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Expression Profiling Reveals Developmentally Regulated lncRNA Repertoire in the Mouse Male Germline[1]

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ABSTRACT

In mammals, the transcriptome of large noncoding RNAs (lncRNAs) is believed to be greater than that of messenger RNAs (mRNAs). Some lncRNAs, especially large intergenic noncoding RNAs (lincRNAs), participate in epigenetic regulation by binding chromatin-modifying protein complexes and regulating protein-coding gene expression. Given that epigenetic regulation plays a critical role in male germline development, we embarked on expression profiling of both lncRNAs and mRNAs during male germline reprogramming and postnatal development using microarray analyses. We identified thousands of lncRNAs and hundreds of lincRNAs that are either up- or downregulated at six critical time points during male germ cell development. In addition, highly regulated lncRNAs were correlated with nearby (<30 kb) mRNA gene clusters, which were also significantly up- or downregulated. Large ncRNAs can be localized to both the nucleus and cytoplasm, with nuclear lncRNAs mostly associated with key components of the chromatin-remodeling protein complexes. Our data indicate that expression of lncRNAs is dynamically regulated during male germline development and that lncRNAs may function to regulate gene expression at both transcriptional and posttranscriptional levels via genetic and epigenetic mechanisms.

epigenetics, fertility, germ cell, reproduction, spermatogenesis

INTRODUCTION

Analyses of cellular RNA contents using next-generation deep-sequencing technologies have revealed that >90% of the eukaryotic genome is transcribed into RNA transcripts, which are subsequently processed into messenger mRNAs (mRNAs), small (<100 nt; sncRNAs), and large (>100 nt; lncRNAs) noncoding RNAs [1–3]. Messenger RNAs encode proteins that execute physiological functions, whereas sncRNAs act mostly at posttranscriptional levels by affecting mRNA stability and translational efficiency [4]. Several lncRNAs are well documented as critical epigenetic regulators acting to modulate the transcriptional status of individual mRNA genes or even the entire chromosome [5–8]. One of the best-known examples of lncRNA-induced epigenetic silencing through chromatin remodeling is X chromosome inactivation [5]. An lncRNA called *Xist* is expressed on the targeted X chromosome, attracting and binding polycomb group protein complexes, which in turn trimethylate H3K27 *in cis*, thus initiating the panchromosomal epigenetic silencing [6]. Methylation of *H19*, a paternally imprinted gene, requires another large intergenic noncoding RNA (lincRNA) *H19* transcribed from the *H19* locus [7, 8]. This lincRNA is essential for maintaining the hypermethylation status of the paternal copy of the *H19* gene. Recently, it has been demonstrated that the differential expression patterns of the *HOXC* gene cluster depend on a lincRNA called *HOTAIR* [9–11]. *HOTAIR* is transcribed in a region close to the *HOXC* gene cluster, and it enables tethering of two distinct repressive complexes to chromatin for coupled H3K27 methylation and H3K4 demethylation [9, 10]. In this way, the *HOXC* gene cluster displays a wide variety of expression profiles in multiple organs of the body.

Current data indicate that the number of unique lncRNAs may be greater than that of mRNAs. This was not elucidated until recently because most lncRNAs are in low abundance and lack polyA tails; thus, they could not be easily cloned and identified with the past conventional Sanger sequencing [6, 12]. The latest RNA-Seq-based lncRNA profiling analyses suggest that lncRNAs, especially lincRNAs, are transcribed from sites within or close to genomic regions that are destined to be silenced [9, 10, 13, 14]. These lncRNAs can serve as sequence guides, directing chromatin-remodeling complexes or other epigenetic machineries to the genomic loci and inducing regional silencing through DNA methylation or histone modifications [9, 10, 14].

Male germ cell development is a complex process driven by unique gene expression programs that are, in part, controlled by epigenetic mechanisms involving histone modifications and DNA methylation [15, 16]. During primordial germ cell (PGC) development, a wave of global reprogramming is achieved by genome-wide DNA demethylation followed by remethylation as well as extensive histone modifications, including gradual loss of H3 lysine-9 dimethylation (H3K9me2) and global gain of H3K27me3 [15, 17]. The postnatal development of male germ cells can be divided into three phases: mitotic, meiotic, and haploid [18]. In the mitotic phase, prospermatogonia differentiate into differentiating spermatogonia that undergo multiple rounds of mitotic cell divisions before entering the meiotic phase and becoming spermatocytes. During meiosis, homologous chromosomes pair and cross over through recombination. This process requires a histone methyltransferase PRDM9, which catalyzes H3K4me3 and the subsequent recruitment of SPO11 and other proteins of the recombination machinery [15]. Unpaired X and Y chromosomes are sequestered in the sex body, and the chromosomes are silenced through a process termed meiotic sex chromosome inactivation

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(MSCI), which involves phosphorylation of histone variant H2AX at serine-139 (r-H2AX) and ubiquitination of H2A and H2B [19, 20]. After meiotic cell divisions, spermatocytes become spermatids that undergo a differentiation process termed spermiogenesis. During spermiogenesis, round spermatids differentiate into elongated spermatids and, eventually, spermatozoa [21]. At chromatin levels, during spermiogenesis, spermatids undergo extensive chromatin remodeling, which requires histone H4 hyperacetylation and probably the incorporation of various histone variants (e.g. H1, H2A, H2B) [15]. In spermatozoa, paternal chromatin is heavily packed and mostly associated with protamine. However, recent data have identified that many domains of the paternal genome retain nucleosomal histones, which are enriched with H3K4me3 and associated with hypomethylated/unmethylated DNA [22–24]. These include loci containing key developmental genes, imprinted genes, microRNAs, and homeotic genes [22–24]. Taken together, dynamic epigenetic regulations are involved in male germline reprogramming and all three phases of postnatal development. Given that lncRNAs have been implicated in these epigenetic regulations [25, 26], we hypothesized that there should be abundant lncRNAs that are significantly regulated at each of these critical time points during the male germline development.

To test this hypothesis, we profiled lncRNA expression during PGC reprogramming (Embryonic Day 15.5 [E15.5] vs. E12.5) and during postnatal germ cell development from the mitotic to the meiotic phase (Postnatal Day 14 [P14] vs. P7), the meiotic to the haploid phase (P21 vs. P14), and early to late spermiogenesis (P21 vs. adult) using microarrays that allow for simultaneous profiling of both lncRNAs and mRNAs. We report here that thousands of lncRNAs and hundreds of lincRNAs were either up- or downregulated at each of six critical time points during male germ cell development. Furthermore, most of these highly regulated lncRNAs were correlated with mRNA gene clusters located nearby (<30kb), which were also significantly up- or downregulated at the same time points. Additionally, these lncRNAs were localized to the nuclei of developing male germ cells and were associated with key components of the chromatin-remodeling protein complexes.

MATERIALS AND METHODS

Animal Use and Sample Collection

Adult mice (C57BL/6J) were purchased from the Jackson Laboratory, and young mice were from in-house breeding in the vivarium of the University of Nevada, Reno. The Institutional Animal Use and Care Committee of the University of Nevada, Reno, approved the animal use protocol. For the collection of embryonic (E12.5 and E15.5) testes, timed, natural mating was performed by placing two wild-type adult female mice (6–12 wk old) into a cage with one wild-type adult male mouse (8–24 wk old). Plugs were checked every morning; the day that a plug was seen was designated as E0.5. Pregnant female mice at E12.5 and E15.5 were euthanized, and the fetuses were dissected in order to collect E12.5 and E15.5 testes under a dissection microscope, respectively. Embryonic testes were stored in 1 ml Trizol reagent (Invitrogen) at −80°C freezer until RNA isolation. Twenty P7, eight P14, four P21, and two adult testes were used for RNA isolation.

Large ncRNA Microarray Analysis

For microarray analyses, three sets of testes at each of the six time points (E12.5, E15.5, P7, P14, P21, and adult) were used for mRNA purification after removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit; Epicentre). Each sample was then reverse-transcribed into cDNAs without 3′ bias using a Superscript Double-Strand Synthesis Kit (catalog #11917-020; Invitrogen). The cDNAs were then labeled with Cy3 using the Quick-Amp labeling kit (Agilent). The labeled cDNAs were hybridized to the Mouse LncRNA Array (version 2.0; 8 × 60 K; Arraystar) using the SureHyb Hybridization Chambers according to the One-Color Microarray-Based Gene Expression Analysis protocol (Agilent). After washing, the arrays were scanned by the Agilent Scanner G2505C. The GeneSpring GX version 12.0 software package (Agilent) was employed for quantile normalization and data processing. Differentially expressed lncRNAs and mRNAs between two time points were identified through volcano plotting and fold change filtering ($P < 0.05$, fold change > 2.0). Raw and processed microarray data have been deposited in the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI) and can be accessed by the GEO accession number, GSE46896.

Cell Purification

Spermatogonia were purified from P7 testes, and spermatocytes and round spermatids were purified from adult testes using the STA-PUT method as described previously [27]. Purity of the three germ cell types was >90%, based on microscopic observation of morphology. For purification of Sertoli and Leydig cells, a Sertoli cell-specific (*Amh-Cre*) and a Leydig cell-specific (*Cyp17-iCre*) Cre line were crossed with a dual-fluorescence reporter line (*Rosa26-mTmG*$^{tg/tg}$) to obtain *Amh-Cre; mTmG*$^{+/tg}$ and *Cyp17-iCre; mTmG*$^{+/tg}$ mice, respectively. In these two lines, Sertoli and Leydig cells expressed membrane-tagged eGFP (mG); thus, the green Sertoli and Leydig cells were purified using FACS as described previously [28–30]. The purity was >95% based upon quantitative PCR (qPCR) detection of Sertoli cell and Leydig cell marker genes.

Real-Time qPCR Analyses of lncRNAs

Large ncRNA fractions were isolated using the Trizol reagent following the manufacturer's instructions. Potential genomic DNA contamination was removed by DNase I treatment (TURBO DNA-free Kit; Ambion) according to the manufacturer's protocol. RNA quantity and quality were assessed using NanoDrop 2000 (Thermo Scientific). For real-time qPCR assays, first-strand cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). Total RNA (1 μg) was first reverse-transcribed into cDNAs using a combination of random hexamers and oligo(dT)$_{20}$ primer for each sample. To increase PCR sensitivity, the RNA complement strand from cDNA:RNA hybrids was removed by RNase H treatment. Quantitative PCR was performed as described previously [28, 29]. *Gapdh* was used as a housekeeping control. All primer sequences are listed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org).

Cross-Link RNA Immunoprecipitation Followed by Real-Time qPCR

Cross-link RNA immunoprecipitation was performed using an RNA ChIP-IT kit (catalog #53024; Active Motif) with minor modifications. In brief, testes were dissected, and the tunica albuginea was removed. Seminiferous tubules were dispersed in 10 ml Dulbecco modified Eagle medium by gentle pipetting. The dispersed seminiferous tubules were then fixed in 1% formaldehyde for 7 min at room temperature. The fixed tubules were incubated in the Complete Lysis Buffer provided by the kit, and cell nuclei were purified. To facilitate RNA immunoprecipitation, sheared chromatin was prepared by sonication for 12 min using the Bioruptor UCD-200 (Diagenode) with the following settings: OFF, 30 sec; ON, 30 sec; Intensity, H. DNase I was added to the sonicated lysate to remove genomic DNA contamination. Immunoprecipitation was performed overnight at 4°C in a 200-μl volume containing 5 μl EZH2 or LSD Antibody (Abcam) or IgG (control), and 30 μl protein-G-conjugated magnetic beads for each sample. Beads were washed four times with the Complete RNA-ChIP Wash Buffer 1, and two times with Complete RNA-ChIP Wash Buffer 2, respectively. The RNA-protein complex was finally recovered by incubation with Complete RNA-ChIP Elution Buffer, and the protein components were eliminated by digestion with Proteinase K. RNA was extracted and qPCR was performed as described above.

Large ncRNA In Situ Hybridization

Subcellular localization of lncRNAs in adult mouse testis was analyzed using the RNAscope 2.0 FFPE Assay-Brown kit (Advanced Cell Diagnostics, Inc.) according to the manufacturer's instructions. The kit employs a novel in situ hybridization (ISH) method that can amplify signals from numerous synthetic probes hybridized to their specific RNA target for additional sensitivity and specificity [31]. Briefly, adult mouse testes were fixed in freshly prepared 10% formalin for 20 h at room temperature with gentle agitation. After dehydration, testes were embedded in paraffin, and sections (5-μm thickness) were cut. Section slides were incubated at 60°C for 1 h and then

deparaffinized with xylene and 100% ethanol. Sections then underwent three steps of pretreatment: pretreat 1 to inhibit endogenous peroxidase, pretreat 2 to allow access to target RNA, pretreat 3 to digest proteins (1:12 was used for AK016507 and 1:15 was used for AK082424). Subsequently, the sections were hybridized to prewarmed (40°C) target probes for 18 h at 40°C using a hybridization chamber (Dako). The DapB probe provided by the kit was used as negative control [31]. Slides were then sequentially treated with Amp1 (preamplifier), Amp2 (signal enhancer), Amp3 (amplifier), Amp4 (probe labeling), Amp5 (signal Amp), and Amp6 (horseradish peroxidase-linked labeling molecule). Following amplification steps, diaminobenzidine substrate was added for colorimetric detection of target RNA. Finally, slides were counterstained with hematoxylin (Sigma) and then dehydrated with serial gradient ethanol, cleared with xylene, mounted with whole-mount medium (Fisher), observed, and photographed using a bright field microscope (Olympus) equipped with a digital camera (Pixelink).

Quantification of lncRNA Levels Based on Staining Scores of ISH

The ISH signals are represented as punctuated dots, and individual dots represent individual copies of the RNA target, allowing easy subcellular localization and quantification [31]. The number of brown punctuated dots in each cell type was counted. At least 500 cells for each cell type and five seminiferous tubules at each of the 12 stages were counted. The staining scores were categorized into five grades (0, 1, 2, 3, 4), which were determined according to the criteria described previously [31].

Large ncRNA Northern Blot Analysis

Total RNA was isolated from testes at different time points (P7, P14, P21, P35, and Postnatal Week 8) using the Trizol reagent as described above. An aliquot of total RNA (20 μg) was fractionated on 1.2% formaldehyde-agarose gels and transferred to positively charged nylon membrane (Genescreen Plus). Digoxigen (DIG)-labeled, single-strand RNA probes were generated using DIG Northern Starter Kit (Roche) according to the manufacturer's instructions.

RESULTS

Dynamic Expression of lncRNAs During Testicular and Male Germline Development

To gain insights into the role of lncRNAs during testicular development, we performed microarray-based profiling analyses using fetal and postnatal testes (Fig. 1A). Aims were to identify lncRNAs and mRNAs that were significantly up- or downregulated during the six critical male germline development time points and to explore genome-wide positional correlations between regulated lncRNAs and mRNAs. In fetal murine testes, PGCs undergo a wave of reprogramming characterized by nearly global demethylation between E10.5 and E13.5, followed by genome-wide remethylation to establish male germline methylome during E14.5 and E16.5 [15, 17] (Fig. 1B). We chose E12.5 and 15.5 testes because they represent the hypomethylated and hypermethylated statuses of the genome in PGCs [15, 17]. In P7 testes, male germ cell development is at the mitotic phase, and spermatogonia are the only germ cell type (Fig. 1B) within the testis. At P14, male germ cells develop into the meiotic phase, and the most advanced germ cells are midpachytene spermatocytes (Fig. 1B). Completion of the first meiotic division occurs at ~P20, and by P21 in the testes, the male germ cells have developed into round spermatids (Fig. 1B). In adult murine testes, full spermatogenesis is established and adult testes contain all three types of developing male germ cells (i.e., spermatogonia, spermatocytes, and spermatids; Fig. 1B).

The microarrays used contained probes detecting 31 423 murine lncRNAs and 25 376 murine mRNAs simultaneously [32]. By comparing the expression profiles between two adjacent time points, we identified thousands of lncRNAs that are either significantly up- or downregulated (2-fold changes as the cutoff; $P < 0.05$; Fig. 1C and Supplemental Figs. S1 and S2). There appeared to be more upregulated than downregulated lncRNAs during fetal testicular development from E12.5 to E15.5 (2593 upregulated vs. 1125 downregulated lncRNAs). In contrast, more downregulated testicular lncRNAs were detected in P21 compared to adult testes (1976 upregulated vs. 3096 downregulated lncRNAs). In the remaining three time points, there appeared to be similar numbers of up- and downregulated lncRNAs when compared to adjacent time points (Fig. 1C and Supplemental Fig. S3).

We further divided the regulated lncRNAs into four groups, including sense lncRNAs derived from introns or exons of mRNAs, antisense lncRNAs from introns or exons of mRNAs, bidirectional lncRNAs from regions adjacent to mRNA genes with opposite orientations within a 1-kb range, and intergenic lncRNAs (Supplemental Fig. S4). In addition, the relative number of significantly regulated lncRNA subtypes followed the order intergenic lncRNAs > antisense lncRNAs > sense lncRNAs > bidirectional lncRNAs (Fig. 1D).

Validation of lncRNA Expression

To validate the microarray data, we chose 28 lncRNAs, including three sense lncRNAs, six antisense lncRNAs, three bidirectional lncRNAs, and 16 lincRNAs and examined their expression levels in developing testes via qPCR (Fig. 2A and Supplemental Fig. S5). The dynamic expression patterns in all six time points analyzed were consistent with the lncRNA microarray data, which has been deposited in the GEO database at the NCBI and can be accessed by the GEO accession number GSE46896. Other evidence supporting the lncRNA microarray data was the correct expression patterns of spermatogenic cell marker genes, as revealed in our mRNA microarray data (GEO accession number GSE46896). For example, *Nanos2*, *Tex101*, and *Mov10l1* were upregulated from E12.5 to E15.5; *Sycp3*, *Spo11*, and *Piwil1* were upregulated from P7 to P14; *Pgk2*, *Hist1h1t*, and *Crem* were upregulated from P14 to P21; and *Prm2*, *Tnp2*, and *Spem1* were upregulated from P21 to adulthood.

During male germline reprogramming in the fetal testes (from global demethylation at E12.5 to remethylation at E15.5), both up- and downregulated lncRNAs were observed that either ceased expression or continued to be expressed throughout postnatal testicular development. Most of these lncRNAs displayed the highest levels in P14, P21, and adult testes (Fig. 2A), suggesting that they are predominantly expressed in the meiotic and haploid phases of spermatogenesis. This is also supported by qPCR analyses using purified spermatogonia, pachytene spermatocytes, round spermatids, Sertoli cells, and Leydig cells (Fig. 2B). Some lncRNAs were expressed in both germ (spermatogonia, spermatocytes, and spermatids) and somatic (i.e., Sertoli and Leydig cells) cell types, whereas many were detected in either somatic or germ cells in the testis. Using a DIG+-labeled RNA probe, we also performed Northern blot analyses on *Tsx*, a lincRNA known to be expressed in the testis [33]. Consistent with previous findings [33] and our qPCR data (Fig. 2A), *Tsx* appeared to be predominantly expressed in P14 and older testes (Fig. 2C).

Many lncRNAs Are Associated with Components of Chromatin-Remodeling Complexes

Many lincRNAs have been shown to be associated with chromatin-remodeling complexes, including the polycomb repressive complex 2 (PRC2) and CoREST/REST repressor complexes [10, 14, 34]. EZH2 is an H3K27 methylase, which binds core components Suz12 and EED to form PRC2 [35].

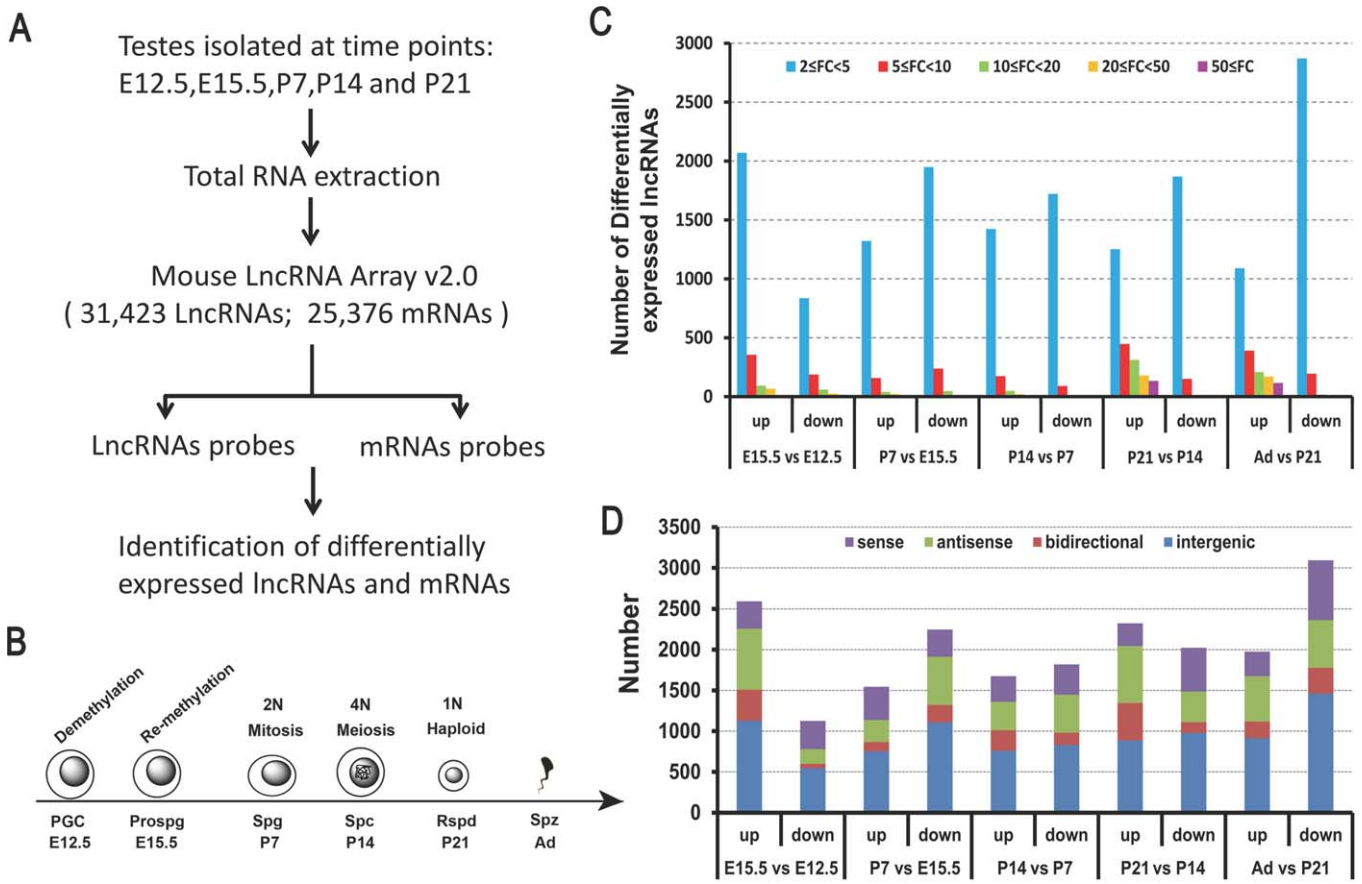


FIG. 1. Microarray-based lncRNA expression profiling analyses on murine testes at six time points during male germline development. **A**) Schematic of the experimental workflow for lncRNA microarray analyses. **B**) Diagram of major cellular events occurring at the six time points during male germ cell development in mice. E, embryonic day; P, postnatal day; PGC, primordial germ cell; Spg, spermatogonia; Spc, spermatocytes; Rspd, round spermatids; Ad, adult; 1N, haploid; 2N, diploid; 4N, tetraploid. **C**) Summary of the total number of lncRNAs that were up- or downregulated at each of the six time windows during male germline development in mice. The regulated lncRNAs were subdivided into five groups based on fold change (FC). **D**) Distribution of the four types of lncRNAs (sense, antisense, bidirectional, and intergenic) in the regulated lncRNAs at each of the six time windows analyzed.

LSD1 is a demethylase that mediates enzymatic demethylation of H3K4me2 in the CoREST/REST complexes [36]. Using cross-link RNA immunoprecipitation followed by qPCR, we found that 10 out of 26 lncRNAs tested interacted with EZH2, and one lincRNA, uc009cxn.1, bound both EZH2 and LSD1 (Fig. 3). These lncRNAs included six lincRNAs (ENSMUST172055, AK005728, NR_027848, NR_003270, BC049716, and ENSMUST153774), two antisense lncRNAs (uc009fxb.1, ENSMUST161823, and uc009cxn.1), and one bidirectional lncRNA (ENSMUST131790). These results suggest that both lincRNAs and nonintergenic (i.e., sense, antisense, and bidirectional) lncRNAs can interact with chromatin remodeling complexes, and some lncRNAs (e.g., uc009cxn.1) can be associated with multiple chromatin-remodeling complexes.

LncRNAs Can Be Localized to Both the Nucleus and Cytoplasm

Supporting an epigenetic role, earlier studies have reported that lincRNAs are mainly localized to the nucleus of the cell [14]. Given that nonintergenic lncRNAs also appeared to interact with chromatin-remodeling protein complexes (Fig. 3), we chose one antisense lncRNA (AK016507) and one bidirectional lncRNA (AK082424) and analyzed their subcellular localization using ISH. ISH analyses detected hybridization signals specific to the bidirectional lncRNA AK082424 exclusively in the nuclei of spermatocytes and round spermatids (Fig. 4, A–E, and Supplemental Fig. S6). The highest levels of AK082424 were detected in late pachytene spermatocytes, and this result is consistent with the qPCR analyses showing that expression of this particular lncRNA was significantly upregulated at P14 and thereafter and was predominantly detected in spermatocytes and round spermatids (Fig. 2). Interestingly, hybridization signals specific to an antisense lncRNA AK016507 were detected mainly in the cytoplasm of spermatocytes and round (steps 1–8) and elongating spermatids (steps 9–12; Fig. 4, F and G, and Supplemental Fig. S6). The highest levels were observed in round and elongating spermatids, and these results are consistent with the qPCR analyses (Fig. 2). The distinct localization patterns and narrow developmental expression windows imply that expression of the two lncRNAs is under tight regulation during spermatogenesis. The nuclear localization suggests a role either at the DNA level through interacting with nuclear factors or at the RNA level by affecting transcription and/or pre-mRNA processing and transport, whereas lncRNAs with a cytoplasmic localization may function mainly at posttranscriptional levels.

Correlations Between Regulated lncRNAs and mRNAs

Large ncRNAs can regulate gene expression at both transcriptional and posttranscriptional levels by binding and

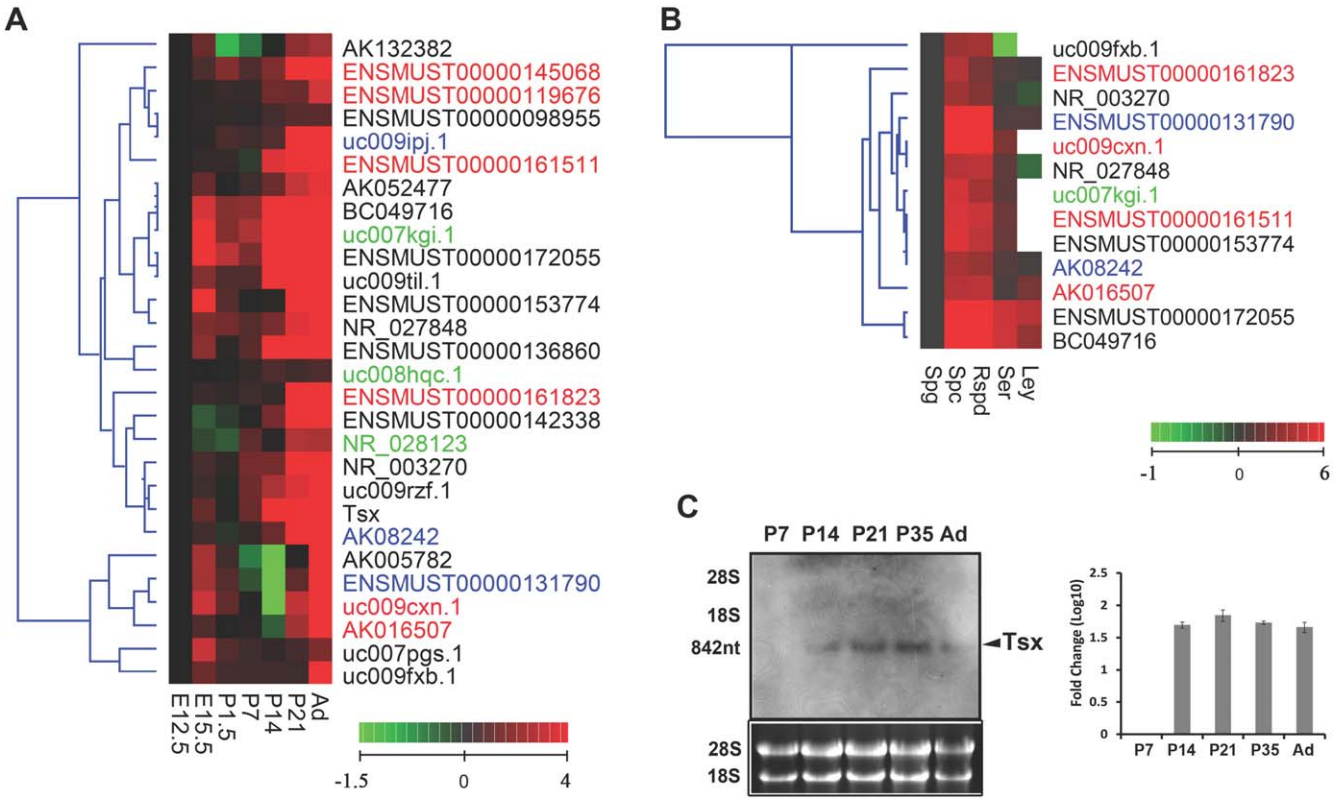
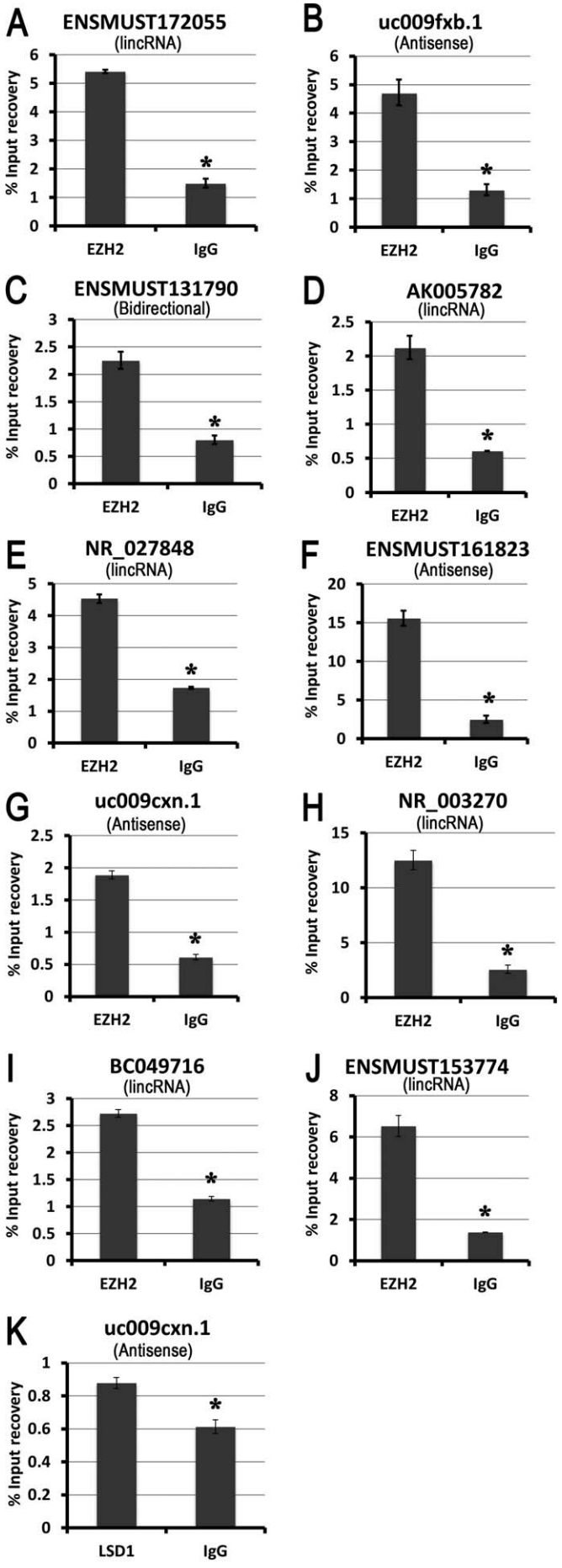


FIG. 2. Quantitative PCR validation of lncRNA expression in developing testes and in purified testicular cell types of mice. **A**) Heat map showing relative expression levels of 28 lncRNAs in developing testes at E12.5, E15.5, P1.5, P7, P14, P21, and adulthood (Ad). The lncRNAs analyzed include 16 intergenic (gene symbols in black), six antisense (in red), three sense (in green), and three bidirectional (in blue). **B**) Heat map representing relative expression levels of 13 lncRNAs in purified testicular cell types, including spermatogonia (Spg), spermatocytes (Spc), round spermatid (Rspd), Sertoli cells (Ser), and Leydig cells (Ley). **C**) Northern blot analyses of the expression of *Tsx*, a lincRNA, in developing testes at P7, P14, P21, P35, and adulthood (Ad). The left panel is a representative Northern blot, and the right panel represents the quantitative analyses of Northern blot results from three independent experiments. Data are presented as mean ± SEM (n = 3).

guiding protein complexes to either the genomic sites (at DNA levels) or their target RNAs (at RNA levels) [25, 37]. Many lincRNAs bind chromatin-modifying complexes and target specific genomic loci through DNA methylation or histone modifications [25, 26, 38, 39]. The action of lncRNAs can take place both *in cis* and *in trans* [25, 38]. To identify potential lncRNA targets *in cis*, we first mapped all lincRNAs that displayed significant up- or downregulation (>2-fold change) in four critical time windows of male germline development (E15.5 vs. E12.5, P14 vs. P7, P21 vs. P14, and adult vs. P21). By mapping lincRNAs and mRNAs that were significantly regulated and <30 kb in distance, we identified numerous correlations between lincRNAs and mRNAs (Fig. 5, Supplemental Fig. S7, and Supplemental Dataset S1), with the following five major patterns: 1) upregulated lincRNAs correlated with upregulated mRNAs, 2) downregulated lincRNAs correlated with downregulated mRNAs, 3) upregulated lincRNAs correlated with downregulated mRNAs, 4) downregulated lincRNAs correlated with upregulated mRNAs, 5) up- or downregulated lincRNAs associated with both up- and downregulated mRNAs (Supplemental Fig. S2 and Supplemental Dataset S1). From E12.5 to E15.5, one of many inverse correlations involved one significantly downregulated lincRNA (MM9LINCRNAEXON11331) and three significantly upregulated mRNAs (*Cthrc1*, *Slc25a32*, and *Dcaf13*), which are located <30 kb up- or downstream of the lincRNA on chromosome 15 (Fig. 5A). From P7 to P14, an interesting correlation was identified between two downregulated *HoxD* genes, *Hoxd9* and *Hoxd10*, and four lincRNAs (MM9LINCRNAEXON10950, MM9LINCRNAEXON10960, MM9LINCRNAEXON10964, and MM9LINCRNAEXON10965) on chromosome 2 (Supplemental Fig. S7 and Supplemental Dataset S1). One of the two upregulated lincRNAs, MM9LINCRNAEXON10950, from P7 to P14 is ~18 kb upstream of *Hoxd10*, and the other (MM9LINCRNAEXON10960) is ~3 kb downstream of the *Hoxd9*. The other two downregulated lincRNAs (MM9LINCRNAEXON10964 and MM9LINCRNAEXON10965) were located ~11 kb and 18 kb downstream, respectively, of the two *HoxD* genes (Fig. 5B and Supplemental Dataset S1).

From P14 to P21, midpachytene spermatocytes further develop into late pachytene and diplotene spermatocytes, followed by two consecutive meiotic cell divisions to become round spermatids [18]. In this particular time window, almost all X-linked mRNAs are downregulated due to MSCI in pachytene spermatocytes [19, 20]. Six X-linked lincRNAs (NR_027445, NR_027446, MM9LINCRNAEXON10009, MM9LINCRNAEXON10013, MM9LINCRNAEXON10032, and AK014868) were significantly downregulated (Fig. 5C). Interestingly, these six lincRNAs were <30 kb away from five X-linked mRNAs (*Uxt*, *C330007P06Rik*, *Pdk3*, *Las1l*, and *Prdx4*), all of which were downregulated as well (Fig. 5C and Supplemental Dataset S1). Moreover, while the testicular expression of *Uxt* and *Uxt*-associated lincRNAs (NR_027445 and uc009suh.1) remained to be suppressed from P21 to adulthood, *Pdk3* became upregulated, and this upregulation coincided with the upregulation of its associated lincRNA



(MM9LINCRNAEXON10032), which is located ~9 kb upstream of *Pdk3* (Fig. 5C and Supplemental Dataset S1).

We further mapped regulated nonintergenic (sense, antisense, and bidirectional) lncRNAs to the chromosomes and analyzed mRNAs that were up- or downregulated at the same time windows and <30 kb away from lncRNAs (Supplemental Fig. S8 and Supplemental Dataset S2). Similar to lincRNAs, the same five correlation patterns were found between nonintergenic lncRNAs and mRNAs (Supplemental Fig. S8 and Supplemental Dataset S2). During testicular development from P21 to adulthood, round spermatids elongate and chromatin becomes heavily packed through nuclear condensation, which involves replacement of histones with transition proteins followed by protamine [18]. *Tnp1*, *Tnp2*, *Prm1*, *Prm2*, and *Prm3* mRNAs levels were all increased by >100-fold in our microarray analyses (GEO accession number GSE46896 and Supplemental Dataset S2). Interestingly, we found two lncRNAs derived from both upstream (humanlincRNA0830) and downstream (DQ072380) of the *Tnp2* gene that were upregulated as well (Fig. 5E). More interestingly, the lncRNA DQ072380 is in between *Prm3* and *Prm2/1* (Fig. 5E). The close proximity of this lncRNA to the *Tnp-Prm* gene cluster, similarities in biological functions between the gene cluster members, and the coregulation of these lncRNA-mRNA pairs may suggest an interaction at either transcriptional or posttranscriptional levels.

Like the *Hox* gene cluster that is under the regulation of a lincRNA HOTAIR [9, 10], expression of the *Rhox* gene cluster may also be controlled by lncRNAs, based on our chromosomal mapping analyses (Fig. 5F). The *Rhox* gene cluster is located in a single region on the X chromosome, and it has been shown that this cluster of homeobox genes is predominantly expressed in reproductive organs [40, 41]. From E12.5 to E15.5, six *Rhox* genes (*Rhox4c*, *Rhox4d*, *Rhox4f*, *Rhox4g*, *Rhox12*, and *Rhox13*) were upregulated 2- to 8-fold (Fig. 5F and Supplemental Dataset S2). Interestingly, three lncRNAs (Rhox3-ps, Rhox3g-ps, and AK007253) that are 1–20 kb away from the six *Rhox* genes were also upregulated 9- to 14-fold. From P7 to P14, five *Rhox* genes (*Rhox4c, Rhox4d, Rhox4f, Rhox4g*, and *Rhox12*) became downregulated 2- to 3-fold, whereas the two lncRNAs (*Rhox3-ps* and *Rhox3g-ps*) that were close to these *Rhox* genes remained upregulated (Fig. 5F and Supplemental Dataset S2). The differential expression patterns between the five *Rhox* genes (*Rhox4c, Rhox4d, Rhox4f, Rhox4g*, and *Rhox12*) and the two lncRNAs (*Rhox3-ps* and *Rhox3g-ps*) suggest that those two pseudogene-like lncRNAs may not be the direct regulators of the five *Rhox* genes. However, the consistent inverse expression patterns between *Rhox12* and *Rhox13* and their neighboring lncRNA Ak007253 may suggest a regulatory relation (Fig. 5F). Although lincRNAs may act *in trans* by guiding chromatin-remodeling complexes

FIG. 3. RNA immunoprecipitation followed by real-time qPCR analyses demonstrating that many lncRNAs are associated with key components of chromatin-modifying complexes. **A**–**J**) Ten out of 26 lncRNAs analyzed were associated with EZH2, a H3K27 methylase that binds the core components Suz12 and EED to form polycomb repressive complex 2 (PRC2). The 10 lncRNAs include six lincRNAs, three antisense lncRNAs, and one bidirectional lncRNA. **K**) One antisense lncRNA, uc009cxn.1, interacted with LSD1, a demethylase that mediates enzymatic demethylation of H3K4me2 in the CoREST/REST complexes. Data were obtained from experiments in biological triplicates and presented as mean ± SEM. $^*P < 0.05$.

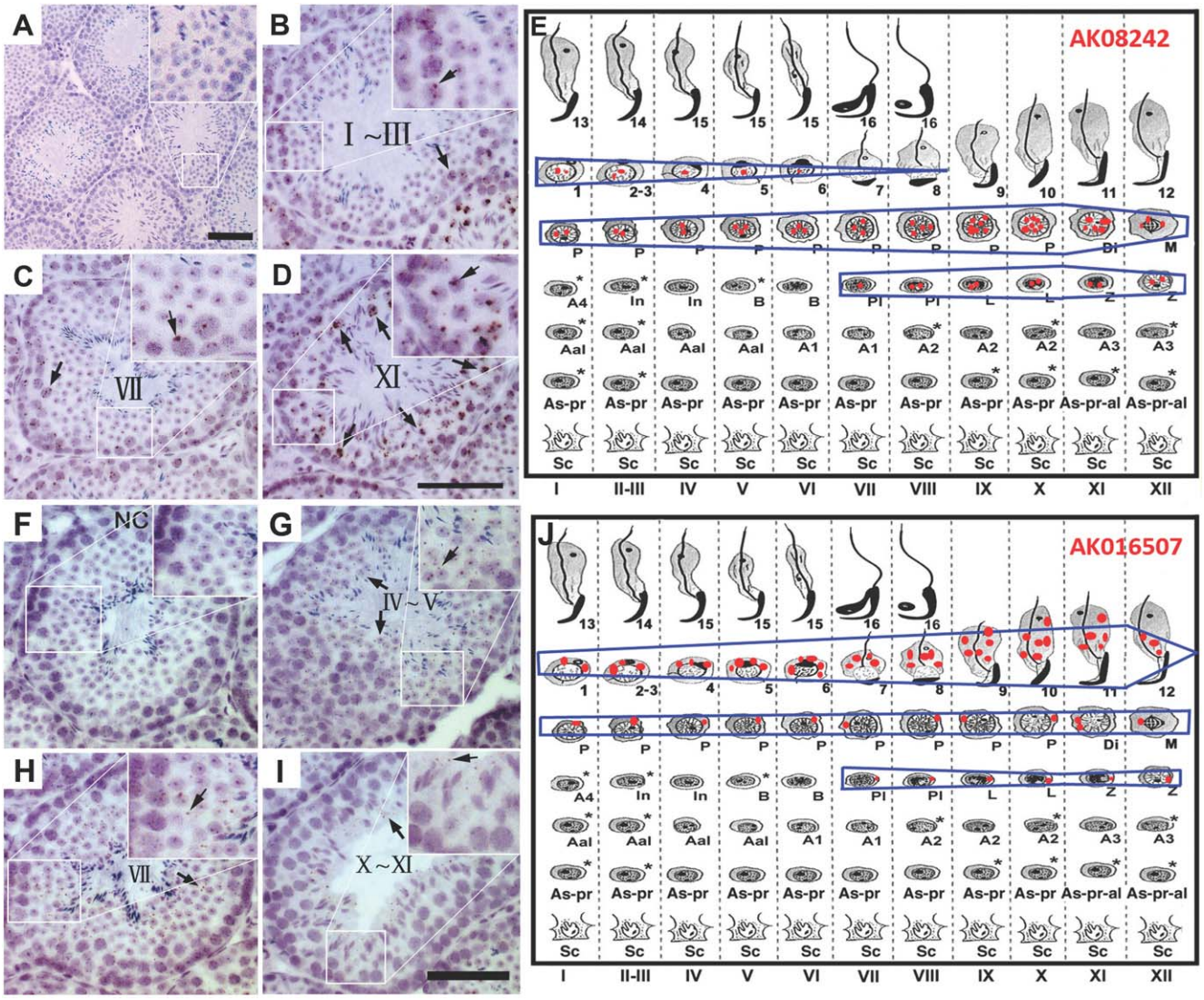


FIG. 4. Subcellular localization of two lncRNAs in adult murine testes by ISH assays. **A**–**D**) ISH-based localization of a bidirectional lncRNA (AK082424) in adult murine testes. Brown, punctuated dots representing specific hybridization signals are confined to the nuclei of spermatocytes and round spermatids in seminiferous tubules at stages I–III, VII, and XI. Arrows point to representative positive cells (**B**–**D**). Sections hybridized to control probes show no specific hybridization signals (**A**). Insets show the digitally magnified view of the framed regions. Panels **B**–**D** are in the same magnification. Bar = 50 μm. **E**) Schematic of the localization of lncRNA AK082424 during spermatogenesis in adult murine testes. Cells expressing this lncRNA are framed, and the height of the frame represents relative expression levels. Note that specific hybridization signals are confined to the nuclei of spermatocytes and round spermatids (red dots). Roman numerals (I–XII) mark stages of the seminiferous epithelial cycle, and steps of spermatid differentiation (i.e., spermiogenesis) are labeled with Arabic numerals (1–16). Sc, Sertoli cells; As, single type A spermatogonia; As-pr, paired type A spermatogonia; Aal, aligned type A spermatogonia; A1-4, type A1-A4 spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; P, pachytene spermatocyte; Di, diplotene spermatocyte; M, meiotically dividing. **F**–**I**) ISH-based localization of an antisense lncRNA (AK016507) in adult mouse testes. Brown, punctuated dots representing specific hybridization signals are confined mainly to the cytoplasm of spermatocytes and round and elongating spermatids in seminiferous tubules at stages IV, V, VII, X, and XI. Arrows point to representative positive cells (**G**–**I**). Sections hybridized to control probes show no specific hybridization signals (**F**). Insets show the magnified views of the framed regions. Panels **F**–**I** are all in the same magnification. Bar = 50 μm. **J**) Schematic of localization of lncRNA AK016507 during spermatogenesis in adult murine testes. Cells expressing this lncRNA are framed, and the height of the frame represents relative expression levels. Note that specific hybridization signals are mainly confined to the cytoplasm of spermatocytes and round and elongating spermatids (red dots). Labels are the same as in **E**.

to the various genomic loci, we did not analyze the potential distal targets of lincRNAs in the present study.

Identification of lncRNAs Associated with miRNA and piRNA Clusters

We also analyzed positional relations between significantly up- or downregulated lncRNAs at the six time windows in relation to known miRNAs and piRNA clusters. In total, 342 regulated lncRNAs were found to be <30 kb away from one or more miRNAs, and 498 miRNAs appeared to have one or more lncRNAs nearby (<30 kb). Of all lncRNA-miRNA pairs, two large miRNA clusters located on distal chromosome 12 are of great interest because 108 miRNA genes are clustered in two 100-kb regions, and ~14 lncRNAs were mapped to these regions, all of which displayed dynamic changes during the male germline development (Fig. 6A and Supplemental Dataset S3). Gene clusters of miRNA on the distal chromosome 12 are located in a parentally imprinted region designated the *Dlk1-Gtl2* domain in mice or the *Dlk-Dio3* domain in humans [42].

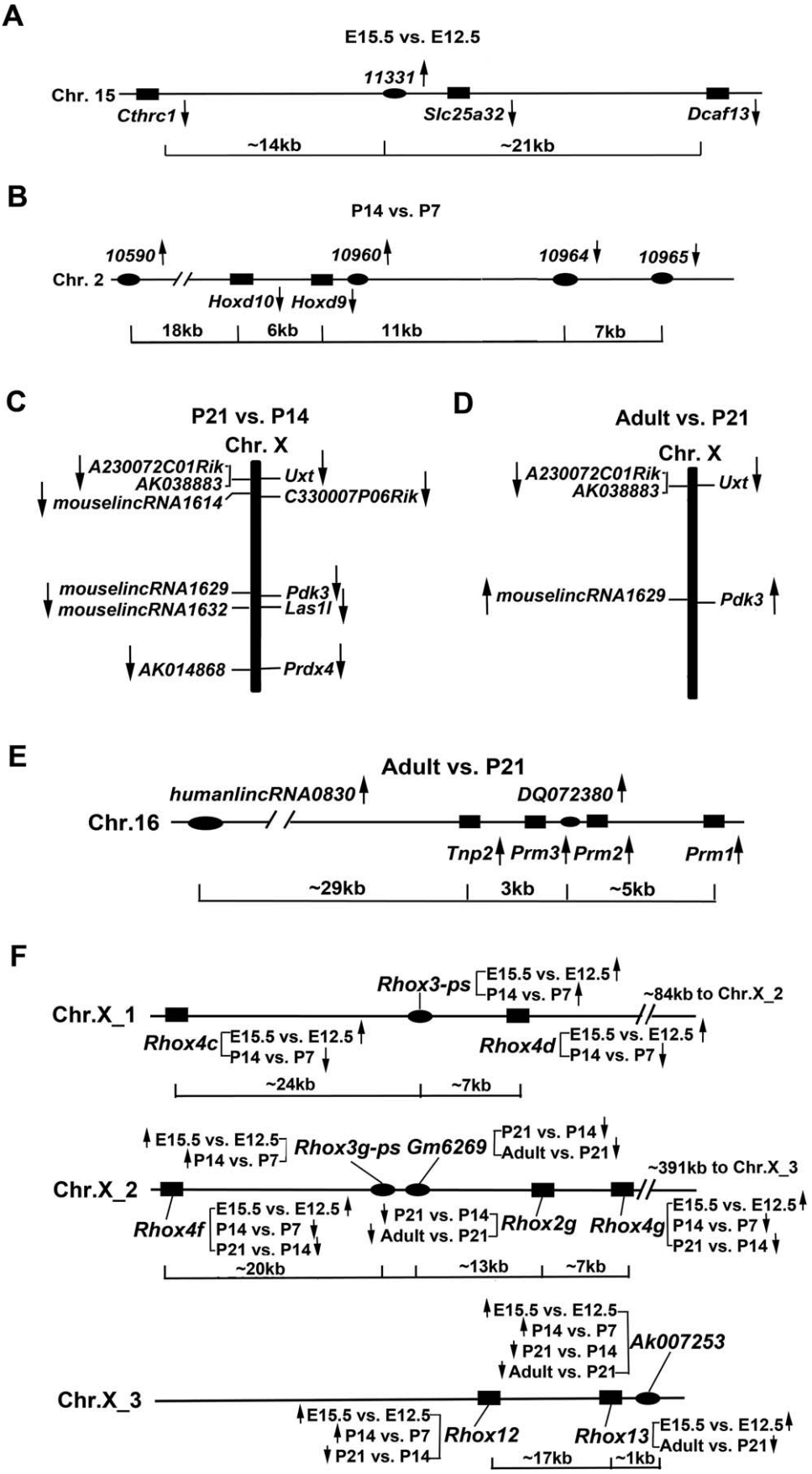


FIG. 5. Many lncRNAs and their neighboring mRNA genes (<30 kb) are either co-regulated or display inverse expression patterns during the six time windows of male germline development. Up and down arrows denote up and downregulation, respectively, throughout. **A**) An example of an inverse correlation between a significantly upregulated lincRNA (MM9LINCRNAEXON11331) and three of its neighboring mRNA genes (*Cthrc1, Slc25a32*, and

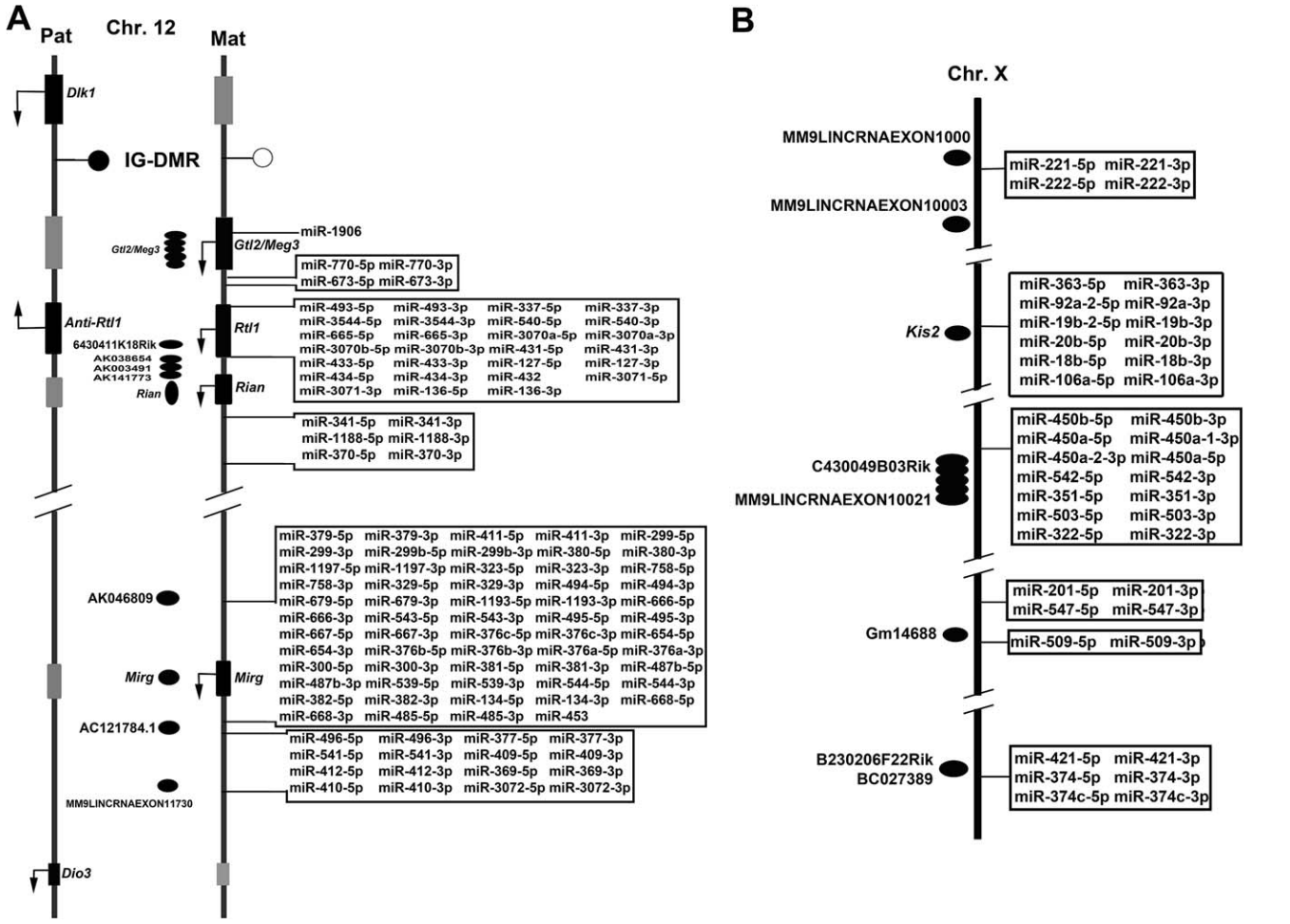


FIG. 6. Many lncRNAs appear to be associated with genomic regions enriched with miRNA clusters and/or under epigenetic regulations. **A**) Schematic representation of the distal chromosome 12 in mice, which is a parentally imprinted region enriched with miRNAs and lncRNAs. More than 10 lncRNAs (black oval shapes) derived from this region were significantly upregulated during fetal testicular development, and their levels remained high but became gradually downregulated with the progression of postnatal testicular development. Solid black bars represent active alleles, whereas gray bars denote inactive alleles. The transcriptional direction is marked with an arrow. Five major miRNA clusters containing >100 miRNAs are believed to be transcribed mainly from the maternal allele and, thus, are listed in rectangular boxes next to the maternal copy of chromosome 12. Some of the lncRNAs (e.g., *6430411K18Rik, Rian, Mirg*, and *Gtl2/Meg3*) contain sequences overlapping with miRNAs, and some (e.g., *AK003491, AK046809*, and *AC121784.1*) are derived from sequences in between those large miRNA clusters. **B**) Several of the X-linked miRNA clusters (rectangular boxes) known to escape the MSCI have lncRNAs (black oval shapes) expressed from their neighboring regions.

This region contains several maternally expressed (*Gtl2*, *Rtl1*, *Rian*, and *Mirg*) and paternally expressed (*Dlk1*, *anti-Rtl1*, and *Dio3*) genes (Fig. 6A). Many of the miRNAs in these clusters have been shown to be predominantly expressed from the maternal allele [43–45]. Some of the lncRNAs have been suggested to be primary miRNA transcripts, which are further processed by DROSHA-DGCR8 in the nucleus into pre-miRNAs followed by mature miRNA production by DICER-mediated cleavage in the cytoplasm [44]. However, many lncRNAs are out of the miRNA production region (e.g., AK046809, AC121784.1, AK141773). The X chromosome is also enriched with miRNAs, and many of the X-linked miRNAs escape the MSCI via an unknown mechanism [20, 27]. Interestingly, six X-linked miRNA clusters containing 48 miRNAs were found to have one or two nearby (<30 kb) lncRNAs (Fig. 6B).

Two types of piRNAs have been identified: one is termed prenatal piRNA, which is mainly expressed in PGCs and gonocytes in fetal testes; the other is called postnatal/pachytene piRNA and is predominantly expressed in spermatocytes and

Dcaf13) that were drastically downregulated during PGC reprogramming in the fetal testes (E15.5 vs. E12.5). **B**) Two *Hoxd* genes (*Hoxd10* and *Hoxd9*) are <30 kb away from four lincRNAs (MM9LINCRNAEXON-10590, −10960, −10964, and −10965) and are co-regulated with two distal lincRNAs (MM9LINCRNAEXON-10964 and −10965), but inversely regulated with one proximal (−10960) and one distal (−10590) lincRNA during transition from the mitotic to meiotic phases of spermatogenesis (P14 vs. P7). **C**) Co-regulation of five X-linked mRNA genes known to be suppressed during MSCI and six neighboring X-linked lncRNAs during late meiotic and early haploid phases of spermatogenesis (P21 vs. P14). **D**) While some X-linked mRNA genes (e.g., *Uxt*) remained suppressed after meiosis, some (e.g., *Pdk3*) started to be activated, and expression levels, thus, increased postmeiotically (Adult vs. P21). X-linked lncRNAs associated with these mRNA genes displayed similar changes in expression patterns. **E**) Co-regulation of distal and proximal lncRNAs with the *Tnp-Prm* gene cluster on chromosome 16 during spermiogenesis (Adult vs. P21). **F**) Dynamic correlations in expression patterns of seven *Rhox* genes and their neighboring lncRNAs during the four time windows of testicular development.

spermatids in postnatal testes [46, 47]. Both types of piRNAs consist of numerous large piRNA clusters located on several chromosomes [48–50]. We mapped the regulated lncRNAs and piRNA clusters from both types and identified 42 lncRNAs that were significantly up- or downregulated between E12.5 and E15.5 <30 kb away from 29 of the 97 prenatal piRNA clusters (30%; Supplemental Dataset S4). We also found that 93 lncRNAs that were regulated during postnatal testicular development (from P7 to adult) appeared to be <30 kb away from 123 out of 267 (46%) piRNA clusters (Supplemental Dataset S4). Given that many piRNAs are derived from repetitive sequences, repeat-associated piRNAs have been suggested to act *in trans* to induce DNA methylation, which in turn silences the repetitive sequences [51, 52]. The effects of these piRNA cluster-associated lncRNAs on piRNA function would be an interesting topic for future studies.

DISCUSSION

It is estimated that >40 000 unique lncRNAs are expressed in murine and human cells [25, 53, 54]. The microarray employed in this study covered 31 423 lncRNAs, some of which are overlapping in sequence; thus, the actual number of lncRNAs assessed was less. One of the advantages of using the ArrayStar lncRNA microarrays is that they detect both lncRNAs and mRNAs simultaneously; in addition, mRNA data can serve as a quality control and also can be used for correlation analyses for potential interactions between lncRNAs and mRNAs. The six time points chosen represent milestones critical for the male germline development, which include PGC reprogramming (E12.5 to E15.5); the mitotic (P7), meiotic (P14), and haploid (P21) phases of spermatogenesis; and full spermatogenesis (adult). Comparison of adjacent time points allowed us to identify both lncRNAs and mRNAs that were significantly up- or downregulated. Interestingly, most of the lncRNAs with >10-fold changes were detected in two time windows (P21 vs. P14 and adult vs. P21), suggesting that the regulation of lncRNAs is the most dynamic in the late meiotic and haploid phases of spermatogenesis. The timing coincides with the peak of both transcriptional activities and epigenetic modifications [15], implying a potential involvement of lncRNAs in the regulation of meiosis and spermatid differentiation during postnatal testicular development and spermatogenesis. Moreover, the opposite regulation patterns between PGC reprogramming (more upregulated than downregulated lncRNAs) and the haploid phase of spermatogenesis (more downregulated than upregulated lncRNAs) are consistent with the general transcriptional activities at the two time windows, suggesting either that the production of lncRNAs is transcriptionally controlled or that lncRNAs may have a role in the control of transcription of mRNA genes.

The origin and subcellular localization of lncRNAs may give a hint to their molecular actions. Large ncRNAs derived from introns or exons of mRNA genes in either sense or antisense orientation allow them, in theory, to target their host mRNA genes at both the transcriptional and posttranscriptional level. Recent data have shown that cytoplasmic lncRNAs can act as molecular decoys for miRNAs [55], suppress translation [56], or affect mRNA stability [57]. Therefore, with the identification of numerous nonintergenic lncRNAs expressed and highly regulated in the testis in this study, such evidence should become available in the near future. If derived from DNA and primary mRNA transcript promoter regions, nuclear lncRNAs can affect transcriptional activities and interact with transcriptional machineries [4, 25]. There are also reports supporting the role of nuclear lncRNAs in the regulation of splicing [54, 58].

Many lncRNAs neighbor mRNA genes, some of which have similar or related functions and, thus, constitute functionally related mRNA gene clusters. A significant proportion of lincRNAs has been shown to interact with chromatin-remodeling complexes and, thus, may function to induce epigenetic changes, which in turn can affect mRNA gene expression. Our data suggest that not only lincRNAs, but also nonintergenic lncRNAs, can bind chromatin-modifying complexes. If these lncRNAs act at the DNA level in the nucleus, they may induce epigenetic modifications, which can influence not only their host genes, but also neighboring genes. Given that most of the lncRNAs act *in cis*, we conducted the chromosomal mapping analyses to unveil lncRNAs and mRNAs that are nearby (<30 kb) and that are both significantly regulated during critical time points in the male germline development. Co-regulation of lncRNA-mRNA pairs may suggest that they both are under the same transcriptional control, and if they function locally (*in cis*), these lncRNAs may support the expression of their host mRNAs. Alternatively, they may act *in trans* by targeting genes located on other chromosomes. Inverse expression patterns (i.e., opposite changes during a specific time window) between lncRNAs and their neighboring mRNAs/mRNA clusters may imply a local action at both transcriptional and posttranscriptional levels. Interestingly, some lncRNAs are proximal to both upregulated and downregulated mRNAs. This may suggest that one lncRNA can have suppressive effects on a number of its target genes and at the same time can promote the expression of other genes nearby. Given that lncRNAs can directly interact with epigenetic machineries, the inverse regulation between lncRNAs and their neighboring mRNAs or mRNA clusters may represent a common mechanism through which lncRNAs act *in cis* to control the expression of their target mRNA genes. The present study identified numerous such positional correlations, providing researchers in the field with abundant clues that may lead to mechanistic studies in the future.

The abundance of miRNAs and lncRNAs derived from a region on distal chromosome 12 is of great interest because this region harbors several known lncRNA genes, e.g., *Dlk1, Gtl2, Rtl1, Rian, Mirg*, and *Dio3*, and is highly conserved in humans (i.e., human 14q32) [42]. These lncRNA genes are either paternally (*Dlk1* and *Dio3*) or maternally (*Gtl2, Rtl1, Rian*, and *Mirg*) imprinted, and an intergenic germline-derived differentially methylated region between *Dlk1* and *Gtl2* loci was suggested to control the differential methylation patterns in this region. More than 100 miRNAs are derived from this region, and these miRNA genes constitute five miRNA clusters based on their proximity [42, 44]. Although these lncRNAs have been suggested to be precursors for those miRNAs, several lncRNAs identified in this study are located in between miRNA clusters, and no miRNAs are directly derived from these lncRNAs. Moreover, many lncRNAs appear to exist as shorter sequences, instead of full-length transcripts of those lncRNA genes, and their sequences do not overlap with known miRNAs, suggesting that these lncRNAs may have a role beyond miRNA biosynthesis. Indeed, the enrichment of lncRNAs in an imprinted region may imply potential involvement of these lncRNAs in the establishment and/or maintenance of the imprinting patterns.

The X chromosome is also enriched with miRNAs, and many X-linked miRNAs escape the MSCI [20, 27]; the expression of these X-linked miRNAs persists in late pachytene spermatocytes even though almost all mRNA genes are silenced and display minimal or no transcriptional activity

[19]. While DNA methylation has been excluded as the cause of panchromosomal silencing on the X chromosome in late pachytene spermatocytes, the exact molecular mechanism underlying MSCI and selective active transcription of miRNA loci remains a mystery (20). The fact that these X-linked lncRNAs were all downregulated during the late pachytene and early haploid phases (P21 vs. P14) suggests that these lncRNAs undergo normal MSCI. Therefore, it is unlikely that they are involved in the MSCI escape of X-linked miRNAs.

Piwi-interacting RNAs expressed in PGCs during reprogramming between E10.5 and E15.5 have been shown to be essential for remethylation of repetitive sequences after global demethylation [51, 52]. However, these fetal testicular piRNAs do not directly bind the methylation machineries [51, 52], and it remains elusive as to how those piRNAs direct the remethylation of the repetitive sequences from which they are derived. The discovery that many lncRNAs are significantly upregulated during the reprogramming and are associated with those fetal piRNA clusters suggests that lncRNAs may interact with the methylation machinery and, thus, achieve silencing of those repetitive loci at the epigenetic level. Targeted deep sequencing in search of more lncRNAs derived from those regions that are subject to demethylation, followed by piRNA-dependent remethylation, may shed more light on this issue.

Our study represents the first systematic profiling analyses aimed at identification of lncRNAs that are expressed and strictly regulated during male germline development. Our data support the notion that the lncRNA transcriptome is highly heterogeneous, and our expression profiling data and correlation study suggest that lncRNAs are likely involved in gene regulation at both transcriptional and posttranscriptional levels. This study revealed numerous novel links between lncRNAs and their potential targets, which is of great interest if a cause-effect relation is confirmed experimentally. However, it is also possible that many of the lncRNAs may represent transcriptional ''noises'' and are, thus, nonfunctional. Further study is warranted to test these hypotheses.

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