Mili, a mammalian member of *piwi* family gene, is essential for spermatogenesis

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Summary

The *piwi* family genes, which are defined by conserved PAZ and Piwi domains, play important roles in stem cell self-renewal, RNA silencing, and translational regulation in various organisms. To reveal the function of the mammalian homolog of *piwi*, we produced and analyzed mice with targeted mutations in the *Mili* gene, which is one of three mouse homologs of *piwi*. Spermatogenesis in the MILI-null mice was blocked completely at the early prophase of the first meiosis, from the zygotene to early pachytene, and the mice were sterile. However, primordial germ cell development and female germ cell production were not disturbed. Furthermore, MILI bound to MVH, which is an essential factor during the early spermatocyte stage. The similarities in the phenotypes of the MILI- and MVH-deficient mice and in the physical binding properties of MILI and MVH indicate a functional association of these proteins in post-transcriptional regulation. These data indicate that MILI is essential for the differentiation of spermatocytes.

Key words: *Mili*, *Miwi*, *piwi*, *Mvh*, Spermatogenesis

Introduction

Germ cells, which are highly specialized cells that transmit genetic information from generation to generation, are segregated from somatic lineages very early in embryonic life. Primordial germ cells (PGCs), which are the first germ cells specified during embryogenesis. They eventually differentiate into gametes in the adult gonad (Wylie, 1993). In *Drosophila*, gametes of both sexes are produced continuously from germline stem cells in the adult gonad. In mammals, the continuous production of germ cells by the stem cell system proceeds only in the male gonad via spermatogenesis. Genetic studies in *Drosophila* have identified several genes that are involved in germ cell development and germline stem cell division. The former include the *oskar*, *vasa*, *nanos*, *tudor* and *germ cell-less* genes (Rongo and Lehmann, 1996), and the latter include the *piwi* (Cox et al., 1998) and *Yb* (King et al., 2001) genes. However, the homologs of these gene products are not necessarily functional in the mammalian germ cell system. One reason is that the molecular mechanisms in *Drosophila* germ cell development and differentiation are quite different from those in mammals. Gene targeting analyses of *Mvh* (mouse vasa homolog; *Ddx4* – Mouse Genome

Informatics), *Mgcl-1* (mouse germ cell-less-1; *Gcl* – Mouse Genome Informatics) and *Miwi* (mouse piwi; *Piwil1* – Mouse Genome Informatics) have shown that although these genes are dispensable for PGC formation, they are essential for spermatogenesis (Deng and Lin, 2002; Kimura et al., 2003; Tanaka et al., 2000).

Spermatogenesis is one of the most dramatic examples of cell proliferation, differentiation and morphogenesis. The mouse spermatogenic cycle can be divided into 12 stages, with each stage consisting of a specific complement of male germ cells (Russell et al., 1990). The entire process occurs in three phases: mitosis (spermatocytogenesis), meiosis and spermiogenesis. A cascade of mitoses, which are initiated by the self-renewing division of germline stem cells (a subset of type A spermatogonia), gives rise to the primary spermatocytes. Subsequently, meiosis of primary spermatocytes leads to the production of haploid round spermatids. The prophase of the first meiotic division progresses in the following order: leptotene, zygotene, pachytene, diplotene and diakinesis. Various genes that are involved in cell cycling, DNA replication and RNA processing are essential for this process.

One of the germ cell determinant genes in *Drosophila*, *vasa* encodes an ATP-dependent RNA helicase of the DEAD-box protein family that is essential for the assembly and function of the germ plasm (Hay et al., 1988; Hay et al., 1990; Lasko and Ashburner, 1988). Based on structural conservation data, homologs of *vasa* have been identified in many animal species, such as *C. elegans*, *Xenopus*, zebrafish, chicken and mouse (Fujiwara et al., 1994; Komiya et al., 1994; Olsen et al., 1997; Roussell and Bennett, 1993; Tsunekawa et al., 2000; Yoon et al., 1997). All of these *vasa* homologs are expressed exclusively in the germ lineage. The expression and function of the *Mvh* gene were analyzed by immunostaining and gene-targeting analysis, respectively (Tanaka et al., 2000; Toyooka et al., 2000). The MVH protein appears initially in PGCs that colonize the embryonic gonads at 10.5-11.5 dpc, and is maintained in both male and female germ cells until the development of postmeiotic spermatids and primary oocytes, respectively. Sperm are absent from the testes of *Mvh*-null male mice, in which premeiotic germ cells cease differentiation by the zygotene stage and undergo apoptotic death.

The *piwi* gene of *Drosophila* belongs to a novel class of evolutionarily conserved genes (the *piwi* or *Argonaute* family) (Benfey, 1999). The *piwi* family genes encode basic proteins that contain a highly conserved PAZ domain of 110-amino acid residues in the middle region of the proteins and a 300-amino acid Piwi domain in the C-terminal region (Cerutti et al., 2000), even though the function of these domains remains elusive. In *Drosophila*, the loss of *piwi* function leads to the failure of germline stem cell self-renewal as well as downstream gametogenic functions such as germline cyst formation, egg polarity and possibly meiosis (Cox et al., 1998; Lin and Spradling, 1997). Based on database analyses, three *piwi* homologs have been identified in the mouse genome. Two of these genes, *Miwi* and *Mili* (*Miwi like*; *Piwil2* – Mouse Genome Informatics), have been examined in detail. *Miwi*-null mice do not complete spermatogenesis, but arrest occurs at the beginning of the round spermatid stage (Deng and Lin, 2002), significantly downstream of the germline stem cell division stage. To reveal the functional of *Mili*, here we report the generation and analysis of the *Mili*-null mice. The *Mili*-null mice showed arrest of spermatogenesis at the spermatocyte stage, which is reminiscent of the phenotype of *Mvh*-null mice. Furthermore, we demonstrate the physical association between MILI and MVH, which may account for the similarities between the phenotypes of the *Mili*- and *Mvh*-null mice.

Materials and methods

Generation and genotyping of *Mili*-deficient mice

Genomic DNA clones of the *Mili* locus were isolated from a mouse (129/Svj) genomic library (Stratagene) using full-length *Mili* cDNA as a probe. The 5.2-kb *Bam*HI fragment that encompasses exon 2 to exon 5 was replaced with a neomycin resistance cassette in the pPNT vector (Tybulewicz et al., 1991). The 2.0-kb *Bgl*II-*Bam*HI fragment was inserted between the neomycin resistance cassette and the HSV thymidine kinase gene, both of which are driven by the *PGK1* promoter. The 4.5-kb *Bam*HI-*Kpn*I fragment was inserted upstream of the neomycin resistance gene. The resulting targeting vector, pKO-Mili2, was electroporated into D3 embryonic stem (ES) cells, and selected with G418 and gancyclovir. The targeting events were screened by PCR and confirmed by Southern blot analysis using digestion with restriction enzymes and both 3′- and 5′-end probes. The

recombinant cells were karyotyped to ensure that 2N chromosomes were present in the majority of the metaphase spreads. Chimeric mice, which were derived from correctly targeted ES cells, were mated to C57BL/6 mice to obtain F1 *Mili*$^{+/-}$ mice.

The oligonucleotide PCR primers that were used to distinguish the insertion of the *neo* gene from the wild-type allele were as follows: pPNT-1, 5′-CCTACCCGGTAGAATTGACC-3′; Mili-Int4, 5′-GTCC-TGTGTAGAGCCAAG-3′; and Mili-Int5, 5′-TGACAAGGTGC-GAGTCT-3′. The pPNT-1 and Mili-Int5 primers gave a 410 bp DNA fragment that identified the targeted allele, while the Mili-Int4 and Mili-Int5 primers yielded the 800 bp fragment of the wild-type allele. The PCR was carried out for 40 cycles of 94°C for 1 minute, 65°C for 1 minute and 72°C for 1 minute.

Antibodies

The GST-Mili-26F plasmid was constructed by inserting the *Mili* cDNA fragment that encodes the sequence from Ile357 to Phe502 into the pGEX3X expression vector (Pharmacia Biotech). The purified GST-fusion proteins were used to immunize rabbits. The affinity-purified polyclonal antibody directed against Mili-26F recognized both the MIWI and MILI proteins in western blots. The anti-MILI-N1 and anti-MIWI-C polyclonal antibodies (against MILI and MIWI) were generated by immunization with the MILI N-terminal peptide (DPVRPLFRGPTPVHPSQC) and MIWI C-terminal peptide (CHHEPAIQLCGNLFFL), respectively. The affinity-purified antibodies against the peptides (produced by MBL, Japan) were used for immunohistochemical analysis. The anti-MVH (Toyooka et al., 2000) and anti-SYCP3 (Chuma and Nakatsuji, 2001) antibodies were donated by Drs. T. Noce and N. Nakatsuji, respectively. The rabbit B antiserum against whole synaptonemal complexes, which reacts both COR1 and SYN1 proteins (Moens and Spyropoulos, 1995), were kindly provided by Dr P. B. Moens. The anti-RAD51 (Ab-1, Oncogene, San Diego, CA), the anti-γ-H2AX [anti-phospho-H2A.X(Ser139), Upstate, Lake Placid, NY], the anti-MYC antibody 9E10 (BIOMOL Research Laboratories, Plymouth Meeting, PA) and the anti-FLAG M2 antibody (Sigma, St Louis, MO) were used for the immunohistochemical and immunoprecipitation assays.

Western blotting

The testes were homogenized in TNE buffer [50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 20 mM EDTA] that contained protease inhibitors. Equal amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking, the filters were incubated with the affinity-purified anti-Mili26F antibody. Peroxidase-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA) was used as the secondary antibody, and the signals were detected using the ECL kit (Amersham Pharmacia).

Histological analysis

Littermate embryonic male gonads at 14.5 dpc and postnatal testes were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated progressively and embedded in methyl methacrylate. Subsequently, 5 μm rehydrated sections were used for Hematoxylin-Eosin staining, for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) reaction, and for immunohistochemical staining. For the TUNEL reactions, the rehydrated sections were treated with methanol that contained 0.3% H_2O_2 for 10 minutes at room temperature. Apoptotic cells were detected using the In Situ Cell Death Detection Kit, POD (Roche). After the detection of apoptotic cells with DAB, the sections were stained with 1% Methyl Green. For the immunohistochemical analysis, the rehydrated slides were boiled in 10 mM tri-sodium citrate (pH 6.0) in a microwave oven for 8 minutes, to retrieve the antigens. After blocking with 5% normal goat serum in PBS, the slides were incubated with the anti-Mili-N1 (15 μg/ml), anti-Miwi-C (5 μg/ml) and anti-MVH (1:500) antibodies. For SYCP3, RAD51 and γ-H2AX staining, the testes were embedded

directly in OCT compound and cut at 10 μm. The sections were fixed with 2% paraformaldehyde for 20 minutes, permeabilized with acetone for 20 minutes, and blocked for 1 hour with 3% BSA and 10% normal goat serum in PBS. The staining was carried out as described previously (Chuma and Nakatsuji, 2001). As negative controls, pre-immune sera or isotype matched immunoglobulins were used and essentially no positive signals were detected in the control staining. Positive signals were detected with Alexa568- or Alexa488-conjugated anti-rabbit IgG (H+L) antibodies (Molecular Probes, Eugene, OR).

Surface spreading methods and chromosome preparation

For immunocytological analysis of SC formation at meiotic prophase was performed as described by Matsuda et al. (Matsuda et al., 1992). The images of immunocytochemical staining were captured with 550CW-QFISH application program of Leica Microsystems Imaging Solutions (Cambridge, UK) using a cooled CCD camera (MicroMAX 782Y, Prinston Instruments) mounted on a Leica DMRA microscope. The preparation of chromosomes and nuclei for light microscopy of the spermatogenic cells of the *Mili*-deficient mouse was performed using the air-drying method (Imai et al., 1981), with the omission of colchicine treatment. The preparations were stained with 4% Giemsa in phosphate buffer (pH 7.0), followed by counting of the three types of spermatogenic cells, i.e. spermatogonia, spermatocytes and spermatids, as well as the Sertoli cells.

RT-PCR analysis

Total RNA samples were isolated from the testes using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). Single-stranded cDNA was prepared from 3 μg of total RNA using random hexamers and the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). Each PCR reaction was performed with a 1/30 dilution of the RT products and using HotStar Taq (Qiagen, Valencia, CA). The PCR was carried out for 25-28 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute. The following primer pairs were used for the PCR: *Mili*, 5′-AGTGTGTGGGAGGA-3′ and 5′-AGAGCCATCAAAAGCAG-3′; and *Miwi*, 5′-ATGATCGTGGGCATC-3′ and 5′-AGGCCACTGC-TGTCATA-3′. The sequences of the other primers have been described previously (Tanaka et al., 2000).

Transient transfection and immunoprecipitation

The *Myc*-tagged *Mvh* expression plasmid was constructed by inserting the full-length fragment of *Mvh* (pQE32-Mvh; donated by Dr T. Noce) into the pcDNA3 vector. The 293T cells were transfected with plasmid DNA by the calcium phosphate method, and cultured for 48 hours. The cells were lysed in TNE buffer that contained protease inhibitors. For anti-MVH immunoprecipitation, testes were lysed with the same buffer. The lysates were pre-cleared with protein G Sepharose (Pharmacia), and immunoprecipitated with the anti-MYC, anti-FLAG or anti-MVH antibody. The immunoprecipitates were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking, the filters were incubated with the anti-FLAG, anti-MYC or anti-26F antibody. The secondary antibody was peroxidase-conjugated goat anti-mouse IgG (Sigma) or anti-rabbit IgG (Zymed).

GST-pull down assay

The pGEX4T-*Mvh* plasmid was donated by Dr T. Noce. Deletions of the GST-Mvh plasmid were produced using the appropriate restriction enzymes, as shown in Fig. 7B. The GST-fusion proteins were purified from *E. coli* lysates using glutathione-Sepharose 4B beads. The GST-fusion proteins that were bound to the Sepharose beads were then incubated with testis lysates. The GST precipitates were separated by SDS-PAGE, and transferred to PVDF membranes. After blocking, the filters were incubated with the affinity-purified anti-Mili26F antibody.

Results

Generation of mice with targeted mutations in the *Mili* gene

To investigate the in vivo function of MILI, we generated *Mili*-deficient mice by homologous recombination. The gene-targeting scheme that was used to generate the targeted embryonic stem (ES) cells is shown in Fig. 1A. In the mutant allele, the 5.2 kb fragment encompassing exons 2 to 5 was deleted, which corresponds to the deletion of the 1-209 residue region of the MILI protein. Southern blot analysis of genomic DNA identified the targeted homologous recombination event (Fig. 1B). Targeted ES cell lines were transferred into germ lines, and the subsequent F1 heterozygous mutant (*Mili*^{+/–}) crosses produced homozygous mutant (*Mili*^{–/–}) mice. The mutant mice were maintained in a mixed genetic background (129/sv_C57BL/6) in the subsequent studies. To confirm the lack of production of a functional MILI protein in the *Mili*^{–/–} testes, western blot analysis was carried out with an antibody that recognized both the MILI and MIWI proteins. MILI was detected in the testes of wild-type (*Mili*^{+/+}) and *Mili*^{+/–} mice. By contrast, MILI protein was not detected in the testes of *Mili*^{–/–} mice (Fig. 1C). MIWI protein was not observed in *Mili*^{–/–} testes, owing to the lack of the MIWI-expressing cell population, as shown below.

Impaired spermatogenesis in the *Mili* mutant mice

The *Mili*^{–/–} mice were viable and showed apparently normal development. Interbreeding of *Mili*^{+/–} mice produced offspring of normal litter size, and yielded the Mendelian ratios of *Mili*^{+/+}, *Mili*^{+/–} and *Mili*^{–/–} mice. These findings indicate that MILI is not required for embryonic somatic cell division. The female *Mili*^{–/–} mice were fertile, and histological analysis revealed that there were no abnormalities in the *Mili*^{–/–} ovaries (data not shown). However, the male *Mili*^{–/–} mice were sterile. Although *Mili* was expressed in PGCs, PGC development was normal and the numbers of germ cells in *Mili*^{–/–} mice of both sexes were not different from those in the control mice at 14.5 dpc (Fig. 2A).

In adulthood, the *Mili*^{–/–} testes were strikingly smaller than the control testes (Fig. 2B). The testes from 10-week-old *Mili*^{–/–} males were only one-fifth of the weight of those from control males. During development, the testis weights of *Mili*^{–/–} mice were almost the same as those of the *Mili*^{+/–} and *Mili*^{+/+} mice until 2 weeks after birth, which suggests normal growth of the spermatogonia. Thereafter, the testes of the *Mili*^{–/–} mice ceased to grow, and the weight difference became significant (Fig. 2C). Sperm were completely absent from the epididymides of the *Mili*^{–/–} mice (data not shown). Histological analysis of the adult testes revealed complete depletion of post-meiotic germ cells in the *Mili*^{–/–} testes (Fig. 2D, parts II, IV). By contrast, the full component of spermatogenic cells, including spermatogonia, spermatocytes and spermatids, was detected in the *Mili*^{+/–} adult testes (Fig. 2D, parts I, III).

Increased apoptosis in *Mili*-null mice

TUNEL labeling was used to analyze the timing of abnormal cell death in the first wave of spermatogenesis and the continuous degeneration of early spermatocytes in adulthood. In mice, spermatogenesis is initiated on day 3 after birth and progresses as a synchronous wave during the first week of life.
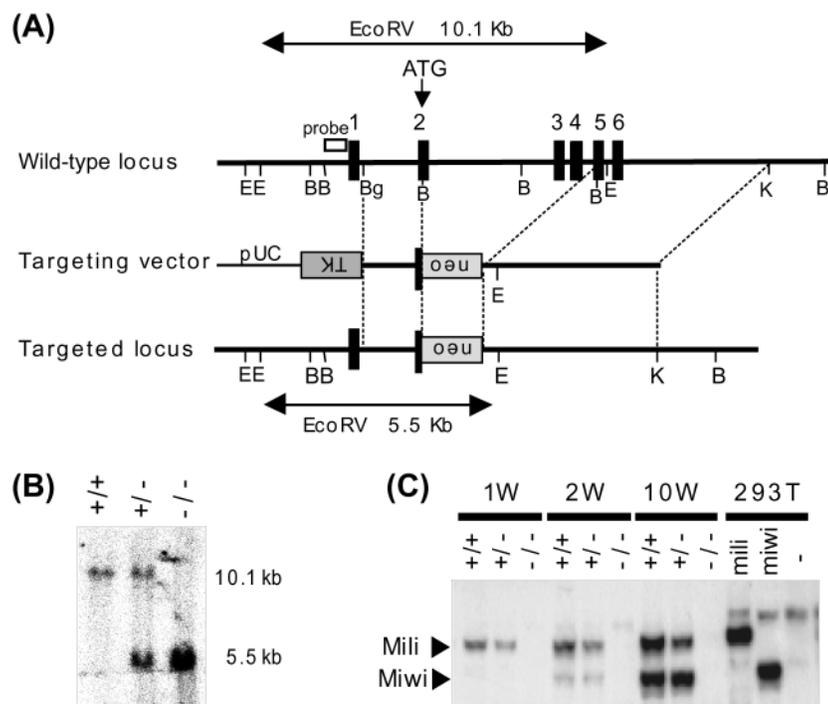
Fig. 1. Targeted disruption of the *Mili* gene. (A) Schematic representation of the wild-type allele, the targeting vector and the mutated alleles. The numbered boxes (1-6) denote the 5′-end non-coding exon (exon 1) and the five coding exons (exons 2-5). The targeting vector includes the PGK-*neo* gene (*neo*) and the thymidine kinase gene (TK). (B) Southern blot analysis of representative offspring from heterozygous mating. The wild-type allele produces a 10.1 kb *Eco*RV product, while the disrupted allele gives rise to a 5.5 kb band with the 5′-end hybridization probe. (C) Western blot analysis of the MILI and MIWI proteins from testes using antibody 26F, which recognizes both MILI and MIWI. Lysates (10 μg of protein in each lane) of the testes were loaded on the gel. The +/+, +/– and –/– designations indicate samples from wild-type, heterozygous and homozygous mutant testes, respectively. The 293T cells that were transfected with *Mili*- or *Miwi*-expressing plasmids and mock plasmids are shown as the controls.

The most mature germ cell type observed 8 days after birth is the type B spermatogonium; the preleptotene/leptotene, zygotene, and pachytene spermatocytes appear on days 10, 12, and 14, respectively (Bellve et al., 1977). TUNEL-positive cells were rarely detected in *Mili*+/– and *Mili*–/– testes on days 7 and 9 (Fig. 3A-D). On days 11 and 14, there were some TUNEL-positive cells in the heterozygous testes (Fig. 3E,G). However, significant numbers of apoptotic cells were observed in the homozygous testes (Fig. 3F,H). The apoptotic cells in the homozygous testis were located in the inner layers of the seminiferous tubules, where the most mature cells exist during the first wave of spermatogenesis. Virtually no TUNEL-positive cells were detected in the adult *Mili*+/– testis. By contrast, in most of the seminiferous tubules of the *Mili*–/– testes, significant numbers of TUNEL-labeling cells were detected in the spermatocyte layers (Fig. 3I,J). Thus, spermatogenesis in the *Mili*–/– mice is blocked during the early stages of meiosis, probably at the zygotene or early pachytene stage of the meiotic prophase, and apoptosis occurs subsequently.
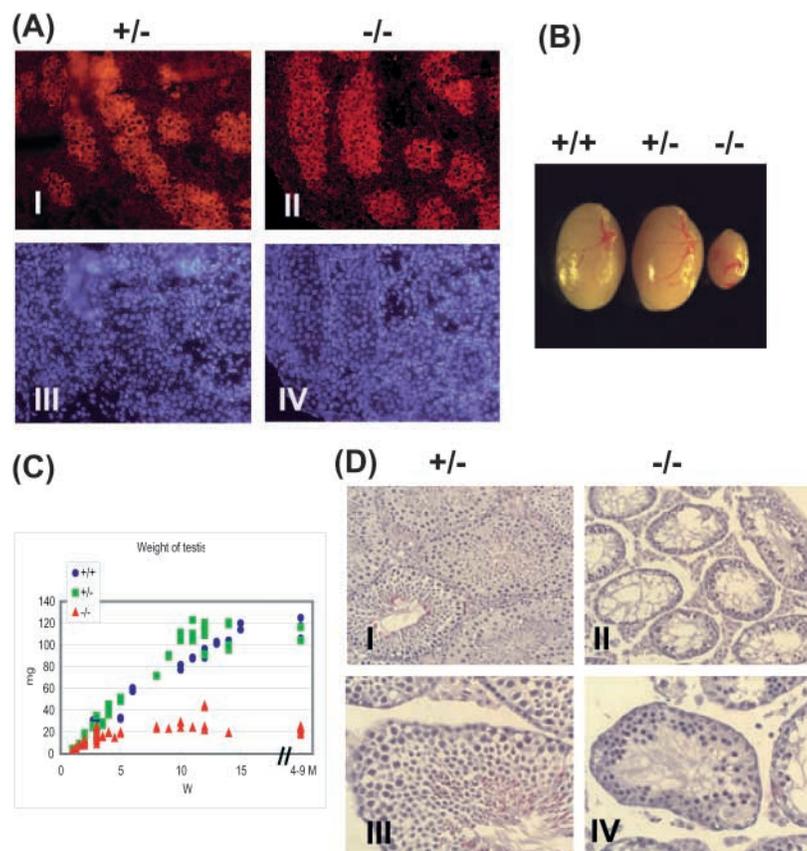

Fig. 2. Defective spermatogenesis in *Mili*-deficient mice. (A) Immunostaining of 14.5-dpc gonads with the anti-MVH antibody. Sections from heterozygous (+/–) and homozygous mutant (–/–) littermate embryos were stained with the anti-MVH antibody (I, II) or DAPI (III, IV). (B) Comparison of the testes from 10-week-old wild type (+/+), heterozygous (+/–) and homozygous mutant (–/–) mice. The testis from the *Mili*–/– mouse is smaller. (C) Weights of testes from wild-type (+/+), heterozygous (+/–) and homozygous mutant (–/–) mice. (D) Hematoxylin and Eosin-stained sections of testes from 10-week-old mice. Sections from the heterozygous (I, III) and homozygous mutant (II, IV) mouse testes are shown.

Meiotic arrest in *Mili*⁻/⁻ testes

RT-PCR analysis of the genes expressed at the different spermatogenic stages was carried out to determine the stage of arrest (Fig. 4). Genes that are expressed before the pachytene spermatocyte stage, such as the DNA mismatch repair gene
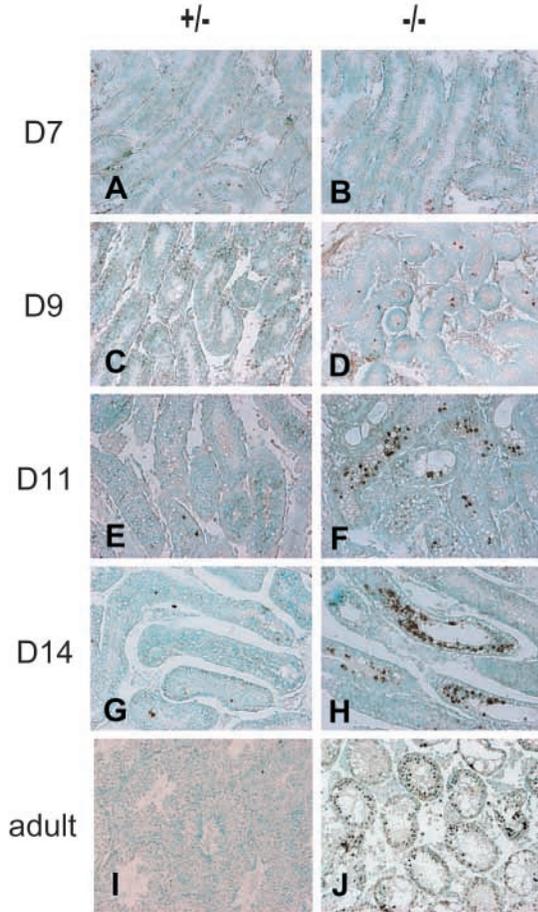

Fig. 3. Apoptosis in the *Mili*⁺/⁻ and *Mili*⁻/⁻ testes. TUNEL-labeled testes during the first wave of spermatogenesis (A-H) and in adulthood (I,J). Testes from heterozygous (A,C,E,G,I) and homozygous mutant (B,D,F,H,J) littermates were stained using the TUNEL technique. Testis sections are shown from 7-day-old (A,B), 9-day-old (C,D), 11-day-old (E,F), 14-day-old (G,H) and adult (I,J) mice. The nuclei were counterstained with Methyl Green.

Mlh1 (Baker et al., 1996; Edelmann et al., 1996), and *Dmc1*, which is the mouse homolog of *E. coli RecA* (Habu et al., 1996), did not show significantly different expression patterns between the *Mili*⁺/⁻ and *Mili*⁻/⁻ genotypes. By contrast, the genes that are expressed in pachytene spermatocytes and at later stages, such as calmegin (Watanabe et al., 1994), *Hoxa4* (Rubin et al., 1986), Cyclin A1 (Sweeney et al., 1996), and the gene encoding the cyclic AMP-responsive element modulator isoform Crem-τ (Foulkes et al., 1992), were not detectable in the *Mili*⁻/⁻ testis. In addition, the expression of *Miwi*, which is present in midpachytene-stage spermatocytes, was not detected in the *Mili*⁻/⁻ testes. Although *Sycp1* and *Sycp3* (Dobson et al., 1994; Lammers et al., 1994; Meuwissen et al., 1992), both of which encode synaptonemal complex proteins and are restricted to zygotene to diplotene spermatocytes, were detected in the *Mili*⁻/⁻ testes, their expression levels were much lower than those in heterozygous or wild-type testes. The expression level of *Mvh* was significantly lower in the *Mili*⁻/⁻ testes. Similarly, the expression of *A-myb* (Mybl1 – Mouse Genome Informatics), which is a *myb*-family transcription factor gene that is expressed in type B spermatogonia and leptotene- to pachytene-stage spermatocytes (Mettus et al., 1994; Trauth et al., 1994), decreased in the *Mili*⁻/⁻ testes. The expression patterns of these genes in 3-week-old testes were essentially similar to those in the adult testes (data not shown).

The expression patterns of *Bmp8a* and *Bmp8b* differed between the 3-week-old testes and adult testes of the various genotypes. These two genes are expressed in a tightly regulated temporal and spatial manner in the placenta and in male germ cells during postnatal development (Zhao and Hogan, 1996). Before 3.5 weeks of age, both genes are expressed at low levels in the spermatogonia and primary spermatocytes. Thereafter, high levels of expression are observed in the spermatids. In adulthood, the expression levels of *Bmp8a* and *Bmp8b* were lower in the *Mili*⁻/⁻ testes. However, the expression levels were higher in the testes of 3-week-old mice, which may reflect a yet unknown feedback mechanism that responds to the deficiency in spermatogenesis in the mutant mouse.

To define further the meiotic defect in the *Mili*⁻/⁻ mice, sections stained with anti-SYCP3, RAD51 and γ-H2AX (phosphorylated form of H2AX, a histon H2A variant) antibodies were examined (Fig. 5). SYCP3 expression in *Mili*⁻/⁻ testis was weaker than that in *Mili*⁺/⁻ testis as expected from RT-PCR (Fig. 5A,B). Axial core formation was observed in the SYCP3-expressing meiotic cells in *Mili*⁻/⁻ testis (Fig. 5C,D), suggesting the entry to the first meiotic division; however, pachytene chromosome formation was not completed in *Mili*⁻/⁻ testis. Expressions of RAD51 and γ-H2AX were essentially same in *Mili*⁻/⁻ and *Mili*⁺/⁻ testes (Fig. 5E-P). During meiotic prophase, the X and Y chromosomes condense to form the sex body or XY body, and in late zygotene/ early pachytene spermatocyte, γ-H2AX
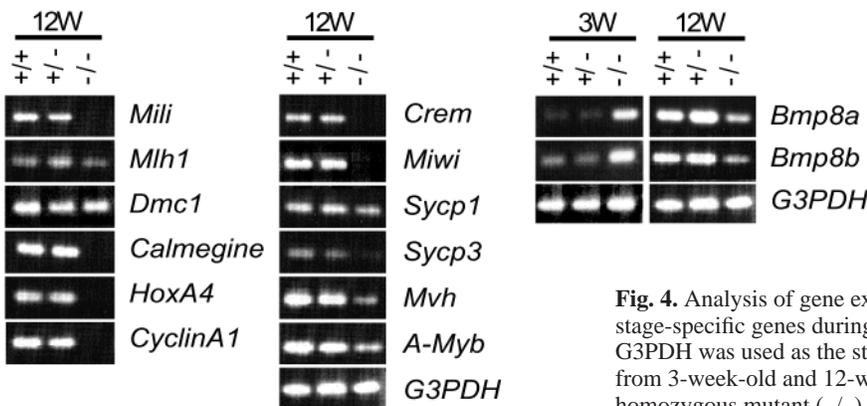
Fig. 4. Analysis of gene expression in *Mili*⁻/⁻ mouse testes. The expression of stage-specific genes during spermatogenesis was analyzed by RT-PCR. G3PDH was used as the standard. Total testis RNA samples were prepared from 3-week-old and 12-week-old wild-type (+/+), heterozygous (+/−) and homozygous mutant (−/−) mice.

accumulates in the sex body (Mahadevaiah et al., 2001). Many γ-H2AX condensed spots were observed in *Mili*^{–/–} testes as well as *Mili*^{+/–} testes (Fig. 5K,N arrow). Furthermore, we analyzed synaptonemal complex formation in more detail using B antibody that recognizes both COR1 and SYN1 of synaptonemal complex core. Synaptonemal complex core was detected until early pachytene stage in the *Mili*^{–/–} testis (Fig. 6A-E). Similarly, nuclei at and after the mid-pachytene spermatocyte stage were not detected in the *Mili*^{–/–} mice by Giemsa staining (Fig. 6F,G). The mitotic metaphases of the spermatogonia were observed in *Mili*^{+/–} and *Mili*^{–/–} testes. By contrast, the first meiotic metaphase of the primary spermatocyte was not detected in the *Mili*^{–/–} testis. Table 1 shows the quantification of spermatogenic cells and Sertoli cells. Although a lot of cells can be observed in Fig. 6F,G, the majority of the cells were Sertoli cells as shown in the Table 1. Nuclei at and after the mid-pachytene spermatocyte stage were not detected in the *Mili*^{–/–} mice. The histological analysis
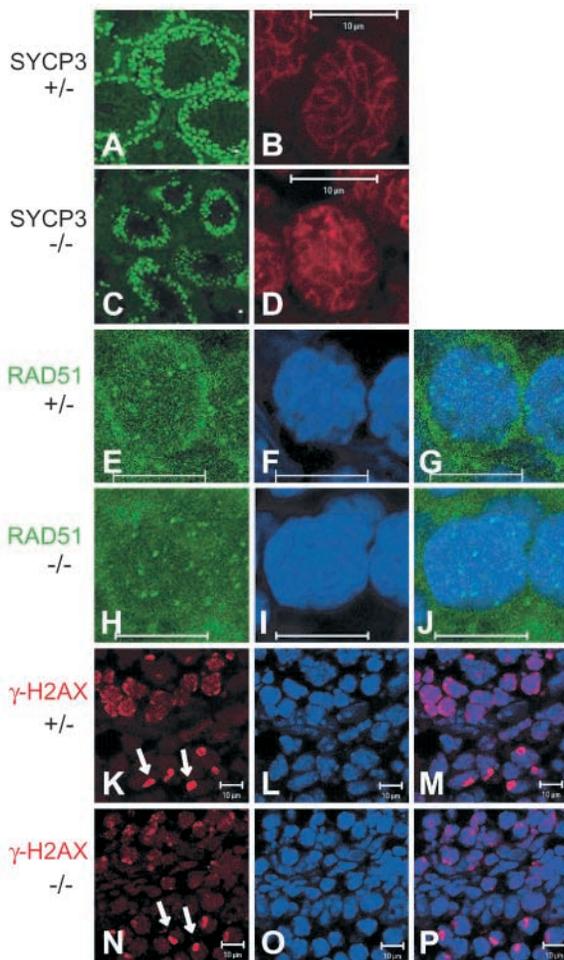
clearly demonstrates that spermatogenesis after the postmeiotic stage was completely absent in all the seminiferous tubules of adult *Mili*^{–/–} testes. Taken together, the spermatogenesis arrest in adult *Mili*^{–/–} testes occurs during the early stages of meiosis, probably at the zygotene or early pachytene stages of the meiotic prophase.

Binding of *Mili* and *Miwi* to MVH

The stages of spermatogenesis arrest in the MILI-deficient and MVH-deficient mice were similar. To test the potential synergistic action of MILI and MVH, we examined the physical association between the proteins using 293T cells that expressed MYC-tagged MVH and FLAG-tagged MILI or MIWI. The lysates were immunoprecipitated with anti-MYC and anti-FLAG antibodies and detected with the anti-FLAG and anti-MYC antibodies. As shown in Fig. 7A, both MILI and MIWI were associated with MVH. Binding of MILI and MIWI was confirmed by immunoprecipitation assay of testicular lysates using anti-26F antibody recognizing both MILI and MIWI (Fig. 7B). GST fusion proteins of full-length and truncated mutants of MVH were produced (Fig. 7C,D). In addition, pull-down assays were carried out with lysates of the wild-type testes (Fig. 7D). MILI and MIWI bound to the full-length MVH, and deletion mutant analysis showed that the N-terminal of MVH was necessary for this binding.
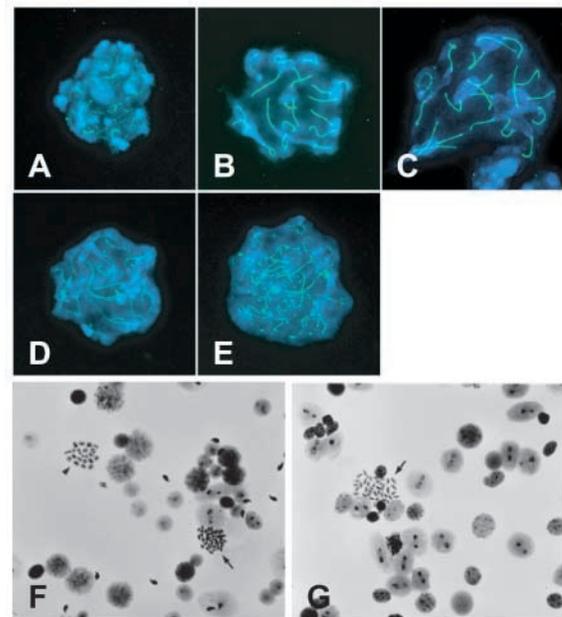

Fig. 5. Expression of SYCP3, RAD51 and γ-H2AX in *Mili*^{–/–} spermatocytes. Anti-SYCP3 immunostaining (A-D), anti-RAD51 staining (E,H), anti- γ-H2AX staining (K,N) and DAPI staining (F,I,L,O), and the merged images of RAD51 or γ-H2AX and DAPI (G,J,M,P) in *Mili*^{+/–} (upper panels of each pair) and *Mili*^{–/–} (lower panels of each pare) testes. Arrows show the γ-H2AX restricted to the sex bodies in early pachytene spermatocyte. All images except A and C were observed by confocal microscopy. Scale bars: 10 μm.

Fig. 6. No mid-pachytene spermatocyte in the *Mili*^{–/–} testes. Merged image of synaptonemal complexes stained with the B antiserum recognizing both COR1 and SYN1 (green) and DNA stained with DAPI (blue) (A-E) in mid-zygotene spermatocytes (A,D), early-pachytene spermatocytes (B,E) and mid-pachytene spermatocyte (C). The images of FITC-stained synaptonemal complexes and DAPI-stained nuclei were captured with L5 and A4 filters, respectively. The surface spreading samples were prepared from control testes (A-C) and the homozygous mutant testes (D,E). The micrographs of spermatogenic cell nuclei in Giemsa-stained preparations of heterozygous (F) and homozygous mutant (G) mice are shown. The arrows indicate mitotic metaphases of spermatogonia, and the arrowhead indicates the first meiotic metaphase of a primary spermatocyte.

Table 1. Stage of *Mili*-deficient mouse testis cells

Genotype	SG	M	L	Z	EP	MP	LP to DP	DA to MI	SC	MII	ES	LS	SZ	S	Total cell numbers
Mili$^{+/-}$	9.9	0.1	6.6	5.7	7.3	4.9	1.5	0.3	1.2	0.3	31.8	8.1	16.9	5.5	779
Mili$^{-/-}$	35.1	1.1	5.7	6.0	1.8	0	0	0	0	0	0	0	0	50.3	616

Percentages of the cells in each stage and the numbers of the counted testis cells are shown. Nuclei were categorized into spermatogonium (SG) and spermatogonium at metaphase (M), primary spermatocytes at leptotene (L), zygotene (Z), early pachytene (EP), midpachytene (MP), late pachytene to diplotene (LP to DP) and diakinesis to metaphase I (DA to MI), secondary spermatocyte (SC) and secondary spermatocyte at metaphase (MII), early spermatid (ES), late spermatid (LS) and spermatozoon (SZ), and sertoli cell (S).

To analyze the localization of MILI, MIWI and MVH, immunohistochemical analyses of the wild-type, *Mili*$^{-/-}$ and *Miwi*$^{-/-}$ mice were carried out with specific antibodies (Fig. 8). The MILI protein was detected until pachytene-stage spermatocytes. Although *Drosophila* Piwi was localized in the nucleoplasm (Cox et al., 2000), MILI, like MIWI, was found in the cytoplasm (Kuramochi-Miyagawa et al., 2001). MILI was not detected in the *Mili*$^{-/-}$ testes, but its expression was normal in the *Miwi*$^{-/-}$ testes. MIWI was not detected in either the *Mili*$^{-/-}$ or *Miwi*$^{-/-}$ testes. The lack of MIWI expression in the *Mili*$^{-/-}$ mice is presumably due to the paucity of MIWI-expressing cells, i.e. midpachytene-stage spermatocytes and round spermatids. MVH was detected in pre-pachytene spermatocytes in the *Mili*$^{-/-}$ testes, which indicates that MILI is not essential for MVH expression. Although MVH was detected in the *Miwi*$^{-/-}$ testes, the subcellular localization of MVH was different from that in the wild type. Anti-MVH staining showed the granulo-fibrillar and granular distribution of MVH in spermatocytes and round spermatids, respectively, in wild-type testes, and anti-MIWI showed the similar staining pattern. As shown in Fig. 8, MVH was not distributed in a granular pattern in the cytoplasm of the *Miwi*$^{-/-}$ round spermatids.

Discussion

MILI is an essential factor for meiotic differentiation during spermatogenesis

The disruption of various genes brings about meiotic arrest and subsequent apoptotic cell death in the testis. These genes include DNA repair genes such as *Mlh1*, *Mlh3*, *Msh4*, *Msh5* and *Hrb6* (de Vries et al., 1999; Edelmann et al., 1996; Edelmann et al., 1999; Kneitz et al., 2000; Lipkin et al., 2002). Mice with null mutations in these genes show defective meiosis with aberrant chromosomal synapsis and postmeiotic apoptosis. Similarly, null mutations in HSP70-2, which is an essential factor for CDC2 kinase activity in meiosis I (Dix et al., 1996), and Cyclin A1 cause apparent structural abnormalities of the male germ cells by late prophase (Liu et al., 1998). Disruption of the other genes that are involved in homologous chromosome synapsis formation, such as *Dmc1*, *Sycp3*, *Spo11* and *Atm*, leads to failed synapsis formation and causes meiotic cell death (Romanienko and Camerini-Otero, 2000; Scherthan et al., 2000; Yoshida et al., 1998; Yuan et al., 2000). Two transcription factors, A-Myb and Egr4, are essential for the early-mid pachytene stage, as mice with targeted disruptions in these genes exhibit apoptotic cell death during this stage (Toscani et al., 1997; Tourtellotte et al., 1999). MVH-null mice also demonstrate meiotic arrest and apoptosis during the early prophase of meiosis (Tanaka et al., 2000). Considering that deficiencies in several genes with
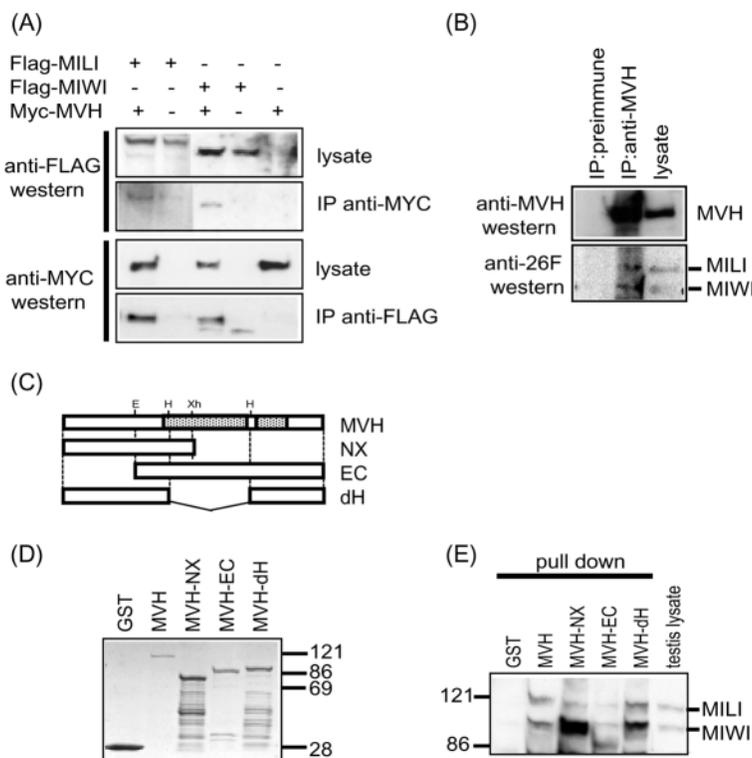

Fig. 7. Association of MVH with MILI and MIWI. (A) Co-immunoprecipitation of MILI and MIWI with MVH. The 293T cells were transfected with plasmids that expressed FLAG-tagged MILI or MIWI and either Myc-tagged MVH or mock plasmids. The lysates were immunoprecipitated with the anti-Myc or anti-FLAG antibodies and detected with the anti-FLAG or anti-Myc antibodies. (B) Co-immunoprecipitation of MILI and MIWI with MVH in the testis lysate. Testis lysate of normal mice was immunoprecipitated with pre-immune serum or anti-MVH antibody, and detected with the anti-MILI-26F antibody recognizing both MILI and MIWI. (C) Schematic structure of the truncated MVH protein. Broken boxes show the helicase domains. E, *Eco*RI; H, *Hind*III; Xh, *Xho*I. (D) CBB staining of the GST-fusion proteins. GST-fusion proteins that were purified with glutathione-Sepharose 4B were loaded on 4-20% SDS-PAGE. (E) GST pull-down assay. Testis lysates were incubated with GST or the various GST-MVH fusion proteins. The MILI and MIWI proteins that were pulled down with glutathione-Sepharose were detected by western blotting using antibody 26F.
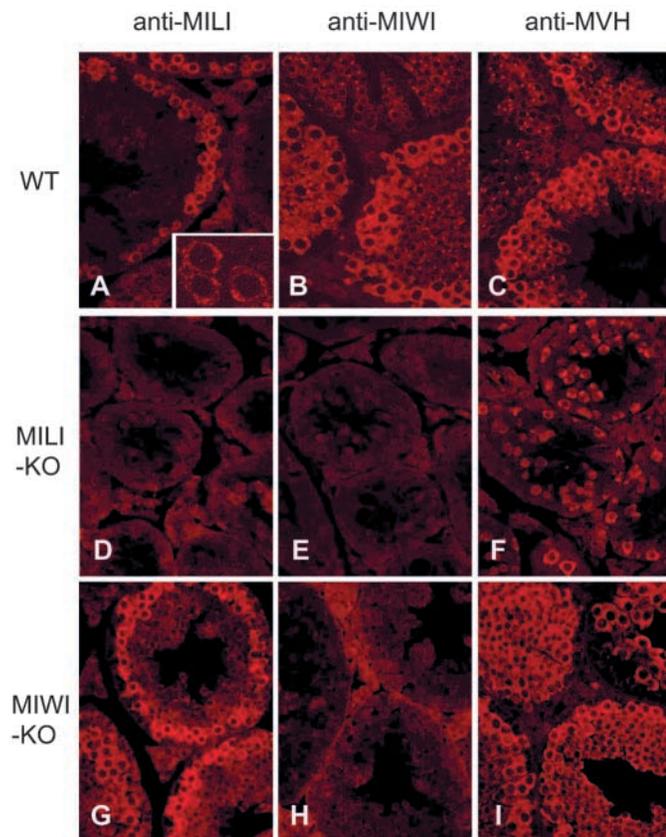
anti-MILI   anti-MIWI   anti-MVH

WT   A   B   C

MILI-KO   D   E   F

MIWI-KO   G   H   I

Fig. 8. Immunohistochemical localization of the MILI, MIWI and MVH proteins. Sections from 10-week-old wild-type (A-C), *Mili*$^{-/-}$ (D-F) and *Miwi*$^{-/-}$ (G-I) mouse testes were stained with the anti-MILI (A,D,G), anti-MIWI (B,E,H), and anti-MVH (C,F,I) antibodies. MILI expression was detected in the cytoplasm of zygotene to pachytene spermatocytes in the wild-type and *Miwi*$^{+/-}$ testes (A,G). MIWI expression in wild-type testis was from pachytene to the round spermatid stage, and granular staining of MIWI was detected in the round spermatids (B). No MIWI-positive cells were observed in the *Mili*$^{-/-}$ testis (E). MVH protein was detected in the zygotene spermatocyte to round spermatid stages of the wild-type testis (C). Although granular distribution of MIWI was detectable in the wild-type testis (C), this granular pattern was not detectable in the *Miwi*$^{-/-}$ testis (I).

distinct functions result in meiotic arrest and apoptotic cell death, it seems plausible that a sensitive checking mechanism for differentiation progression operates during spermatogenesis. Thus, it is conceivable that MILI plays an important role in meiotic prophase progression.

In the *Mili*$^{-/-}$ testis, the expression of genes that are involved in spermiogenesis was undetectable. The *Dmc1* gene, the expression of which is restricted in leptotene- to zygotene-stage spermatocytes (Yoshida et al., 1998), was expressed normally in the *Mili*$^{-/-}$ testis. By contrast, the expression levels of *Sycp1* and *Sycp3*, both of which are transcribed predominantly in the zygotene to diplotene stages (Dobson et al., 1994; Lammers et al., 1994; Meuwissen et al., 1992), were slightly reduced. *A-myb*, which is expressed in early primary spermatocytes (Mettus et al., 1994; Trauth et al., 1994), showed similar low-level expression. Taken together with the data from the chromosome analysis, these results suggest that

spermatogenesis in *Mili*$^{-/-}$ mice is arrested from the zygotene to early pachytene stages.

Differential expression of MILI and MIWI

Although MILI and MIWI are expressed in germ cells, their expression kinetics are different (Kuramochi-Miyagawa et al., 2001). The expression of MILI was detected up to the pachytene spermatocytes, whereas that of MIWI was detected from the mid-pachytene stage to the emergence of elongated spermatids. Thus, MILI is expressed at an earlier stage than MIWI, and the expression of MILI overlaps somewhat with that of MIWI in the mid-pachytene stage. According to the kinetics of expression, the stages of spermatogenetic arrest differ between the *Mili*$^{-/-}$ and *Miwi*$^{-/-}$ testes. Arrest was observed at the early pachytene spermatocyte and round spermatid stages in *Mili*$^{-/-}$ and *Miwi*$^{-/-}$ testes, respectively.

MILI and MIWI share the molecular characteristics such as binding to RNA and MVH. This raised an interesting question whether molecular function(s) of MILI could be same as that of MIWI. To zero in on this point, we are producing the transgenic mice in which MILI or MIWI is expressed under the promoter of *Mili* gene and are examining whether these transgenic mice could rescue the *Mili*$^{-/-}$ phenotype. Our preliminary data of the rescue study suggests that *Mili* but not *Miwi*, transgenic mice can rescue the *Mili*$^{-/-}$ phenotype (data not shown). Thus, the functional differences between MILI and MIWI are not solely dependent on the different expression timing but would be due to their distinctive molecular roles.

Physical association between MILI and MVH

When MILI and MIWI were overexpressed with MVH in 293 cells, the majority of MVH was co-localized with MILI or MIWI as large perinuclear granule-like structure (data not shown). The data of overexpression analysis suggested that MILI and MIWI would define the localization of MVH at least to some extent. MVH is localized exclusively in the cytoplasm of spermatogenic cells from spermatogonia to round spermatids, with the highest expression in early spermatocytes (Toyooka et al., 2000). The stage of meiotic arrest in the *Mili*$^{-/-}$ testis was similar to that in the *Mvh*$^{-/-}$ testis, and the gene expression profiles were essentially the same, as assessed by RT-PCR analysis. MILI and MVH were found to be cytoplasmic proteins, as discussed below, and MILI and MIWI were co-expressed with MVH throughout spermatogenesis. These data led us to analyze the association of MILI and MIWI with MVH. Although physical association does not necessarily bear functional relevance, the similar phenotypes of *Mili*$^{-/-}$ and *Mvh*$^{-/-}$ spermatogenesis imply cooperative molecular functions for these molecules.

In round spermatids, MVH is predominantly localized in a single large granule with a spherical shape that is located in the perinuclear site (Toyooka et al., 2000). The perinuclear granule that stained with anti-MVH antibody was the chromatoid body, which is a perinuclear electron-dense body in the male germ cells of mammalian testicular germ cells classified as a nuage like structure. The binding analysis showed that the affinity of MIWI for MVH was presumably higher than that between MILI and MVH (Fig. 6D). In pull-down analysis of MILI and MIWI with GST-MVH fusion protein, binding of MILI to MVH was competed out by the excessive amount of MIWI. The round spermatids showed similar staining patterns with the

anti-MIWI and anti-MVH antibodies. In addition, the round spermatids of the *Miwi*⁻/⁻ mice did not show perinuclear granular staining by anti-MVH antibody. Given that *Miwi* is required for the stability of its target mRNAs (Deng and Lin, 2002), this further suggests that chromatoid body could be the subcellular structure essential for controlling the mRNA stability for spermiogenesis.

Are Mili and Miwi Functional homologs of piwi?

The functions of the *C. elegans* homologs of *piwi*, *prg-1 and prg-2*, have been studied using RNA interference (RNAi) to be important for the mitotic ability of the germline nuclei and are essential for germline proliferation and maintenance (Cox et al., 1998). In this regard, the *prgs* are not only structural, but also functional homologs of *piwi*. It is intriguing to investigate whether the mammalian MILI and MIWI are functional homologs of *Drosophila* Piwi. A couple of lines of evidence suggest that the two mammalian structural homologs of Piwi, i.e. MILI and MIWI, only inherit a subset of *piwi* functions. One line of evidence comes from the analysis of gene targeting. In addition to its crucial roles in germline stem cells, Piwi has less-well characterized roles in early oogenesis and spermatogenesis, possibly including germline cyst mitosis, meiosis and egg chamber polarity (Cox et al., 1998; Lin and Spradling, 1997). The expression of MIWI and MILI is restricted to germ lineages, and the gene-targeted animals show defective spermatogenesis. However, we have not observed the defect of *Mili*⁻/⁻ nor *Miwi*⁻/⁻ mice at the stage of testicular stem cells. Thus, *Mili* and *Miwi* may only represent a subset of *piwi* functions.

The other line of evidence is subcellular localization of MIWI and MILI. The Piwi protein can be localized either to the nucleoplasm in germline stem cells (Cox et al., 2000) or in the cytoplasm co-localized with polar granules such as Vasa in early embryos (D. N. Cox and H. Lin, unpublished), MILI and MIWI are found in the cytoplasm associated with MVH (Kuramochi-Miyagawa et al., 2001). This again reflects only a subset of the Piwi function. This function is also similar to Aubergine, another *Drosophila* Piwi family protein, that is recruited to the posterior pole in a *vasa*-dependent manner as a polar granule component (Findley et al., 2003). Interestingly, Aubergine remains exclusively in the cytoplasm even after pole cell formation. In addition, the levels of homology between MILI or MIWI and Aubergine (31.0% and 36.6%, respectively) were similar to those seen with Piwi (32.7% and 37.1%, respectively). Taking these data into consideration, it is conceivable that MILI and MIWI might be functionally more similar to Aubergine. Meanwhile, genome analysis has revealed the third mouse homolog of *piwi* and *aubergine* (Accession Number AY135692). It is an unanswered question whether the third member will represent other functions of *piwi* and *aubergine*.

The *piwi* family genes, defined by conserved PAZ and Piwi domains of unknown function, have been implicated in RNAi and related phenomena, such as post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) in several organisms (Doi et al., 2003; Pal-Bhadra et al., 2002; Tijsterman et al., 2002; Vaucheret et al., 2001). AGO1 (*Argonaute*) and QDE-2 are required for PTGS in *Arabidopsis* and *Neurospora*, respectively, and RDE-1 is required for RNAi in *C. elegans* (Catalanotto et al., 2000; Fagard et al., 2000;

Tabara et al., 1999). In *Drosophila*, mutations in *piwi* and *aubergine* block RNAi activation during egg maturation and perturb translational control during oogenesis (Kennerdell et al., 2002). Aubergine has the ability to effect the silencing of *Stellate*, which is a tandemly repetitive gene (Schmidt et al., 1999). Furthermore, in *Drosophila*, *AGO1* and *AGO2* are involved in RNAi, and *piwi* is required for PTGS and for TGS, which is induced by multiple copies of *Alcohol dehydrogenase* (Hammond et al., 2001; Pal-Bhadra et al., 2002; Williams and Rubin, 2002). MILI and MIWI may be involved in similar silencing mechanisms required for spermatogenesis. We are comparing the gene expression profiles between the control and the *Mili*⁻/⁻ testes, which could give some clues about the function on gene silencing.

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