA-a7 sequence [pGL3-TK-delta(ncRNA-a7)], or one with a poly(A) signal in the beginning of the ncRNA-a7 to induce premature polyadenylation [pGL3-TK-ncRNA-a7-p(A)]. See also (D) for analysis of expression from these plasmids. (F) Protein coding sequences were inserted in place of ncRNA-a7 downstream of the ncRNA-a7 promoter. Full-length GTSF1L or ID1 sequences are used. X axes show relative Firefly (FL) to Renilla (RL) luciferase activity. All data shown are mean  $\pm$  SE from six independent experiments. \*\*\* $p < 0.001$  by one-tailed Student's t test.

ncRNA-a7 insert in its endogenous orientation with respect to the regulated gene (Figure 7B).

To show that luciferase expression requires a promoter and that ncRNA-7a cannot act as a proximal promoter, we deleted the TK promoter from the reporter vectors. As shown in Figure 7C, ncRNA-a7 cannot drive transcription of the Firefly luciferase in the absence of a proximal TK promoter. These experiments demonstrate that sequences corresponding to ncRNA-a7 transcription unit can function to activate expression of a heterologous promoter in an orientation-independent manner, but cannot act as a promoter itself.

To further verify that ncRNA-a7 is the active component of the transcriptional enhancement, we constructed two reporters in which ncRNA-a7 sequences are either deleted or shortened by placing a strong polyadenylation signal within the ncRNA-a genomic sequence but close to the transcriptional start site, to induce premature polyadenylation (Figures 7D and 7E). Both modifications result in loss of the increased gene expression (Figure 7E) compared to constructs where ncRNA-a7 is expressed. Finally, to assess whether the RNA corresponding to ncRNA-7a is critical for increased gene expression, we developed constructs where DNA sequences corresponding to two different protein-coding genes were positioned in the place of ncRNA-a7 (Figure 7F), keeping the endogenous ncRNA-a7 promoter. Neither of these constructs displayed an increased gene expression compared to that of the control constructs (Figure 7F). Taken together, these experiments demonstrate that the potentiation of gene expression is signaled by the ncRNA-a and is not merely the result of the transcription of the ncRNA.

## DISCUSSION

We used the annotation of the human genome performed by GENCODE to arrive at a collection of long ncRNAs that are expressed from loci independent of those of protein-coding genes or previously described ncRNAs. GENCODE annotation encompasses both protein-coding and noncoding transcripts and relies on experimental data obtained through the analysis of cDNAs, ESTs and spliced RNAs. Our collection of ~3,000 transcripts correspond to the manual curation of about a 1/3 of the human genome. Analysis of the GENCODE data indicates that nearly all of their noncoding annotated transcripts are spliced (Figure S1A).

Importantly, the median distance of an ncRNA transcript to a protein-coding gene is over a 100 kb making it an unlikely scenario for the ncRNA to be an extension of protein-coding transcripts (Figures S2C and S2D). Moreover, transcriptionally active ncRNAs display similar chromatin modifications seen with expressed protein-coding genes (Figures S1B and S1C). Furthermore, the analyzed ncRNAs display RNA pol(II), p300 and CBP occupancy at levels similar to those of the surrounding protein coding genes, consistent with their transcriptional independence (Figure S4). Although our analysis is focused on understanding the function of a set of ncRNAs annotated by GENCODE, the human transcriptome includes other forms of ncRNAs with important regulatory functions that have not been included in our study. These include the antisense transcripts

arising from protein-coding genes, precursors of microRNAs as well as a wealth of unspliced transcripts described in multiple studies (Guttman et al., 2009; Kapranov et al., 2007; Rinn et al., 2007).

Taken together, the novelty of our work lies in the following. First, we show that at multiple loci of the human genome depletion of a long ncRNA leads to a specific decrease in the expression of neighboring protein-coding genes. Previous studies analyzing the function of long ncRNAs in X-inactivation or the imprinting phenomenon point to their role in silencing of gene expression (Mattick, 2009). Second, we show that the enhancement of gene expression by ncRNAs is not cell specific as we observe the effect in five different cell lines. Third, this enhancement of gene expression is mediated through RNA, as depletion of such activating ncRNAs abrogate increased transcription of the neighboring genes. Fourth, through the use of heterologous reporter assays, we suggest that activating ncRNAs mediate this RNA-dependent transcriptional responsiveness in *cis*. Fifth, we show that similar to classically defined distal activating sequences, ncRNA-mediated activation of gene expression is orientation independent. Sixth, we present evidence that similar to defined activating sequences, ncRNAs cannot drive transcription in the absence of a proximal promoter. Finally, we demonstrate that the activation of gene expression in the heterologous reporter system is mediated through RNA as multiple approaches depleting the RNA levels lead to abrogation of the stimulatory response. Therefore, we have uncovered a new biological function in positive regulation of gene expression for a class of ncRNAs in human cells.

There are previous reports of individual ncRNAs having a positive effect on gene expression. The ~3.8 kb *Evf-2* ncRNA was shown to form a complex with the homeodomain-containing protein Dlx2 and lead to transcriptional enhancement (Feng et al., 2006). Similarly, the ncRNA *HSR1* (heat-shock RNA-1) forms a complex with HSF1 (heat-shock transcription factor 1), resulting in induction of heat-shock proteins during the cellular heat-shock response (Shamovsky et al., 2006) and an isoform of ncRNA SRA (steroid receptor RNA activator) functions to coactivate steroid receptor responsiveness (Lanz et al., 1999). Our findings that activating ncRNAs positively regulate gene expression extend these previous studies and demonstrate that the activation of gene expression by long ncRNA may be a general function of a class of long ncRNAs. Moreover, whether ncRNA effects seen in our study are mediated through association with specific transcriptional activators is not known. However, this is a likely scenario given previous examples of an RNA-mediated responsiveness. Other possibilities include a formation of an RNA-DNA hybrid at the locus of the ncRNA or the protein-coding gene which may result in enhanced binding of the sequence specific DNA binding proteins or chromatin modifying complexes.

A recent study uncovers a set of bidirectional transcripts (termed eRNA) that are derived from sites in the human genome that show occupancy by CBP, RNA polymerase II and are decorated by monomethyl Histone H3 lysine 4 (H3K4) (Kim et al., 2010). Moreover, they show that the expression of such transcripts is correlated with their nearest protein-coding genes. There are fundamental differences between their collection of ~2000 transcripts and our GENCODE set of transcripts. First,

while all their eRNAs are bidirectional, only about 1% of our ncRNAs show evidence of bidirectionality (see the example shown in the TAL1 locus). Second, our analysis of the histone modifications of a subset of ncRNAs that are expressed in lymph (Barski et al., 2007) indicates the presence of H3K4 trimethylation at the transcriptional start sites and H3K36 trimethylation at the body of the gene (Figures S1B and S1C). This is in stark contrast to eRNA loci where there is an absence of H3K4 trimethyl marks and the predominant chromatin signature is the monomethyl H3K4. Third, eRNAs are reported to be predominantly not polyadenylated. The majority of our collection of ncRNAs show evidence of polyadenylation as they were amplified using oligo-dT-primed reactions and furthermore 41% display the presence of a canonical polyadenylation site. Analysis of the protein-coding transcripts revealed that a similar proportion (52%) to that of our ncRNAs contain the canonical polyadenylation sites. Finally, while we show that a set of our ncRNAs function to enhance gene expression, there is no evidence provided for eRNAs exerting a biological function. While we believe that eRNAs designate a different class of ncRNAs than ncRNA-a described in our study, it is tempting to speculate that many of the ncRNA-a and their promoters may correspond to mammalian enhancers or polycomb/trithorax response elements (PRE/TREs). In such a scenario, binding of polycomb or trithorax proteins to proximal promoters of ncRNA-a will regulate the expression of ncRNA-a which in turn impact the expression of the protein-coding gene at the distance.

Another set of recently published ncRNAs were termed long intervening noncoding RNA or lincRNAs (Guttman et al., 2009). The comparison of our ncRNAs and the lincRNAs show that about 13% of the ncRNAs defined by ENCODE overlap the broad regions encoding a set of recently identified human lincRNAs (Khalil et al., 2009). The overlap between our ncRNAs and lincRNAs are even smaller (~4%) if one considers only the exons corresponding to lincRNAs. Importantly, the studies with lincRNAs did not reveal any transcriptional effects in neighboring genes (Khalil et al., 2009). Therefore, it is likely that lincRNAs describe a distinct set of ncRNAs compared to those annotated by GENCODE. Similar to the diverse functions for proteins, ncRNAs such as lincRNAs may play other roles in regulating gene expression.

The GENCODE annotation used in this study encompasses only a third of the human genome. Therefore, the number of ncRNAs in human cells is likely to grow and may equal or even surpass the number of protein-coding genes. Our considerations for selection of ncRNAs excluded all ncRNAs associated with protein-coding genes and their promoters, as well as known ncRNAs. Therefore, the repertoire of the noncoding transcripts in human cells contains many more transcripts than those cataloged in this study. Specifically, there have been reports of pervasive amount of antisense transcription as well as transcription mapping to promoter regions of protein-coding genes (Core et al., 2008; Denoeud et al., 2007; Kapranov et al., 2007; Preker et al., 2008; Seila et al., 2008). Whether such transcripts will have biological functions similar to those described for activating ncRNAs in our study is not known. However, it is clear that future genome-wide genetic analysis of ncRNAs in mammalian cells will begin to shed light on different classes of the ncRNAs.

The precise mechanism by which our ncRNAs function to enhance gene expression is not known. We envision a mechanism by which ncRNAs by virtue of sequence or structural homology targets the neighboring protein-coding genes to bring about increased expression. Our experimental evidence using a heterologous promoter point to the mechanism of action for activating ncRNAs operating in *cis*. However, genome-wide analysis following depletion of ncRNA-a7 suggested changes in gene expression that may not be related to the action of ncRNA-a7 on its local environment and may be a result of wider trans-mediated effects of ncRNA-a7. Such regulatory functions of ncRNAs could be achieved through an RNA-mediated recruitment of a transcriptional activator, displacement of a transcriptional repressor, recruitment of a basal transcription factor or a chromatin-remodeling factor. While we favor a transcriptional based mechanism for ncRNA activation, effects on RNA stability cannot be excluded. Taken together, the next few years will bring about new prospects for the long ncRNAs as central players in gene expression.

## EXPERIMENTAL PROCEDURES

### Extracting Long ncRNA Data

The HAVANA annotation has been downloaded using the DAS server provided by the Sanger institute (version July, 16th 2008). We removed all annotated biotypes or functional elements belonging to specific categories such as pseudogenes or protein-coding genes. We excluded all transcripts overlapping with known protein coding loci annotated by HAVANA, RefSeq or UCSC. Transcripts falling into a 1 kb window of any protein-coding gene were also removed. Finally, we excluded all transcripts covered by known noncoding RNAs such as miRNAs (miRbase version 11.0 April 2008).

To estimate the evolutionary constraints among mammalian sequences we constructed the cumulative distribution of PhastCons scores for ancestral repeats (ARs), RefSeq genes and long ncRNAs. The cumulative distributions of these transcripts or repeats are plotted using a log-scale on the y axis.

### Cell Culture and siRNA Transfections

Human primary keratinocytes from four different biological donors were grown in Keratinocyte medium (KFSM, Invitrogen). Differentiation was induced by 2.5 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) during 48 hr.

HEK293, A549, HeLa, and MCF-7 cells were cultured in complete DMEM media (GIBCO) containing 10% FBS, and 1× Anti/Anti (GIBCO). Jurkat cells were cultured in complete RPMI media (GIBCO) containing 10% FBS and 1× Anti/Anti (GIBCO). Migration assays were performed as previously described (Gumireddy et al., 2009).

For transfections of 293, HeLa, A549, and MCF-7 cells we used Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and an siRNA concentration of 50 nM. Jurkat cells were transfected using HiPerFect (QIAGEN) according to the manufacturer's recommendations and an siRNA concentration of 100 nM.

### RNA Purification, cDNA Synthesis, and Quantitative PCR

Cells were harvested and resuspended in TRIzol (Invitrogen) and RNA extracted according to the manufacturer's protocol. cDNA synthesis was done using MultiScribe reverse transcriptase and random primers from Applied Biosystems. Quantitative PCR was done using SybrGreen reaction mix (Applied Biosystems) and an HT7900 sequence detection system (Applied Biosystems). For all quantitative PCR reactions Gapdh was measured for an internal control and used to normalize the data.

### Cloning of pGL3-TK Reporters and Luciferase Assay

pGL3-Basic was digested with BglII and HindIII and the TK promoter from pRL-TK was inserted into these sites. Inserts were amplified from genomic

DNA and cloned into the BamHI and Sall sites 5' to the luciferase gene. Luciferase assays were performed in 96-well white plates using Dual-Glo (Promega) according to the manufacturer's protocol.

### Microarrays

Custom-made microarrays (Agilent) were designed based on the library of 3019 long ncRNA sequences, with on average six probes targeting each transcript. Human whole genome mRNA arrays were from Agilent (G4112F). Total RNA samples were converted to cDNA using oligo-dT primers. Labeling of the cDNA and hybridization to the microarrays were performed according to Agilent standard dye swap protocols. Data analysis was done using the AFM 4.0 software. All microarrays were done in four biological replicates.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and six tables and can be found with this article online at doi:10.1016/j.cell.2010.09.001.

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