Molecular Cell
Short Article

The Noncoding RNA *Mistral* Activates *Hoxa6* and *Hoxa7* Expression and Stem Cell Differentiation by Recruiting MLL1 to Chromatin

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DOI 10.1016/j.molcel.2011.08.019

SUMMARY

The epigenetic activator Mixed lineage leukemia 1 (MLL1) is paramount for embryonic development and hematopoiesis. Here, we demonstrate that the long, noncoding RNA (lncRNA) *Mistral* (*Mira*) activates transcription of the homeotic genes *Hoxa6* and *Hoxa7* in mouse embryonic stem cells (mESC) by recruiting MLL1 to chromatin. The *Mira* gene is located in the spacer DNA region (SDR) separating *Hoxa6* and *Hoxa7*, transcriptionally silent in mESCs, and activated by retinoic acid. *Mira*-mediated recruitment of MLL1 to the *Mira* gene triggers dynamic changes in chromosome conformation, culminating in activation of *Hoxa6* and *Hoxa7* transcription. *Hoxa6* and *Hoxa7* activate the expression of genes involved in germ layer specification during mESC differentiation in a cooperative and redundant fashion. Our results connect the lncRNA *Mira* with the recruitment of MLL1 to target genes and implicate lncRNAs in epigenetic activation of gene expression during vertebrate cell-fate determination.

INTRODUCTION

In *Arthropods* and *Chordates*, *Hox* genes play pivotal roles in cell-fate determination (Carroll, 1995). The 39 *Hox* genes present in vertebrate genomes are organized into four gene clusters (*Hoxa-d*) and encode for transcription factors, which regulate the activities of specific target genes (Maconochie et al., 1996; Svingen and Tonissen, 2006). Differential *Hox* gene expression involves long, noncoding RNAs (lncRNAs), epigenetic activators of the Trithorax group (TrxG), and epigenetic repressors of the Polycomb group (PcG) (Ringrose and Paro, 2004; Sanchez-Elsner et al., 2006; Rinn et al., 2007). Epigenetic activation of *Hox* transcription can involve trimethylation of lysine 4 in H3 [H3K4(me_3)] by the TrxG activator Mixed lineage leukemia 1 (MLL1), whereas PcG proteins silence *Hox* gene expression (Guenther et al., 2005; Wang et al., 2009; Margueron and Reinberg, 2011). Polycomb repressive complexes maintain the pluripotency and self-renewal of embryonic stem cells (ESCs) by silencing *Hox* genes and other developmental regulators (Boyer et al., 2006; Bracken et al., 2006). Cell differentiation coincides dynamic changes of the epigenetic landscape of *Hox* genes as evident by the exchange of epigenetic factors at *Hox* genes and the transcription of lncRNAs, which originate from the spacer DNA regions (SDR) separating *Hox* genes (Guenther et al., 2005; Boyer et al., 2006; Sessa et al., 2007; Dinger et al., 2008; Ørom et al., 2010). LncRNAs have emerged as important regulators of gene silencing in vertebrates (Rinn et al., 2007; Khalil et al., 2009; Tsai et al., 2010). Although vertebrate lncRNAs have been associated with transcriptional activation and the lncRNA *HOTTIP* is involved in the activation of *Hox* genes by maintaining the association of MLL1 with chromatin, the role of lncRNAs in recruitment of epigenetic activators to target genes remains unclear (Zhang et al., 2009; Ørom et al., 2010; Wang et al., 2011).

Here we show that the lncRNA *Mistral* (*Mira*) mediates MLL1-dependent transcriptional activation of *Hoxa6* and *Hoxa7* by recruiting MLL1 to chromatin. *Mira*-mediated activation of *Hoxa6* and *Hoxa7* instigates the expression of genes involved in germ-layer specification in differentiating mouse ESCs (mESCs). Our results connect lncRNAs with recruitment of epigenetic activators to target genes in differentiating cells.

RESULTS

MLL1 Associates with *Mira* in RA-Induced mESCs

To investigate whether lncRNAs are involved in epigenetic activation of gene expression by recruiting MLL1 to chromatin, we used a native RNA-chromatin immunoprecipitation (RNA ChIP) coupled to DNA microarray (RNA ChIP-on-chip) assay designed to detect the interaction of MLL1 with lncRNAs in differentiating mESCs, which had been treated with all-*trans* retinoic acid (RA) that is known to induce lncRNA transcription (Sessa et al., 2007). RA induced the transcription of *Hox* genes and attenuated the expression of pluripotency markers in mESCs (Figures 1A and S1A; Table S1). We compared the association of MLL1 with lncRNAs in chromatin isolated from undifferentiated mESCs

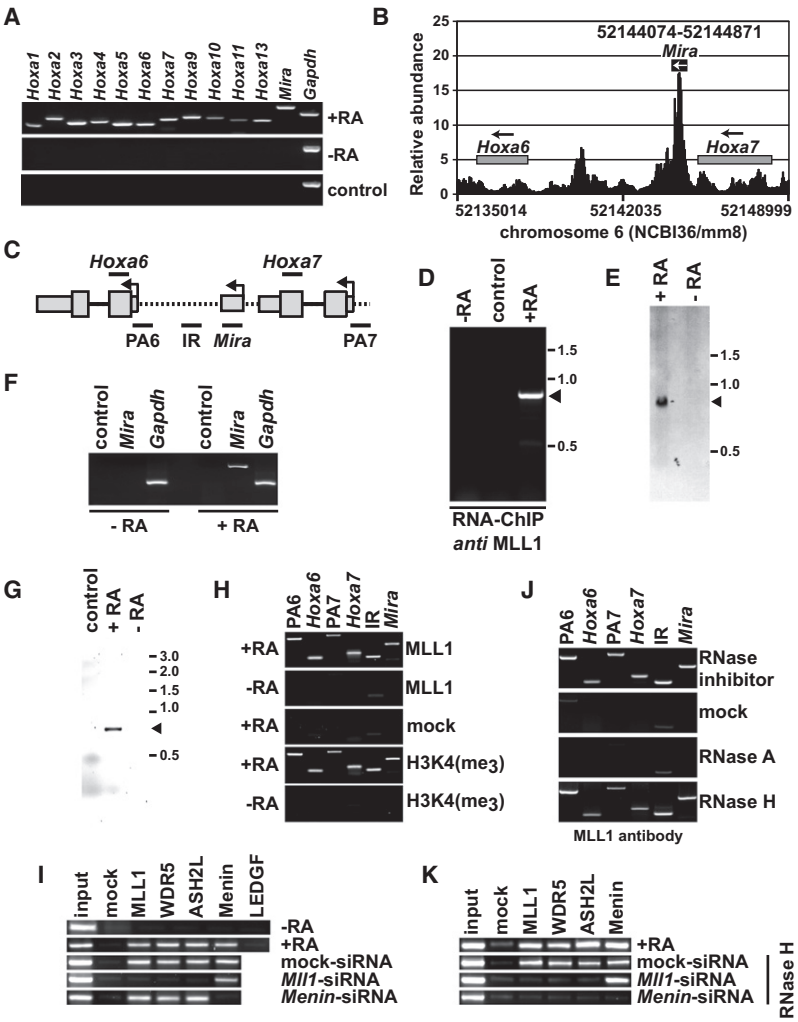

Figure 1. Identification and Characterization of *Mira*

(A) Transcription of *Hoxa* genes, *Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh)*, and *Mira* in undifferentiated (−RA), RA-treated (+RA), and control (differentiated in the absence of RA) mESCs detected by RvT-PCR.

(B) RNA ChIP-on-chip assays detecting the interaction of MLL1 with chromatin-associated *Mira* in +RA mESCs. The relative abundance of RNAs, which associate with MLL1 in chromatin, is plotted over the corresponding template DNA of the *Hoxa* cluster (nucleotides 52135014-5214899) on chromosome 6. The position and transcriptional orientation (arrows) of *Mira*, *Hoxa6*, and *Hoxa7* are indicated.

(C) Structure of the *Hoxa6/a7* cassette. Bars indicate the positions of probes in the *Hoxa6/a7* cassette, which detected the *Mira* gene locus, an untranscribed region (IR), the promoter of *Hoxa6* (PA6) and *Hoxa7* (PA7), and the coding regions of *Hoxa6* and *Hoxa7* in ChIP assays. Arrows indicate the transcriptional start site of genes.

(D) RNA ChIP assays detecting the association of MLL1 with *Mira* in native chromatin isolated from −RA, control, and +RA mESCs.

(E) Primer-extension assays detecting *Mira* transcript (arrowhead) in RNA isolated from +RA and −RA mESCs.

(F) RvT-PCR assays detecting full-length *Mira*, an untranscribed region of the *Hoxa6/a7*-SDR (control) (Table S3), and *Gapdh* mRNA in control, −RA, and +RA mESCs.

(G) Northern blot assays detecting *Mira* (arrowhead) in +RA and −RA mESCs. In (D), (E), and (G), the positions of size markers are indicated to the right.

(H) ChIP assays detecting the presence of MLL1 and H3-K4(me_3) at the *Hoxa6/a7*-SDR in chromatin isolated from −RA and +RA mESCs. Chromatin was precipitated with antibodies to MLL1, H3-K4(me_3), or rabbit serum (mock).

(I) ChIP assays detecting the association of the MLL1 complex, Menin, and LEDGF with the *Mira* gene in −RA, +RA mESCs, and +RA mESCs lacking Menin or MLL1 through RNAi. Chromatin was precipitated with antibodies to the indicated antigens and rabbit serum (mock).

(J) RNase-ChIP assays detecting the association of MLL1 with the *Hoxa6/a7* cassette. Chromatin was isolated from +RA mESCs and treated with BSA and RNase inhibitor (mock), RNase A, or RNase H.

(K) ChIP assays as in (I) except that RNase H treated chromatin was used. See also Figures S1, S2, and S3.

(−RA mESCs) and mESCs, which had been differentiated in the absence (control mESCs) or presence (+RA mESCs) of RA. We uncovered a chromatin-associated RNA (termed *Mistral*), which associated with MLL1 in the chromatin of +RA mESCs and originated from the spacer DNA region (SDR) separating *Hoxa6* and *Hoxa7* (*Hoxa6/a7*-SDR) (Figures 1B–1D and S1B; Tables S2 and S3). Molecular assays revealed that *Mira* is a 798 nt, unspliced, and polyadenylated transcript (Figures 1E–1G; Tables S2 and S3). RA activated *Mira* and *Hoxa* transcription (Figures 1A and 1D–1G). *Mira* transcription preceded *Hoxa6* and *Hoxa7* transcription (Figures S1C and S1D). *Mira* contains only short open-reading frames, which share no significant homology with any known protein, and did not associate with polysomes, the translational entity of the cell (Figures S2A–S2C).

MLL1 Controls *Hoxa6* and *Hoxa7* Transription

MLL1 is an integral subunit of protein complexes, which can contain WDR5, ASH2L, and RBBP5 (Nakamura et al., 2002). We detected MLL1 and H3K4(me_3), at the transcriptionally active but not silent *Hoxa6/a7* cassette, which consists of the *Mira* gene, the *Hoxa6/a7*-SDR, and the promoter and coding regions of *Hoxa6* and *Hoxa7* (Figure 1H; Tables S3 and S4). Numerous factors such as Menin and LEDGF can recruit MLL1 to target genes (Milne et al., 2005; Yokoyama and Cleary, 2008; Wang et al., 2009). We detected the MLL1 complex (MLL1, WDR5, and ASH2L) and Menin at the *Mira* gene locus (Figure 1I). RvT-PCR and ChIP assays using RNA and chromatin, respectively, isolated from cells lacking MLL1 or Menin through RNAi uncovered that Menin and MLL1 cooperatively activated *Hoxa6* and *Hoxa7* but not *Mira* transcription (Figures S2D and S2E; Table S2), and that Menin is not involved in the recruitment of MLL1 to the *Hoxa6/a7* cassette (Figure 1I).

We performed RNase-ChIP experiments to assess whether the association of MLL1 with the *Hoxa6/a7* cassette is RNA dependent. RNase A, which degrades single-stranded RNA (ssRNA), degraded *Mira* (Figure S3A) and abrogated the

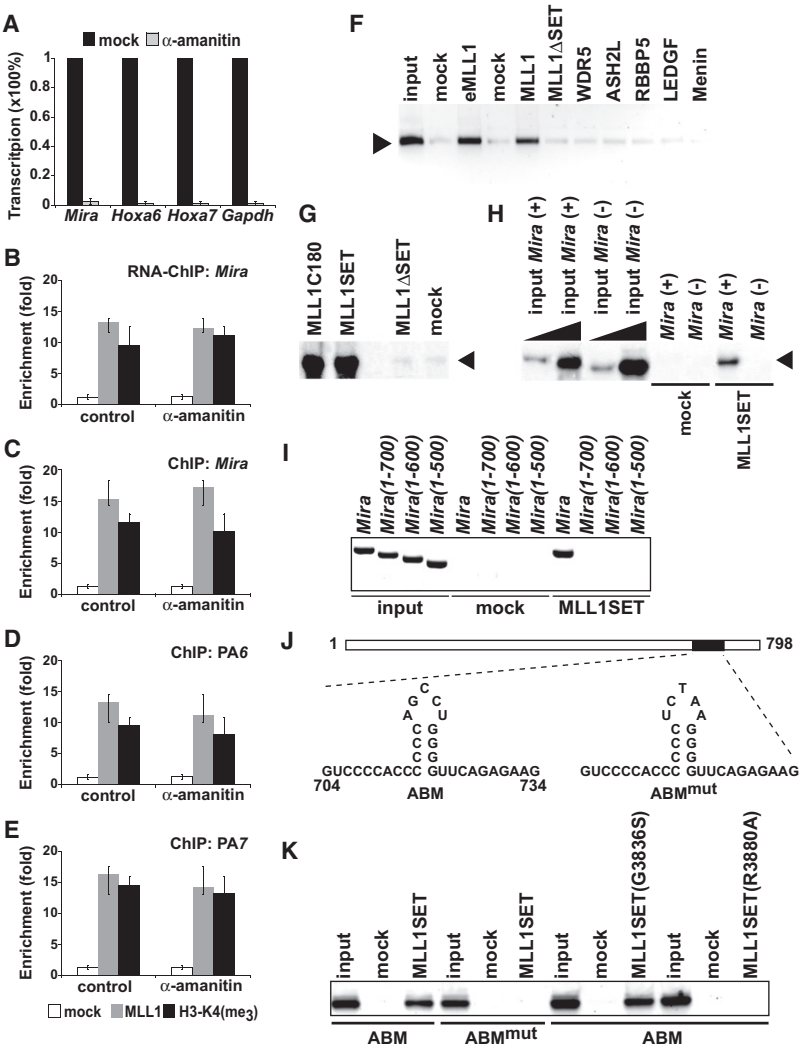

Figure 2. MLL1 Interacts with *Mira*

(A) Nuclear run-on assays detecting *Mira*, *Hoxa6*, *Hoxa7*, and *Gapdh* transcription in nuclei of +RA mESCs, which had been incubated with tetracycline (mock) or α-amanitin.

(B) RNA ChIP assays detecting the association of MLL1 with *Mira* in native chromatin of +RA mESCs, which had been treated with tetracycline (mock) or α-amanitin. Native chromatin was immunoprecipitated with antibodies to MLL1, H3K4 (me_3), and rabbit serum (mock).

(C–E) ChIP assays detecting the association of MLL1 with the *Mira* gene locus (C) and the promoter of *Hoxa6* (D) and *Hoxa7* (E) in +RA cells described in (B). Error bars in (A)–(E) represent the standard error of the mean (SEM).

(F) In vitro protein-RNA binding assays detecting the interaction of *Mira* with endogenous MLL1 (eMLL1), recombinant MLL1C180, MLL1ΔSET, which lacks the SET domain, WDR5, ASH2L, RBBP5, LEDGF, and Menin.

(G) In vitro binding assays detecting the interaction of *Mira* with recombinant MLL1SET, MLL1ΔSET, MLL1C180, and ASH1SET (mock).

(H) In vitro binding assays detecting the interaction of sense (+) and anti-sense (–) *Mira* with MLL1ΔSET and ASH1SET (mock).

(I) In vitro binding assays detecting the interacting of truncated *Mira* transcripts with MLL1SET or ASH1SET (mock).

(J) Schematic representation of (top) *Mira* and (bottom) wild-type and mutant ABM.

(K) In vitro binding assays detecting the interaction of MLL1SET, MLL1SET(G3836S), and MLL1(R3880A) with wild-type and mutant ABM. In (F), (H), (I), and (K), input represents 15% of the radiolabeled RNA present in in vitro binding assays. See also Figures S2 and S3.

association of MLL1 with the *Hoxa6/a7* cassette (Figure 1J). RNase H, which degrades RNA in RNA/DNA hybrids, did not affect the association of MLL1 with the *Hoxa6/a7* cassette (Figures 1J and S3A) in +RA mESCs, but attenuated the association of MLL1 with *Mira* and the *Mira* gene in cells lacking Menin through RNAi, suggesting that Menin prevents degradation of *Mira* by RNase H and maintains a RNA/chromatin structure at the *Mira* gene (Figures 1K and S3A).

We used nuclear run-on and ChIP assays to investigate whether *Mira* or *Mira* transcription per se mediates recruitment of MLL1 to the *Hoxa6/a7* cassette. RNA and chromatin were obtained from nuclei of +RA mESCs, which had been incubated with tetracycline (mock) or the transcription inhibitor α-amanitin. α-amanitin inhibited the transcription of *Mira*, *Hoxa6*, and *Hoxa7* in +RA mESCs (Figure 2A), but did not affect the association of MLL1 with *Mira* (Figure 2B) and the *Hoxa6/a7* cassette (Figures 2C–2E), suggesting that the association of MLL1 with the *Hoxa6/a7* cassette is RNA dependent and implicating *Mira* in the association of MLL1 with chromatin.

Mira Activates *Hoxa6* and *Hoxa7* Transcription by Recruiting MLL1 to Chromatin

To assess whether *Mira* can recruit MLL1 to chromatin, we performed in vitro RNA-protein binding assays to test whether *Mira* interacts with MLL1 complex. *Mira* interacted with the endogenous MLL1 complex and recombinant MLL1C180, but not other MLL1 subunits, Menin, or LEDGF (Figures 2F, S3B, and S3C). The SET domain of MLL1 (MLL1SET) bound *Mira* but not control RNA (Figures 2G, 2H, and S3D). MLL1SET interacted with a hairpin RNA loop (Activator binding motif [ABM]) located in the 3′ region of *Mira* (Figures 2I–2K). To identify amino acid residues involved in the interaction of MLL1SET with *Mira* we compared the interaction of the ABM with mutant MLL1 proteins. The mutant MLL1(R3768A), which does not bind WDR5 (Cosgrove and Patel, 2010), did interact with *Mira* (Figure S3E). The mutant MLL1SET(G3836S), which does not bind single-stranded DNA (ssDNA) (Krajewski et al., 2005), did bind *Mira* (Figure S3F) and the ABM (Figure 2K). MLL1SET(R3880A), which contains a mutation in a nonconserved arginine-residue of the SET domain (Cosgrove and Patel, 2010), failed to bind the ABM, suggesting that specific amino acid residues are involved in the association of MLL1SET with *Mira* (Figure 2K).

Next, we investigated whether the RNAi-mediated destruction of *Mira* affects the recruitment of MLL1 to the *Hoxa6/a7* cassette.

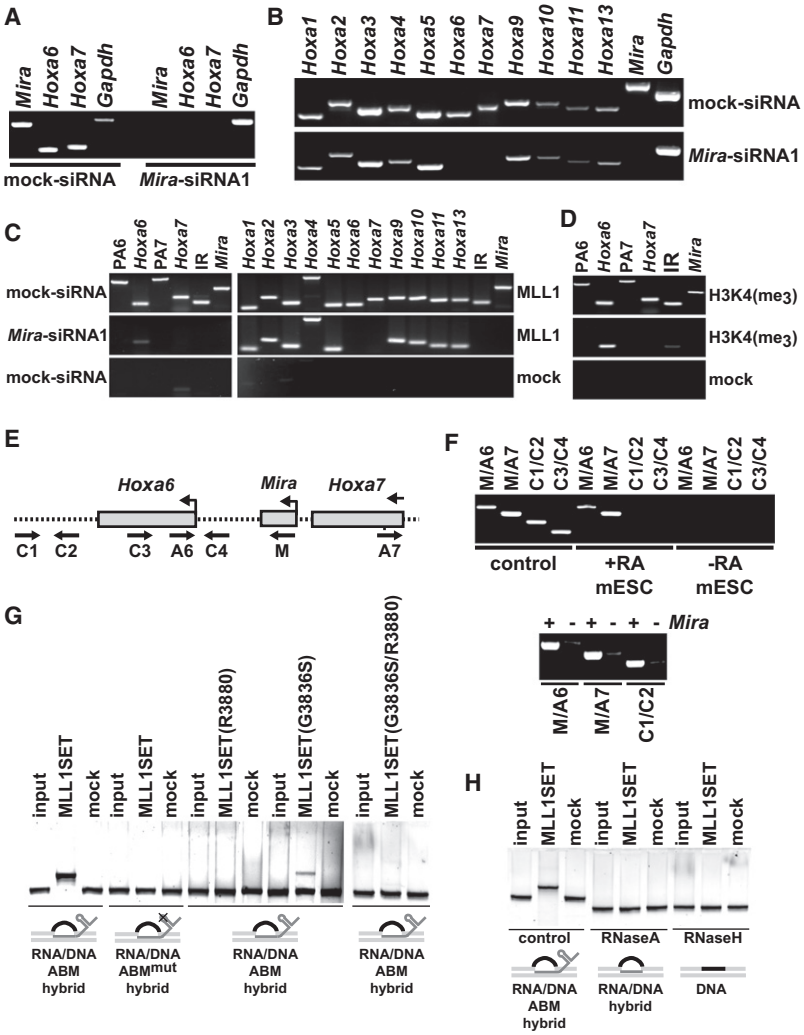

Figure 3. *Mira*-Mediated Recruitment of MLL1 Promotes Transcription of *Hoxa6* and *Hoxa7*

(A) Transcription of *Mira*, *Hoxa6*, *Hoxa7*, and *Gapdh* in +RA mESCs treated with control siRNA (mock-siRNA) or siRNA targeting *Mira* (*Mira*-siRNA1).

(B) Transcription of *Mira*, *Gapdh*, and *Hoxa* genes in mock-siRNA and *Mira*-siRNA1 mESCs.

(C) ChIP assays monitoring the association of MLL1 and a control (rabbit serum) with the Hoxa6/a7 cassette and *Hoxa* genes in mock-siRNA and *Mira*-siRNA1 mESCs. Target DNA regions are described in Figure 2A.

(D) ChIP assays as described in (C) detecting the association of H3K4(me_3) with the *Hoxa6/a7* cassette.

(E) Schematic representation of the *Hoxa6/a7* cassette. The positions of PCR primer pairs used for 3C assays are indicated.

(F) 3C assays detecting the association of the *Mira* gene locus with *Hoxa6* and *Hoxa7* in (top panel) −RA and +RA mESCs, and (bottom panel) +RA mESCs, which contain (+) or lack *Mira* through RNAi (−).

(G) Binding of MLL1SET, MLL1SET(G3836S), and MLL1SET(R3880A) to RNA/DNA complexes containing ssDNA (black) and truncated *Mira* (dark gray) containing a wild-type or mutant ABM.

(H) In vitro protein nucleic acid binding assays as described in (F) except that the RNA/DNA hybrids were preincubated with RNase-A or -H. See also Figure S3.

Destruction of *Mira* by RNAi attenuated the (1) transcription of *Hoxa6* and *Hoxa7* (Figures 3A and 3B), (2) interaction of MLL1 with chromatin-associated *Mira* (Figure S3G), and (3) association of MLL1 and H3K4(me_3) with the *Hoxa6/a7* cassette (Figures 3C and 3D). The knockdown of *Mira* did not abolish H3K4 methylation at the coding region of *Hoxa6*, suggesting that *Mira* is not involved in methylation of H3K4 at this region (Figure 3D). *Mira* knockdown did not affect the transcription of other *Hoxa* genes (Figure 3B), the transcription of the paralogs of *Hoxa6* and *Hoxa7* (Figure S3H), and the association of MLL1 and H3K4(me_3) with these genes (Figures 3C and S3I).

The *Mira*-dependent recruitment of MLL1 to the *Mira* gene and the promoter of *Hoxa6* and *Hoxa7* raised the possibilities that MLL1/*Mira* complexes associate with multiple, different target genes in *cis* and *trans* or that the association of MLL1 with *Mira*, *Hoxa6*, and *Hoxa7* is a result of dynamic changes in chromatin structure. We used 3C assays to analyze the spatial organization of the *Hoxa6/a7* cassette in +RA mESCs. The *Mira* gene, *Hoxa6*, and *Hoxa7* interacted in +RA but not in −RA mESCs (Figures 3E and 3F; Tables S3 and S4). The RNAi-mediated destruction of *Mira* disrupted the *Mira*/*Hoxa6*/*Hoxa7* complex, suggesting that *Mira*-mediated recruitment of MLL1 supports the intrachromosomal association of MLL1 with *Hoxa6* and *Hoxa7* (Figure 3F).

MLL1 Associates with a DNA/RNA Hybrid

The detection of *Mira*/DNA hybrids in chromatin suggested that MLL1 might bind RNA/DNA hybrids. Because the association of MLL1 with *Mira* is RNase A sensitive, ssRNA has to protrude from the RNA/DNA hybrid, suggesting that the 3′ *Mira* region containing the ABM protrudes from a *Mira*/DNA hybrid and remains accessible to MLL1 and RNase A. To test this we assessed whether MLL1 binds a RNA/DNA complex, which contains a *Mira*/DNA hybrid with a protruding ABM motif. MLL1SET interacted with the *Mira*/DNA complex, but failed to bind an RNA/DNA complex containing a mutant ABM (Figure 3G). Treatment of RNA/DNA complexes with RNase-H and -A attenuated the interaction of MLL1SET and the RNA/DNA structure (Figure 3H). MLL1SET(R3880) did not bind the RNA/DNA complex, indicating that the RNA-binding activity of the SET domain is essential for binding the RNA/DNA complex (Figure 3G). We detected a weak interaction of MLL1SET(G3836S), which binds RNA but not ssDNA, with the wild-type RNA/DNA hybrid, whereas the G3836S/R3880A double-mutant protein failed to bind the RNA/DNA complex, suggesting that the ssDNA-binding activity of MLL1 contributes to the interaction of MLL1 with RNA/DNA hybrids (Figure 3G). This hypothesis is supported by the *Mira*-dependent interaction of MLL1SET with a RNA/DNA hybrid, which lacks the ABM and resembles a *Mira* transcription bubble (Figure S4A).

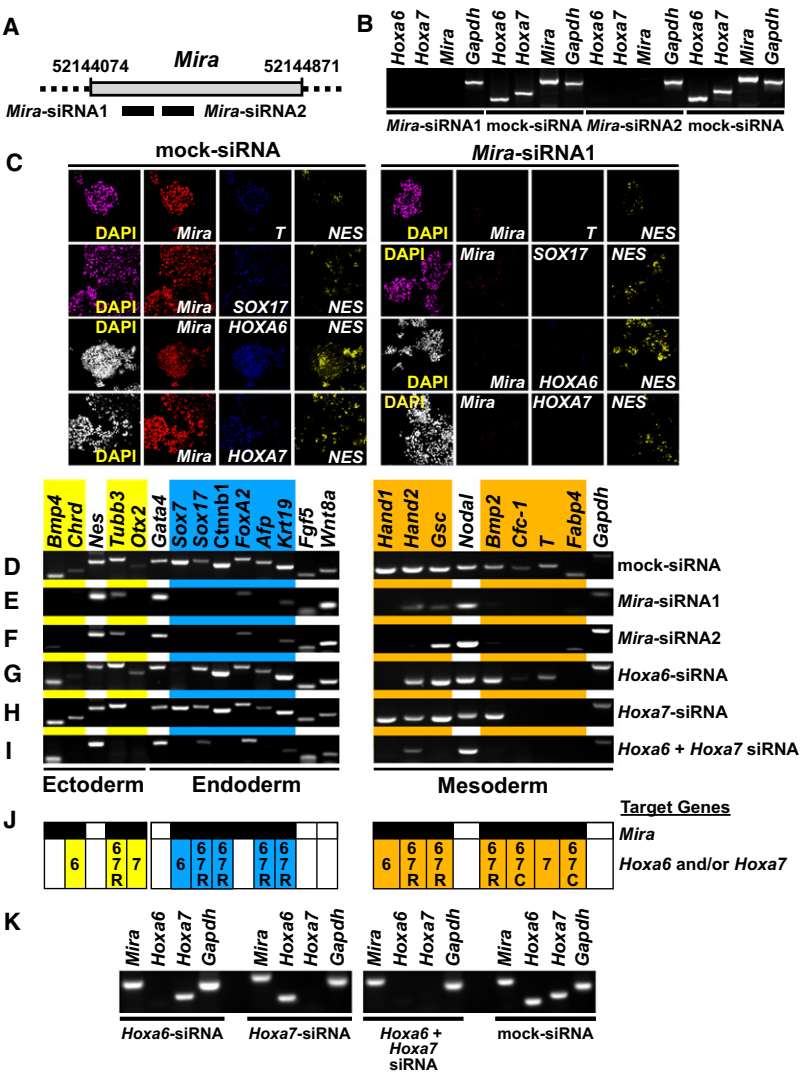

Figure 4. *Mira* Controls the Expression of Germ-Layer Marker Genes

(A) Schematic representation of the *Mira* gene locus. Black boxes indicate the positions of *Mira*-siRNA1 and *Mira*-siRNA2.

(B) Transcription of *Mira*, *Hoxa6*, *Hoxa7*, and *Gapdh* in +RA mock-siRNA, *Mira*-siRNA1, and *Mira*-siRNA2 mESCs.

(C) FISH coupled to immunofluorescence (IF) assays detecting *Mira* and NESTIN (NES) together with T, SOX17, HOXA6, or HOXA7 in +RA mock-siRNA and +RA *Mira*-siRNA1 cells.

(D–F) Transcription of germ-layer marker genes in (D) mock-siRNA, (E) *Mira*-siRNA1, and (F) *Mira*-siRNA2 +RA mESCs. Target genes for *Mira* are highlighted in yellow (ectoderm), blue (endoderm), and orange (mesoderm).

(G–I) Marker gene transcription in +RA mESCs, which had been transfected with (G) *Hoxa6*-siRNA, (H) *Hoxa7*-siRNA, and (I) *Hoxa6* + *Hoxa7* siRNA.

(J) Results of the RvT-PCR assays described in (D)–(I). Dark boxes indicate target genes for *Mira*. The numbers 6 and 7 indicate the target genes for *Hoxa6* and *Hoxa7*, respectively. The symbols (67R) and (67C) indicate genes activated by *Hoxa6* and *Hoxa7* in a redundant fashion and cooperative, respectively, fashion. For gene nomenclature see Table S1.

(K) RvT-PCR assays detecting the transcription of *Mira*, *Hoxa6*, *Hoxa7,* and *Gapdh* in mock-siRNA and +RA mESCs, which lack *Hoxa6* (*Hoxa6*-siRNA), *Hoxa7* (*Hoxa7*-siRNA), or *Hoxa6* and *Hoxa7* (*Hoxa6* + *Hoxa7* siRNA) through RNAi. See also Figure S4.

Mira-Mediated Activation of *Hoxa6* and *Hoxa7* Is Involved in ESC Differentiation

The RA-induced differentiation of mESCs results in the formation of embryoid bodies, containing precursor cells for all three germ layers: ectoderm, endoderm, and mesoderm (Desbaillets et al., 2000). *Hoxa6* and *Hoxa7* are involved in anterior-posterior pattern formation and control the developmental fate of mesoderm-derived organs and tissues (Kessel et al., 1990; Kostic and Capecchi, 1994). To investigate whether *Mira*-dependent activation of *Hoxa6* and *Hoxa7* is involved in germ-layer specification in differentiating mESCs, we compared the transcription of marker genes for germ-layer specification in control and *Mira*-deficient embryoid bodies (Figures 4A and 4B; Table S2). We tested 22 marker genes, whose transcription involves MLL1 (Figure S4B). RNAi-mediated destruction of *Mira* attenuated the transcription of 17 out of 22 marker genes tested (Figures 4C–4F and S4C; Table S1). Identical results were obtained with two different siRNAs against *Mira* (Figures 4A, 4B, 4E, and 4F).

To assess whether *Mira* controls the expression of germ-layer marker genes by activating *Hoxa6* and *Hoxa7* expression, we compared the expression of the marker genes in +RA mESCs, which lack *Hoxa6* protein (HOXA6), and/or *Hoxa7* protein (HOXA7) through RNAi (Figures 4G–4K and S4D). *Hoxa6* and *Hoxa7* activated the transcription of 15 out of 22 marker genes tested and 15 of the 17 identified target genes for *Mira* (Figures 4G–4J and S4E). The majority of tested marker genes (10 out of 15) were activated by HOXA6 and HOXA7 in a cooperative or redundant fashion (Figure 4I and 4J). Our results reveal that by instigating *Hoxa6* and *Hoxa7* transcription *Mira* triggers activation of germ-layer marker gene expression during early mESC differentiation.

DISCUSSION

LncRNAs have been associated with gene silencing, imprinting, and gene-dosage compensation by guiding enzymes involved in chromatin remodeling and posttranslational modification of histones to target genes (Khalil et al., 2009; Tsai et al., 2010). The role of lncRNAs in epigenetic activation of gene expression is only now being dissected.

Our results uncover a role for the lncRNA *Mira* in epigenetic activation and cell differentiation by recruiting the epigenetic activator MLL1 to chromatin. The interaction of MLL1 with chromatin-associated *Mira* triggers dynamic changes in chromosome conformation that mediate activation of *Hoxa6* and

Hoxa7 transcription and culminate in transcriptional activation of genes involved in germ-layer specification.

The RNA and protein motifs involved in the association of epigenetic activators with lncRNAs remain unclear. In *Drosophila*, lncRNA/DNA hybrids are involved in the association of the epigenetic activator ASH1 with chromatin (Sanchez-Elsner et al., 2006). The WDR5 subunit of the MLL1 complex interacts with an unknown RNA motif in *HOTTIP* (Wang et al., 2011). The interaction of MLL1 with *Mira* involves the specific interaction of the SET domain of MLL1 with the ABM of *Mira*. Our results support the model that MLL1 interacts with chromatin-associated *Mira* and that the RNA- and ssDNA-binding activities of the MLL1 SET domain are involved in recognizing and binding the *Mira*/DNA hybrid. The *Mira*-dependent interaction of MLL1 with ssDNA implies that the interaction of MLL1 with *Mira* stimulates the ssDNA-binding activity of MLL1.

Hoxa6 and *Hoxa7* are involved in determining the specification of mesoderm derived tissues and organs (Kessel et al., 1990; Kostic and Capecchi, 1994). Our results suggest that the cooperative/redundant interplay of *Hoxa6* and *Hoxa7* controls the expression of genes involved in early germ-layer specification. The roles of *Hox* genes in early vertebrate development remain unclear and the analysis of mice lacking *Hoxa6*, *Hoxa7*, or the entire *Hoxa* cluster did not support the involvement of *Hoxa6* and *Hoxa7* in early germ-layer specification (Kessel et al., 1990; Kostic and Capecchi, 1994; Kmita et al., 2005); however, because to our knowledge the phenotype of *Hoxa6*/*Hoxa7* double mutants remains unknown and the effects of the *Hoxa*-cluster deletion were analyzed during later stages of development, these studies do not exclude the possibility that *Hox* genes act during early germ-layer specification.

While paramount for development, the anomalous activities of MLL1 and *Hox* genes have been correlated with various human diseases such as cancer (Hess, 2004). The described *Mira*-dependent activation of *Hoxa6* and *Hoxa7* establishes a foundation for the dissection of the functional importance of lncRNAs in epigenetic activation in development and disease.

EXPERIMENTAL PROCEDURES

Cultivation and Differentiation of mESCs

CJ7 mouse embryonic stem cells (mESCs) were maintained and differentiated as described (Sato et al., 2004) (see Supplemental Experimental Procedures for details).

Chromosome Conformation Capture (3C) Assays

The 3C assay was performed as described (Hagège et al., 2007) (see Supplemental Experimental Procedures for details).

Rapid Amplification of cDNA Ends (RACE)

RACE was performed with the FirstChoice RLM-RACE Kit (Ambion) according to the manufacturer's instructions (see Supplemental Experimental Procedures for details).

Crosslinked Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described (Bertani et al., 2008) (see Supplemental Experimental Procedures for details).

Protein-Nucleic Acid Interaction Assays

Protein-nucleic acid interaction assays were performed as described (Sanchez-Elsner et al., 2006) using in vitro transcribed, radiolabeled RNA or fluorescine labeled DNA/DNA hybrids as bait (see the Supplemental Experimental Procedures for details).

RNA ChIP-on-Chip

ChIP using native chromatin was performed as described (Sanchez-Elsner et al., 2006; Bertani et al., 2008), except that native chromatin was isolated from 1×10^8 −RA, control, and +RA CJ7 mESCs. All buffers were supplemented with RNase inhibitor (1000 U/ml). Chromatin was immunoprecipitated with 10 μg antibody to MLL1 or 10 μg rabbit serum (mock). Immunoprecipitated RNA was purified with Trizol and reverse transcribed with Superscript II and random hexamers. The cDNA libraries were amplified using the GenomePLex Whole Genome Amplification kit with 10× Amp Mix (Sigma; WGA1), according the manufacturer's instructions. The amplified DNA pools were labeled using the GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix) and hybridized to GeneChip Mouse Tiling 2.0R F Arrays (P/N900899; Affymetrix), which contain probes for chromosomes 6, 8, and 16 tiled at an average resolution of 35 bp. Hybridization, washes, staining, data acquisition, and data analysis were performed using the GeneChip Workstation (GeneChip Expression Analysis; Affymetrix). Data normalization, background subtraction, and peak detection of the arrays was conducted using Model-based Analysis of Tiling-array (MAT) software (Johnson et al., 2006) and mouse genome version 8 (February, 2006) as a reference sequence (NCBI36/mm8). Peaks with a p value of $\geq 10^{-4}$ were considered to be significant. Processed peak data was extracted using a custom Perl script. The comparison of the pattern of MLL1-associated RNAs in +RA mESCs with the pattern observed in −RA ESCs, control mESCs, and mock assays identified RNAs, which associate with MLL1 and chromatin in +RA mESCs.

RNase ChIP

RNase-ChIP assays were performed as described (Sanchez-Elsner et al., 2006) (see Supplemental Experimental Procedures for details).

RNA Interference

SiRNAs were generated with the Silencer siRNA Construction Kit (Ambion) or purchased from IDT (San Diego) and transfected into mESCs using Lipofectamine 2000 (Invitrogen) (see Supplemental Experimental Procedures for details).

ACCESSION NUMBERS

The *Mistral* sequence has been deposited in the GenBank database under the accession number BankIt1472716 Seq1 JN565285; the RNA ChIP-on-chip have been deposited in the GEO database under the accession number GSE31330.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.08.019.

ACKNOWLEDGMENTS

We thank N. Sato for providing CJ7 mouse embryonic stem cells; V. Sivanandam and A.L.N. Rao for technical help with northern blot assays; M. Rubalcava for technical support; and B. Gadd, D. Parker, Jr., and N. Xia for critical discussions. This work was supported by National Institutes of Health (GM073776) (F.S.) and the California Institute for Regenerative Medicine (CIRM) (RS1-00477-1) (F.S.).

Received: March 29, 2011
Revised: July 20, 2011
Accepted: August 17, 2011
Published: September 15, 2011

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