MIWI-independent small RNAs (MSY-RNAs) bind to the RNA-binding protein, MSY2, in male germ cells

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The germ cell-specific DNA/RNA-binding protein MSY2 binds small RNAs (MSY-RNAs) that are ≈25–31 nt in length, often initiate with a 5′ adenine, and are expressed in both germ cells and somatic cells. MSY-RNA levels do not decrease in *Miwi* or *Msy2* null mice. Most MSY-RNAs map within annotated genes, but some are PIWI-interacting RNA (piRNA)-like and map to piRNA clusters. MSY-RNAs are in both nuclei and cytoplasm. In nuclei, MSY-RNAs are enriched in chromatin, and in the cytoplasm they are detected in both ribonucleoproteins and polysomes.

noncoding RNA | RNA–protein interactions | Y box proteins | spermatogenesis

Noncoding RNAs play important roles in regulating gene expression and development (1). Among the many classes of small noncoding RNAs are micro RNAs (miRNAs), siRNAs, endo-siRNAs, and PIWI-interacting RNAs (piRNAs) (reviewed in refs. 2–4). piRNAs are an extremely abundant group of germ cell RNAs of ≈30 nt that often initiate with a 5′ uracil and require members of the PIWI family of proteins for their biogenesis (4, 5). Based on sequence and their time of expression, piRNAs in mice have been classified as MILI (PIWIL2)-associated, MIWI2 (PIWIL4)-associated, or MIWI (PIWIL1)-associated piRNAs (4, 6). They are believed to be transcribed from long precursor RNAs and processed by a Dicer-independent mechanism (4, 6). Although some mammalian piRNAs share features with the *Drosophila* repeat-sequence repeat associated small interfering RNA (rasiRNAs) that are proposed to control transposable elements (6–8), the large number, variable sequences, and nuclear localization of mammalian piRNAs suggest additional cellular functions (9, 10).

The germ cell-specific DNA/RNA-binding protein MSY2 is a member of a Y box family of cold shock domain proteins (11). It is solely expressed in male and female germ cells and necessary for fertility (12). In the male, the absence of MSY2 leads to precocious destabilization of germ cell mRNAs (12). MSY2 serves as a coactivator of transcription, binding to the DNA sequence CTGATTGGC/TC/TAA in the promoters of genes transcribed in germ cells, thereby linking transcription with mRNA storage/stabilization (11). Suppressive subtractive hybridization studies reveal that of the nearly 100 MSY2 bound/nonbound mRNAs analyzed, many mRNAs encoding male gamete-specific and translationally delayed proteins are bound by MSY2 (11). In addition, MSY2 also binds in vitro to the piRNA, gsRNA10 (13).

Here, we describe a previously uncharacterized class of small RNAs (MSY-RNAs) from the mouse testis that are bound by MSY2. MSY-RNAs are ≈25–31 nt, often initiate with a 5′ adenine, and are expressed throughout germ cell differentiation and in somatic cells. The majority of the MSY-RNAs are derived from annotated genes, and some MSY-RNAs are piRNAs or piRNA-like. MSY-RNA levels are not altered in MIWI or MSY2 null mice.

Results and Discussion

MSY2 Selectively Binds a Previously Uncharacterized Population of ≈30-Nucleotide RNAs. Because in vitro RNA gel shift assays revealed that MSY2 binds to the piRNA, gsRNA10 (13), we used immunoprecipitation with an affinity- purified antibody to MSY2 (14) to assay for other small RNA targets. Analyses of the small RNAs isolated from immunoprecipitates of testicular ribonucleoprotein (RNPs) and polysomes detected a major band of ≈30 nt, a size class of RNA containing piRNAs in germ cells (Fig. 1*A*, lanes 4 and 5) (4). The interaction between MSY2 and the ≈30-nt RNAs appears selective, because a parallel experiment with antibody to Translin, another DNA/RNA-binding protein, did not preferentially immunoprecipitate small RNAs of ≈30 nt (Fig. 1*A*, lane 3).

To determine which small RNAs directly interact with MSY2, an in vivo cross-linking and immunoprecipitation procedure, the CLIP assay, was used. This assay covalently cross-links proteins to RNA, thereby allowing purification of highly specific protein–RNA complexes (15, 16). After the cross-linking and stringent washing of the complexes (SDS and high salt), RNA–protein complexes were resolved by SDS/PAGE (Fig. 1*B*). In contrast to immunoprecipitation procedures where UV cross-linking is not used, the CLIP assay avoids cloning small RNAs bound to other interacting proteins. Based on the predicted electrophoretic mobility of MSY2 bound to small RNAs, RNA was purified and used for cloning and sequencing from RNA–protein complexes of ≈63–68 kDa (Fig. 1*B*, lane 1). (The MSY2 protein migrates at ≈50 kDa, so the more slowly migrating RNA–protein complexes were chosen.) The absence of similarly sized RNA–protein complexes in testis extracts from *Msy2* null mice indicated a specific enrichment between MSY2 and small RNAs (hereafter named MSY-RNAs) (Fig. 1*B*, lane 2).

MSY-RNAs Are Distinct from Other Small Testicular RNAs. Sequence analysis of 231 different clones obtained from the MSY2 cross-linked and immunoprecipitated small RNA fraction yielded unexpected results (Fig. 2 and Table S1). Despite the abundance of piRNAs in the ≈30-nt fraction of the testis (4), only ≈7% of the small RNA sequences matched known piRNAs or mapped to piRNA clusters (Fig. 2*A*). Most of the immunoprecipitating small RNAs were 25- to 31-nt sequences that matched sequences in annotated genes, including exons (65%) and introns (16%) (Fig. 2*A* and Table S1). Other small RNAs mapped to intergenic regions (19%), which included 12% unannotated intergenic sequences and 7% piRNAs. This sequence distribution differs significantly from the predicted genome-wide sequence averages of 2% exonic, 30% intronic, and 68% intergenic (10). Most of the MSY-RNAs within annotated genes were from the sense strand, suggesting that they are not components of an antisense pathway.

Most of the MSY-RNAs had a 5′ A (≈75%), with ≈12% 5′ U, ≈9% 5′ C, and ≈4% 5′ G, in contrast to the strong preference

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DEVELOPMENTAL BIOLOGY

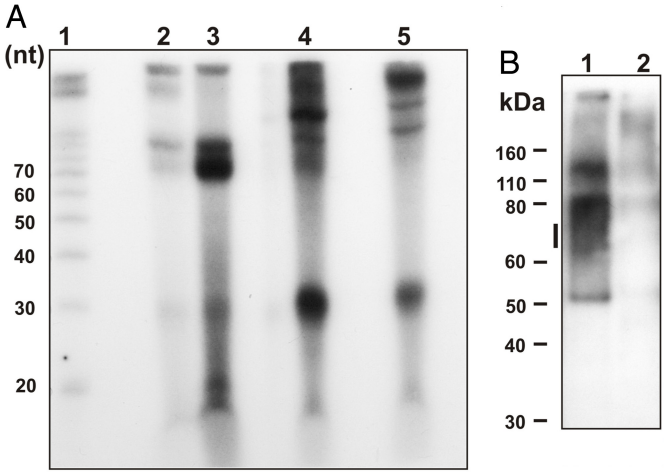

Fig. 1. MSY2 associates with a distinct population of small testicular RNAs. RNPs and polysomes from adult testes were separated by sucrose density gradient centrifugation (28). After immunoprecipitation of pooled fractions (11), RNA was purified, end-labeled with γ-ATP, and electrophoresed in urea-PAGE gels. (*A*) Lane 1, RNA decade markers; lane 2, immunoprecipitation from testis cytoplasmic extract with rabbit IgG; lane 3, immunoprecipitation from testis cytoplasmic extract with anti-Translin; lane 4, immunoprecipitation from pooled testicular RNPs with anti-MSY2; and lane 5, immunoprecipitation from pooled testicular polysomes with anti-MSY2. (*B*) Analysis of CLIP protein–RNA complexes obtained after immunoprecipitation with anti-MSY2. The protein–RNA complexes were separated by SDS/PAGE, and the region indicated with a bar was recovered. Lane 1, immunoprecipitated extract from wild-type mice; and lane 2, immunoprecipitated extract from *Msy2* null mice.

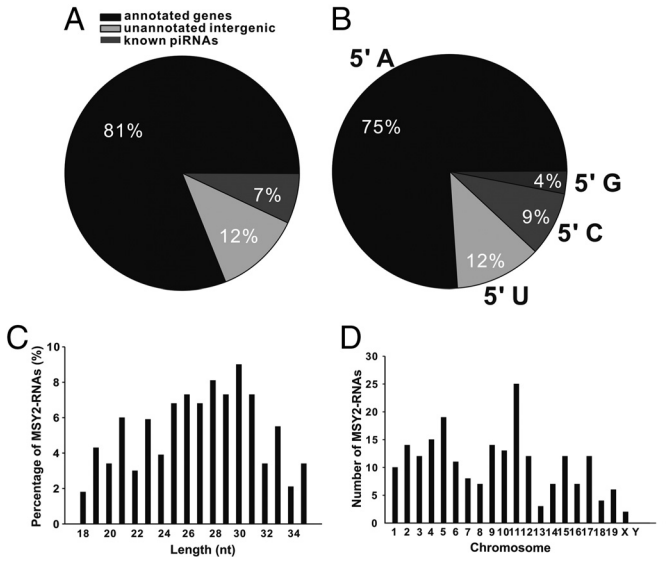

Fig. 2. Properties of MSY-RNAs. (*A*) Genomic annotation of the 231 cloned and sequenced MSY-RNAs. (*B*) Distribution of 5′ termini of the MSY-RNAs. (*C*) Size distribution of the MSY-RNAs. (*D*) Chromosomal distribution of the MSY-RNAs.

for 5′ U in miRNAs and piRNAs (Fig. 2*B*) (4). The initiation of a majority of MSY-RNAs with a 5′ adenosine distinguishes them from most mammalian miRNAs and piRNAs. Similar small RNAs exist in plants. Deep-sequencing studies in *Arabidopsis* reveal that the Argonaute proteins AGO2 and AGO4 bind small RNAs that predominantly have 5′ adenosine, whereas AGO1 and AGO5 bind small RNAs containing predominantly 5′ uridine and 5′ cytosine, respectively (17). Furthermore, we found that 83% of the nucleotides adjacent to the 5′ first nucleotide of MSY-RNAs are C (59%) or U (24%), and 73% of the nucleotides directly following the 3′ nucleotide of MSY-RNAs are G (33%) or A (40%), suggesting that the biogenesis of MSY-RNAs requires an endoribonuclease that cuts after C or U and before A at the MSY-RNA 5′ terminus, as well as an endoribonuclease that cuts after N and before A or G at the 3′ terminus. The conserved sequences adjacent to the MSY-RNAs indicate MSY-RNAs are not random degradation products derived from exonucleases.

MSY-RNA lengths ranged from 18 to 36 nt, with the majority being 25–31 nt (Fig. 2*C*). A 19-nt motif was found in 25 of the cloned MSY-RNAs (Fig. S1). This motif does not contain the preferred RNA-binding sequence, AACAUC, of FRGY2, the *Xenopus* orthologue of MSY2 determined by in vitro RNA Selex assays (18). Most of the MSY-RNAs (93%) were encoded once in the mouse genome (Table S1), and MSY-RNAs were encoded on both plus (52%) and minus (48%) DNA strands. Like piRNAs, MSY-RNAs were found on all chromosomes, with the exception of the Y chromosome (Fig. 2*D*). By the criterion of real-time PCR, MSY-RNAs were detected in rat testes, suggesting that MSY-RNAs are not solely expressed in mice.

The absence of any rRNA, tRNA, or miRNA sequences (0%) among the 231 clones strongly supports the specificity of RNA-MSY2 interactions in the cross-linking and immunoprecipitations. We believe that the MSY-RNAs are binding directly to MSY2, because the CLIP assay selects for RNAs that bind to proteins, avoiding nonspecific, indirectly bound contaminating RNA or protein. The washing and SDS/PAGE electrophoretic separation of the RNA–protein complex avoids adventitious binding to the immunoprecipitated complexes (15, 16). Although interactions between small RNAs and Translin occur in the testis, Translin binds to specific small RNAs, such as miRNA 122a, but not to piRNAs or most miRNAs (Fig. 1) (19).

Although most of the MSY-RNAs did not overlap, 3 overlapping small RNAs with 5′ A were detected from the 3′ UTR of the protamine 1 gene (Fig. S2 and Table S1, nos. 5, 96, and 135). Two nucleotides, TT, were missing from 2 of the *Prm1* small RNAs. This apparent lack of genomic colinearity (1 or 2 nucleotides missing or different, often C to U) was also seen in a few other MSY-RNAs. Antisense transcripts have been reported for the protamine genes and for ≈60% of the loci examined in 10 human chromosomes (20). The 3 MSY-RNAs encoding overlapping sequences from the 3′ UTR of the *Prm1* gene could bind to Prm1 antisense transcripts and serve as siRNAs (Fig. S2) (20).

The Amounts of Most MSY-RNAs Change Little During Testicular Development, Despite Many-Fold Changes in mRNA Levels from the Same Annotated Genes. Notable among the cloned RNAs were MSY-RNAs from annotated genes expressed solely or primarily in germ cells, including protamine 1 (*Prm1*), glyceraldehyde-3-phosphate dehydrogenase-(*S*) (*Gapds*; Table S1, no. 112), phosphorylase kinase 2 (*Phkg2*; Table S1, no. 163), testicular haploid-expressed gene (*Theg*; Table S1, no. 185), and ornithine decarboxylase antizyme 3 (*Oaz3*) (*Oaz3*; Table S1, no. 209). Some of the MSY-RNAs were from annotated genes whose mRNAs are bound by MSY2 (*Prm1* and *Gapds*), whereas others, such as *Tubb2c* (tubulin, beta 2c; Table S1, no. 124) and *Slc12a6* (solute carrier family 12, member 6; Table S1, no. 169) were from annotated genes whose mRNAs are not bound by MSY2 (11).

To determine the developmental regulation of the MSY-RNAs in the testis, MSY-RNAs were quantitated in prepubertal and adult mice by quantitative PCR (Q-PCR). Germ cells differentiate in the first wave of spermatogenesis, with P10 containing premeiotic germ cells, P17 containing meiotic germ cells, and P22 containing postmeiotic germ cells. The MSY-RNAs from the *Prm1*, *Gapds*, *Ppm1l* (Table S1, no. 184), *Phkg2*,

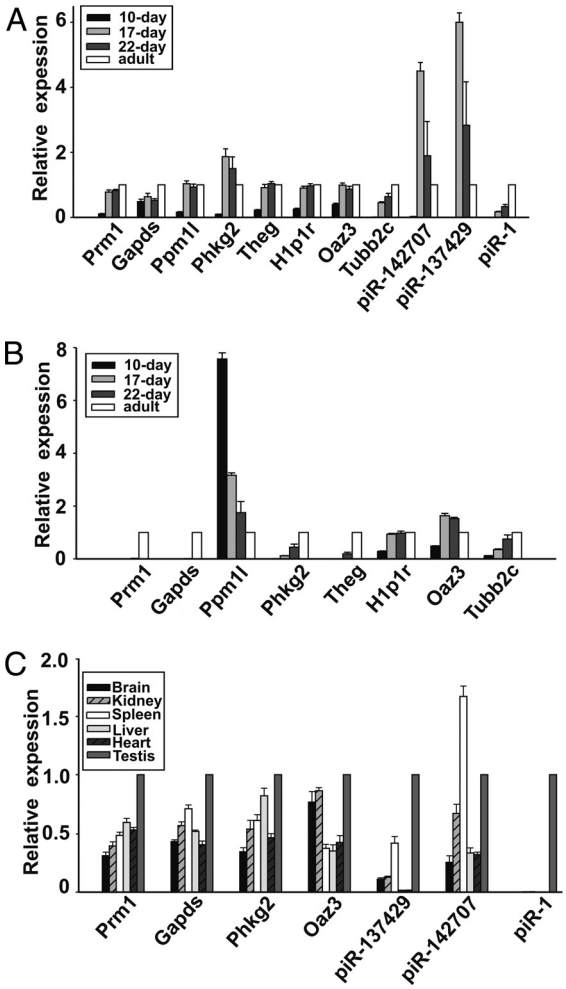

Fig. 3. The amount of MSY-RNAs from the testes of prepubertal and adult mice and somatic tissues differs substantially from mRNA levels. (*A*) MSY-RNA levels are compared in the testes of 10-day, 17-day, 22-day, and adult mice. The relative amounts of MSY-RNAs from prepubertal samples were normalized to those of the adult testis. (*B*) mRNA levels transcribed from the same annotated genes of the testes. The relative amounts of mRNAs were quantitated by Q-PCR, and prepubertal samples were normalized to those of the adult testis. (*C*) MSY-RNAs are expressed in mouse somatic tissues. The relative amounts of MSY-RNAs from adult somatic tissues were normalized to those of the adult testis.

Theg, *Hip1r* (Huntington-interacting protein 1-related protein; Table S1, no. 130), *Oaz3*, and *Tubb2c* loci were present at similar amounts in the testes of 17-day-old and 22-day-old and adult mice (Fig. 3*A*). In contrast, 2 piRNA-like MSY-RNAs whose sequences overlap, piR-142707 (Table S1, nos. 227 and 228) and piR-137429 (Table S1, no. 222), showed substantial decreases between prepubertal and adult ages, whereas a control piRNA, piR-1, which was not cloned as an MSY-RNA, increased, as seen for most piRNAs.

In contrast to the MSY-RNAs, some of the mRNAs encoded by the same annotated genes showed major increases in testes from 22-day-old and adult mice, consistent with their up-regulation in postmeiotic germ cells (Fig. 3*B*). The mRNAs for *Prm1*, *Gapds*, *Phkg2*, and *Theg* were greatly increased in the testes of adult mice, whereas *Ppm11* mRNA decreased. The *Hip1r*, *Oaz3*, and *Tubb2c* mRNAs exhibited more modest changes.

To examine whether the loss of functional mRNAs affects the levels of the MSY-RNAs transcribed from the same annotated gene, the MSY-RNAs for *Gapds* and *Hip1r* were quantitated in mice lacking *Gapds* or *Hip1r*. The deletion of *Gapds* by gene targeting produces infertile male mice with profound defects in sperm motility (21). Generation of *Hip1*/*Hip1r* double-knockout mice leads to accelerated spine abnormalities and testicular degeneration (22). Similar *Gapds* and *Hip1r* MSY-RNA levels were found in the testes of wild-type and *Gapds* or *Hip1*/*Hip1r* null mice, whereas decreases of up to 6-fold were seen for the *Gapds* and *Hip1r* mRNAs by real-time PCR assay. Thus, *Gapds* and *Hip1r* MSY-RNAs are present at wild-type levels in mice lacking *Gapds* or *Hip1r*.

MSY-RNAs Are Present in Somatic Tissues That Do Not Transcribe mRNAs from the Same Annotated Genes. The discrepancy between the amounts of MSY-RNAs and mRNA from the same annotated gene is especially striking in somatic cells. Analyzing a group of MSY-RNAs derived from annotated genes whose mRNAs are solely expressed in germ cells (*Prm1*, *Gapds*, *Phkg2*, and *Oaz3*), MSY-RNAs were detected at comparable amounts in the brain, liver, spleen, kidney, heart, and testis, indicating there is no correlation between MSY-RNAs and mRNA levels (Fig. 3*C*).

We believe that MSY-RNAs are not cytoplasmic degradation intermediates of mRNAs, because (*i*) MSY-RNAs are detected in tissues that do not express the mRNAs (Fig. 3*C*); (*ii*) in germ cells that express mRNAs from the same locus, MSY-RNA levels do not increase with increases in mRNA levels (compare *Prm1* and *Gapds* MSY-RNAs and mRNAs in Fig. 3 *A* and *B*); (*iii*) similar levels of MSY-RNAs for Gapds or Hip1r are seen in the testes of mice lacking either *Gapds* or *Hip1r*; (*iv*) the binding of small RNAs to MSY2 is specific because MSY-RNAs do not form RNA–protein complexes with Translin, another DNA/RNA-binding protein (Fig. 1); (*v*) ≈30% MILI-associated small RNAs are generated from genic sequences, and about 3–4% MIWI-associated small RNAs are from annotated genes (7); (*vi*) strong 5′ end-labeling of MSY-RNAs was seen after incubation with calf intestinal alkaline phosphatase, suggesting that MSY-RNAs have phosphate at their 5′ termini (Fig. S3), as reported previously for miRNAs or piRNAs; and (*vii*) ≈83% of the nucleotides adjacent to the 5′ termini of each MSY-RNA are either a C or U, and ≈73% of the nucleotides adjacent to the 3′ nucleotide of each MSY-RNA are G or A, indicating the MSY-RNAs are not derived by random nonspecific exonuclease degradation. Databases containing numerous small RNAs from HeLa and HepG2 cells that arise from within annotated genes and from intergenic regions also support the general widespread expression of this novel group of small RNAs (23). Moreover, the transsplicing of regions of the normally testis-specific *Oaz3* mRNA in somatic tissues that do not transcribe *Oaz3* mRNA also demonstrates the selective synthesis of parts of mRNAs, as seen with MSY-RNAs (24) The presence of similar amounts of MSY-RNAs in somatic tissues and testes, despite the absence of the corresponding mRNAs, establishes that MSY-RNA levels do not follow the presence or changing amounts of mRNAs.

MSY-RNAs Include piRNAs That Are Expressed in Somatic Tissues. Although piRNA expression is generally thought to be limited to germ cells, low levels of piRNAs have been reported in somatic tissues (25). We found several piRNAs or piRNA-like RNAs that bind MSY2 are expressed in somatic tissues. For example, 2 cloned MSY-RNAs, piR-137429 and piR-142707, are detected in somatic tissues, although a control germ cell-specific piRNA, piR-1, is solely detected in testis (Fig. 3*C*). This indicates that a subpopulation of known and new piRNA-like small RNAs exists in the testis that exhibits the somatic tissue expression patterns of MSY-RNAs. Because MSY2 is only expressed in male and female germ cells, MSY-RNAs in somatic tissues should interact with other Y box proteins, such as MSY1 (YBX1), MSY4

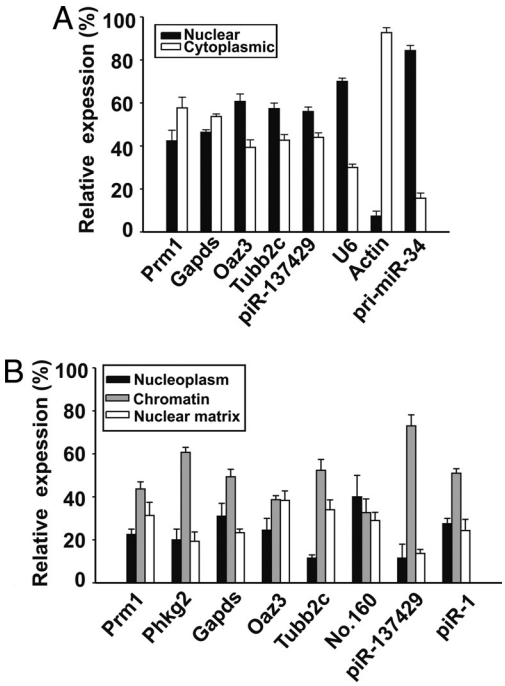

Fig. 4. MSY-RNAs are present in nuclei and cytoplasm in the testis. (*A*) MSY-RNAs were quantitated by Q-PCR using small RNA preparations from purified nuclei and cytoplasm from adult testes. To quantitate the pri-miR-34 and actin mRNA, large RNA preparations from the same samples were used to prepare cDNAs. The relative amounts (percentages) of MSY-RNAs in nuclei were normalized to cytoplasm. (*B*) MSY-RNAs were quantitated by Q-PCR in nuclei subfractionated into nucleoplasm, chromatin, and nuclear matrix.

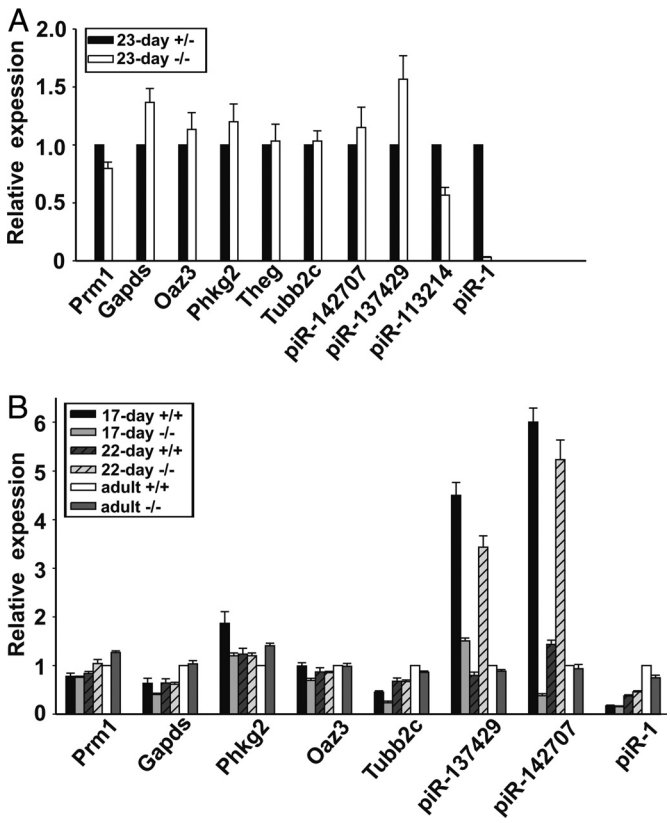

Fig. 5. MSY-RNA levels are independent of MIWI or MSY2. (*A*) MSY-RNAs were quantitated by Q-PCR in the testes of *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ 23-day-old mice. The relative amounts of MSY-RNAs in *Miwi*$^{-/-}$ were normalized to those of the *Miwi*$^{+/-}$ testes. (*B*) MSY-RNAs were quantitated by Q-PCR in the prepubertal and adult testes of wild-type and *Miwi*$^{-/-}$ mice. The relative amounts of MSY-RNAs in prepubertal mice were normalized to those of the wild-type adult testes.

(CSDA), or related proteins. Immunoprecipitation assays with anti-MSY1 have confirmed the binding of MSY1 to many MSY-RNAs, including *Phkg2*, *Theg*, *Prm1*, *Oaz3*, *Gapds*, and piR-142707 in such tissues as the brain. The identification of the endoribonucleases and other enzymes involved in the processing of MSY-RNAs should help elucidate their functions.

MSY-RNAs Are Present in Testicular Nuclei and Cytoplasm. To help define possible function(s) of MSY-RNAs, the subcellular and subnuclear amounts of representative MSY-RNAs were quantitated in testes by Q-PCR. Assaying equal percentages of the total small RNAs purified from each subcellular fraction, around half of the MSY-RNAs for *Prm1*, *Gapds*, *Oaz3*, *Tubb2c*, and piR-137429 were detected in nuclei (Fig. 4*A*). Assays for 2 nuclear markers—the U6 small nuclear RNA and the precursor for miR-34 (pri-miR-34)—and actin mRNA as a cytoplasmic marker confirmed proper subcellular fractionation of the extracts. RT-PCR assays for *Prm1*, *Gapds*, and *Phkg2* MSY-RNAs in a small RNA library prepared from testicular extracts fractionated over sucrose gradients found similar amounts of these MSY-RNAs in pooled RNP and polysomal fractions (Fig. S4).

To better localize MSY-RNAs in nuclei, purified nuclei were fractionated into nucleoplasm, chromatin, and nuclear matrix. Assays for the *Prm1*, *Phkg2*, *Gapds*, *Oaz3*, *Tubb2c*, and no. 160 MSY-RNAs (no.160 is an MSY-RNA that is partially complementary to Prm1; Table S1), as well as piR-137429 revealed that MSY-RNAs are generally enriched in the chromatin fraction (Fig. 4*B*). Similarly, MSY2 is present in both nuclei and cytoplasm, with both transcriptional and posttranscriptional functions (11, 12). In meiotic and postmeiotic male germ cells MSY2 identifies specific mRNAs in the nucleus and facilitates their storage/translational repression in the cytoplasm. The abundance of the testicular MSY-RNAs in chromatin raises the possibility that they play a role targeting MSY2, as seen with PIWI–piRNA complexes, which promote the euchromatization of 3R-TAS heterochromatin, thereby facilitating its transcription (26). The presence of MSY-RNAs in both RNP and polysomal fractions (Fig. 1*A* and Fig. S4) suggests functions in translation and its regulation, as proposed for piRNAs (27). We do not know whether the MSY-RNAs contribute to the spermatogenic arrest seen in Msy2 null mice. In the absence of MSY2, there is a precocious movement of many mRNAs from RNPs to polysomes, leading to their decreased stability (28). Possible contributions of MSY-RNAs to mRNA storage and stabilization/destabilization remain to be determined.

MSY-RNA Levels Are Not Altered by the Absence of MIWI or MSY2. Members of the PIWI family of proteins are required for piRNA biogenesis in a Dicer-independent mechanism (4, 6). Because MSY-RNAs are similar to piRNAs in size, and at least 7% of our cloned MSY-RNAs are piRNAs, we investigated whether the biogenesis of MSY-RNAs requires the MIWI protein. No differences in amount were seen for the MSY-RNAs for *Prm1*, *Gapds*, *Oaz3*, *Phkg2*, *Theg*, and *Tubb2c* between *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ mice. Although most piRNAs are not detectable in *Miwi* null mice (10), the levels of several tested MSY2-binding piRNAs and piRNA-like MSY-RNAs (piR-142707, piR-137429, and piR-113214) were not altered by the absence of *Miwi* (Fig. 5*A*). Control piRNAs that are not immunoprecipitated by anti-MSY2, such as piR-1, were not detectable in testes from *Miwi*$^{-/-}$ mice. This suggests that piRNA-like RNAs that associate with

MSY2 differ from the PIWI family-associated piRNAs. This group of MIWI-independent piRNAs appears to be a distinct subpopulation of piRNAs. Moreover, in contrast to MIWI-requiring piRNAs, whose amounts increase as germ cells differentiate, these MSY2-binding piRNAs decrease in adult testes. The expression of 2 randomly chosen piRNAs (piR-103716 and piR-130724) from the National Center for Biotechnology Information piRNA database that are in the same piRNA genomic cluster as MSY-RNA piR-142707 also appears to be MIWI-independent. Furthermore, immunoprecipitation assays with anti-MILI have demonstrated that MSY-RNAs are not associated with this other members of the PIWI family, suggesting that the synthesis of MSY-RNAs is also MILI-independent.

Mice lacking MSY2 are infertile because spermatogenesis terminates during the haploid phase of germ cell differentiation (12). MSY-RNAs were quantitated in prepubertal and adult testes from MSY2 null mice by Q-PCR. No differences were detected for the *Prm1*, *Gapds*, *Oaz3*, *Phkg2*, *Theg*, and *Tubb2c* MSY-RNAs (Fig. 5*B*). However, 2 MSY2-interacting piRNAs (piR-137429 and piR-142707) showed dramatic changes in prepubertal testes from MSY2 null mice in contrast to the control piR-1. Thus, neither MIWI nor MSY2 appears to be crucial for the processing or survival of most of the MSY-RNAs (Fig. 5). In the testis, other Y box proteins, such as MSY1 or MSY4, may substitute for MSY2 in selectively binding small RNAs.

In contrast to most testicular piRNAs and miRNAs, the majority of MSY-RNAs (≈81%) are derived from sites within annotated genes. A significant number (≈30%) of the MILI-associated piRNAs are also derived from annotated genes (7). This subpopulation of MILI-associated piRNAs is mostly derived from the sense strand, and unlike many MILI-associated piRNAs, their expression is not reduced in *Mili* null mice (7). MSY-RNAs such as the one from the repeat domain in mouse ubiquitin C (Table S1, no. 182) are similar in sequence to these piRNAs, raising the possibility that certain MILI-associated piRNAs and the MSY-RNAs are processed by a similar but unknown mechanism. We do not think Dicer is involved in the processing of MSY-RNAs, because of the size difference between miRNA/siRNAs and the MSY-RNAs, the lack of obvious stem-loop structures for most MSY-RNAs, the nucleotides adjacent to most MSY-RNAs, and the Dicer independence of most piRNA biogenesis.

In summary, we have identified a population of ≈30-nt RNAs that selectively bind to MSY2. They are transcribed from annotated genes and unannotated intergenic regions throughout the mouse genome and are expressed in germ cells and somatic cells in the absence of MIWI or MSY2. Among the MSY-RNAs are a number of piRNAs that show novel temporal patterns of expression and whose synthesis appears to be MIWI-independent. Possible contributions of MSY-RNAs to mRNA storage and stabilization/destabilization or directing MSY2 to specific cellular sites remain to be determined.

Materials and Methods

Mouse Sources. Adult CD-1 mice were purchased from Charles River Laboratories. A breeding colony of *Msy2* null mice was maintained at the University of Pennsylvania. Testes from *Gapds*, *Hip1r*, and *Miwi* null mice were kindly provided by D. A. O'Brien, T. S. Ross, and H. Lin, respectively. All investigations were conducted in accordance with the Guide for Care and Use of Laboratory Animals (1966), and the institutional animal care and use committee approved all animal procedures in advance.

Cross-Linking and Immunoprecipitation of MSY2. A CLIP analysis of MSY2 was performed as described previously (15, 16), with minor modifications. Seminiferous tubules from adult wild-type or *Msy2* null mice were UV cross-linked at 500 mJ/cm^2 in 10-cm dishes and were lysed by sonication in ice-cold lysis buffer (1× PBS, 0.125% SDS, 0.5% deoxycholate, and 0.5% Nonidet P-40) containing 20 units/μL of the RNase inhibitor Superase-in (Ambion). After centrifugation at 10,000 × *g*, the soluble extracts were treated with 60 units of RQ DNaseI (Promega) for 30 min at 37 °C and then centrifuged at 160,000 × *g* for 30 min. The supernatants were incubated with a polyclonal affinity-purified anti-MSY2 coupled to protein A-Dynabeads (Invitrogen) for 1 h at 4 °C. After intensive washing with lysis buffer, wash buffer (3× PBS, 0.125% SDS, 0.5% deoxycholate, and 0.5% Nonidet P-40) and PNK buffer (50 mM Tris·HCl, pH 7.4; 10 mM $MgCl_2$; and 0.5% Nonidet P-40), the cross-linked RNAs were dephosphorylated and ligated to a 3′ RNA linker. After 5′ end labeling, the samples were separated in a 10% Bis-Tris gel, and a labeled region (≈62–68 kDa) was cut from the gel. The RNA was purified, ligated to a 5′ linker, treated with Turbo DNA-free kit (Ambion), and amplified by RT-PCR. Concatemerization and TOPO cloning were performed as described previously (16).

Isolation of Small and Large RNAs. Small and large RNAs were isolated from prepubertal and adult testes [P10, P17, P22, and adult (8–10 weeks)] and somatic tissues by using the mirVana miRNA Isolation Kit (Ambion).

5′ End-Labeling Assay. Total RNAs immunoprecipitated after cross-linking were treated with calf intestinal alkaline phosphatase (Roche) and 5′ end-labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled RNAs were separated in 15% urea-PAGE gels.

Ribonucleoprotein and Polysome Fractionation. Extracts were prepared from the testes of 2 adult wild-type or 3 MSY2 null mice and fractionated over sucrose density gradients (10–30%) as described previously (28). Fractions containing RNPs or polysomes were pooled, and total RNA and small RNA were isolated as described above.

Isolation of Nuclei and Cytoplasm and Subnuclear Fractionation. Nuclei and cytoplasm were isolated as described previously (29). To obtain nuclear subfractions, isolated nuclei were resuspended in 200 μL of buffer (10 mM Hepes, pH 7.4; and 10 mM KCl) containing 30 units of RQ DNaseI (Promega) for 1 h at 4 °C. Chromatin was solubilized in extraction buffer (1% Triton X-100; 50 mM Hepes, pH 7.4; 400 mM NaCl; and 1 mM EDTA) containing protease inhibitors for 10 min at 4 °C and collected at 16,000 × *g* in a microcentrifuge for 10 min. The pellet was extracted in urea buffer (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 8.0), and the nuclear matrix was isolated from the supernatant after centrifugation at 10,000 × *g*. Total RNA was purified with TRIzol, and small RNAs were purified as described above.

Semiquantitative RT-PCR Assays. Construction of small RNA complementary DNA libraries and semiquantitative RT-PCR analyses were performed as described previously (25). Briefly, equal amounts of small RNA were poly(A)-tailed by using the poly(A) tailing kit and purified with the MEGAclear kit (Ambion). Residual DNA contaminants were removed with the Turbo DNA-free kit, and RT-PCR was performed. Q-PCR reactions were performed with SYBR green PCR Master reagents and the 7900HT Sequence Detector (Applied Biosystems). The expression level of small RNAs was normalized to U6 snoRNA and miR-16. All oligonucleotide sequences used in the Q-PCR assays are listed in Table S2. Samples with PCR cycle numbers between 16 and 30 cycles were chosen for analysis to ensure that each of the amplification reactions was in the exponential range.

For mRNA quantitation, total testis RNA was purified with TRIzol, and residual genomic DNA was removed. First-strand complementary DNA was synthesized with random hexamer primers and SuperScript III reverse transcriptase (Invitrogen). The mRNA levels were normalized to actin and 18S rRNA.

Bioinformatic Analyses. The MEME program was used for motif identification (http://meme.sdsc.edu/meme/meme.html). Secondary structure predictions (≈100 bp upstream and downstream of the MSY-RNAs) were carried out with RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). All cloned small RNA sequences were characterized by using the National Center for Biotechnology Information GenBank (www.ncbi.nlm.nih.gov/blast/). The chromosomal locations of MSY-RNAs were determined with the BLAST program of the University of California, Santa Cruz Genome Browser. Data were plotted and graphed by using Sigmaplot (Systat Software).

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