Impaired Spermatogenesis and Fertility in Mice Carrying a Mutation in the Spink2 Gene Expressed Predominantly in Testes*

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Spermatogenesis is a complex process involving an intrinsic genetic program composed of germ cell-specific and –predominant genes. In this study, we investigated the mouse Spink2 (serine protease inhibitor Kazal-type 2) gene, which belongs to the SPINK family of proteins characterized by the presence of a Kazal-type serine protease inhibitor-pancreatic secretory trypsin inhibitor domain. We showed that recombinant mouse Spink2 has trypsin-inhibitory activity. Distribution analyses revealed that Spink2 is transcribed strongly in the testis and weakly in the epididymis, but is not detected in other mouse tissues. Expression of Spink2 is specific to germ cells in the testis and is first evident at the pachytene spermatocyte stage. Immunoblot analyses demonstrated that Spink2 protein is present in male germ cells at all developmental stages, including in testicular spermatogenic cells, testicular sperm, and mature sperm. To elucidate the functional role of Spink2 in vivo, we generated mutant mice with diminished levels of Spink2 using a gene trap mutagenesis approach. Mutant male mice exhibit significantly impaired fertility; further phenotypic analyses revealed that testicular integrity is disrupted, resulting in a reduction in sperm number. Moreover, we found that testes from mutant mice exhibit abnormal spermatogenesis and germ cell apoptosis accompanied by elevated serine protease activity. Our studies thus provide the first demonstration that Spink2 is required for maintaining normal spermatogenesis and potentially regulates serine protease-mediated apoptosis in male germ cells.

Male germ cell development, or spermatogenesis, is a complex process that involves the mitotic proliferation of spermatogonial stem cells, meiotic division of spermatocytes, and dramatic morphological changes from haploid spermatids to highly specialized sperm through spermiogenesis (1, 2). The tightly regulated nature of this process, which occurs in seminiferous tubules in testes, suggests the presence of a highly organized network of genes expressed in germ cells during spermatogenesis. The regulation of gene expression during spermatogenesis occurs at three levels: intrinsic, interactive, and extrinsic (3). The intrinsic program determines which genes are utilized and when the genes are expressed. The interactive process between germ cells and somatic cells is necessary for germ cell proliferation and progression. Extrinsic influences, such as steroid and peptide hormones, regulate the interactive process. The intrinsic component of the genetic program involves germ cell- and stage-specific gene expression patterns that constitute the unique features of male reproduction.

Proteases represent a large group of proteins (~2% of all genes) that share the ability to catalyze the hydrolysis of peptide bonds. Proteases play crucial roles in controlling diverse biological processes such as tissue maintenance, repair, and development, and are essential for the survival of all organisms. Not surprisingly, given the central importance of proteases, deficiencies or alterations in the regulation of proteases underlie important human disease, including cancer, arthritis, and neurodegenerative and cardiovascular diseases (4–8). Accordingly, the activities of proteases must be finely tuned to maintain biological homeostasis. To regulate protease activities and avoid cellular damage, organisms produce protease inhibitors, which are widely distributed in organs. To date, 67 distinct protein families have been classified as protease inhibitors (9).

During the course of our study of unknown or unexplored genes with testis-specific or -predominant expression, we investigated the serine protease inhibitor Kazal-type 2 (Spink2) gene in mice. Previous comparative gene expression profiling between normal and abnormal human testes from patients diagnosed with azoosperma showed that Spink2 expression was decreased 4-fold in abnormal testes compared with those from fertile men (10). Thus, this raises the possibility of Spink2 involvement in male reproduction. Spink2 belongs to the family of Kazal-type serine peptidase inhibitors, which have amino acid sequence homology to bovine pancreatic secretory trypsin inhibitor (11). The first member of the SPINK family, human SPINK1 (mouse Spink3), also known as pancreatic secretory trypsin inhibitor was discovered by Kazal (12). Previous studies and our in silico searches indicate that at least 13 SPINK family members are expressed in diverse tissues (13–18). SPINK2, the only SPINK family member expressed in testes, was first identified in humans (11, 19); however, informa-

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‡ The abbreviation used is: Spink2, serine protease inhibitor Kazal-type 2.
tion on mouse *Spink2* has not yet been reported. In the present study, we report the first investigation of *Spink2* in mice, providing comprehensive information on its expression and function. Notably, we generated mice carrying a gene trap mutation in *Spink2* and found that mutant male mice with reduced SPINK2 levels exhibit impaired fertility.

**EXPERIMENTAL PROCEDURES**

**Trypsin Inhibition Assays**—Trypsin inhibition assays were performed by measuring the trypsin-cleaved products of the synthetic substrate (CBZ-Ile-Pro-Arg)_2-Rhodamine110 (Molecular Probes). Purified His-tagged SPINK2 protein (0.1–30 μM) was preincubated with 42 μM trypsin (Sigma-Aldrich) at 22 °C for 10 min in HEPES-buffered saline (5 mM HEPES, 0.15 mM NaCl, pH 7.35) containing 2 mM EDTA. Activity was determined by measuring the absorbance at 521 nm with a SPECTRAMax Gemini XS microplate reader ( Molecular Devices).

**Reverse Transcription PCR**—The testis-specific expression of the *Spink2* gene was verified by performing reverse transcription-PCR (RT-PCR) using cDNAs from nine different mouse tissues (testis, epididymis, ovary, brain, heart, kidney, lung, liver, and spleen), as well as cDNAs from the testes, and the epididymis of W/W” mutant mice, which lack germ cells, and mature sperm. Total RNA was extracted using the TRIzol reagent (Molecular Research Center) according to the manufacturer’s protocol, and cDNA was synthesized by random hexamer and oligo(dT) priming using Omniscript reverse transcriptase (Qiagen). A specific region of the *Spink2* transcript was amplified with the primers 5’-GTG GGA TTC CCG ACT CTT CCG ATT C-3’ (forward) and 5’-CAT CAA AGG GTC GCA TTT-3’ (reverse) using the following PCR thermocycling conditions: 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The primers for gynecaldehyde-3-phosphate dehydrogenase (*Gapdh*) and Protamine 1 (*Prm1*), used as a control, were amplified under the same conditions using the primers 5’-TGA AGG TCG TCA ACG GAT TTG GT-3’ (forward) and 5’-CAT GTG GGC CAT GAG TCA CAC CAC-3’ (reverse) for *Gapdh* and 5’-GCC GCA GCA AAA GCA-3’ (forward) and 5’-CGG ACC GTG TCA TTT-3’ (reverse) for *Prm1*.

**Immunoblot Analysis**—Proteins, denatured by boiling for 10 min in the presence of 3% SDS and 5% β-mercaptoethanol (1× SDS/β-mercaptoethanol sample buffer), were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (pore size, 0.2 μm; Bio-Rad Laboratories). Membranes were blocked in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature and then washed and incubated with primary antibodies for 1 h. After washing three times with TBS-T (10 min each), membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Following three washes in TBS-T, immunoreactive proteins were detected using an enhanced chemiluminescence kit (Pierce).

**Preparation of Testicular Cells, Testicular Sperm, and Epididymal Sperm from ICR Male Mice and Spink2 Mutant Mice**—All animal investigations were carried out according to the guidelines for animal care and use of Gwangju Institute of Science and Technology. Testicular (spermatogenic) cells, and testicular sperm were isolated by suspending in 52% isotonic Percoll (GE Healthcare), centrifuging for 10 min (27,000 × g, 4 °C), and resuspending in Mg2+–HEPES buffer (20). Sperm from cauda epididymis and vas deferens were released directly into PBS. The sperm suspension was centrifuged twice at 800 × g for 3 min to remove contaminants. The collected cells and sperm were directly resuspended in 1× SDS/β-mercaptoethanol sample buffer followed by boiling for 10 min.

**Immunohistochemistry**—Paraffin sections of mouse testis (Zyagen) were deparaffinized using xylene and rehydrated with a graded series of 100, 95, 70% ethanol and PBS. After blocking with 3% BSA (Bovogen Biologicals) for 1 h, the sections were incubated with primary antibodies and rhodamine-conjugated secondary antibody (Jackson ImmunoResearch), and then stained with Hoechst 33342 dye (Sigma-Aldrich). Fluorescence signals were observed under a microscope (DMLB; Leica Microsystems).

**Generation of Mutant Mice**—A CMHD-GT_327A6-3 embryonic stem (ES) cell line containing a *Spink2* gene trap allele was purchased from CMHD. The gene trap construct contained a splicing acceptor sequence, internal ribosome entry site, enhanced GFP gene, polyadenylation signal, neomycin resistance gene, splicing donor sequence, and β-galactosidase-neomycin resistance gene fusion. ES cells, derived from 129-strain mice, were injected into C57BL/6 blastocysts using standard procedures to produce chimeraic mice. Chimeric males were mated with C57BL/6 females, and germ line transmission in pups was confirmed. The *Spink2*mut line was obtained by crossing heterozygotes. Mice were genotyped by amplifying *Spink2* and GFP in the gene trap vector using the following two sets of primers: 5’-AGA GAA AAG CGG ACC-3’ (forward) and 5’-GGA ATG GAA ACG GGG-3’.
Phenotypic Analyses of Mutant Mice—For fertility test of Spink2 mutant males, each Spink2 mutant and wild-type (WT) male (8 weeks old) was placed with two C57BL/6 females. The females were checked for the presence of vaginal plugs and pregnancy. The number of pups was counted, and fertility rate was calculated. For sperm counting, sperm from cauda epididymis and vas deferens from 8-week-old Spink2 mutant and WT males was collected. Sperm cells were counted in a hemocytometer under a light microscope. In the analysis of testicular integrity, testes were fixed by immersion in Bouin's fixative for 24 h, embedded in paraffin, and sectioned using standard protocols. The morphology of testes was observed after hematoxylin and eosin staining of paraffin-embedded sections. The level of apoptosis in sections of testes was detected by TUNEL assay using the ApopTag Plus Peroxidase in Situ Apoptosis Detection kit (Chemicon), according to the manufacturer's instructions.

Statistics—Results are presented as means ± S.E. or S.D. values. The statistical significance of differences between data means was determined using a two-tailed Student's t test.

RESULTS

Sequence and Phylogenetic Relationships of Mouse SPINK2—As an initial step in characterizing mouse Spink2, we performed sequence analyses using NCBI databases. The mouse Spink2 gene encodes an 86-amino acid protein (Fig. 1A) with a predicted molecular mass of 9.723 kDa. The SPINK2 protein is predicted to contain a signal peptide (residues 1–16, 1.757 kDa) and a Kazal-type serine protease inhibitor-pancreatic secretory trypsin inhibitor domain (residues 44–86). Pro47-Arg48-Asn49-Leu50 residues represent the P2-P2 reactive site. The amino acid sequence of mouse SPINK2 shares 55, 82, and 53% similarity with that of human, rat, and bull, respectively. Notably, cysteine residues were conserved among these species. The phylogenetic relationships of SPINK2 to the other 12 SPINK members in mice are shown in Fig. 1B.

Activity of SPINK2 in Vitro—To explore the functional characteristics of mouse SPINK2, we evaluated SPINK2 for serine protease-inhibitory activity in vitro, assaying the activity of His-tagged SPINK2 in the presence of trypsin and the synthetic substrate (CBZ-Ile-Pro-Arg)2-Rhodamine110. Upon enzymatic cleavage of the two oligopeptide side chains by the serine protease, the nonfluorescent substrate becomes fluorescent. This is a sensitive and selective substrate specific for the serine protease trypsin (21, 22). The results showed that recombinant SPINK2 protein reduced trypsin activity in a dose-dependent manner.

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Spink2 Function in Spermatogenesis

FIGURE 2. Activity of SPINK2 in vitro. Trypsin (42 nM) and His-tagged SPINK2 recombinant protein were incubated with the synthetic substrate (CBZ-Ile-Pro-Arg)2-Rhodamine110, and activity was determined by measuring absorbance at 521 nm. The addition of His-tagged SPINK2 recombinant protein decreased the activity of trypsin. Values are means ± S.D. (error bars, n = 3). Statistical significance (*, p < 0.05; **, p < 0.01) was assessed using Student’s t test. Each experiment was repeated at least three times.

Spink2 Expression Pattern—To investigate the in vivo properties of Spink2, we first examined its expression profile at the transcriptional and protein level. The half-maximal inhibitory concentration (IC50) was 2.73 μM (±0.45), and the maximal decrease in activity was detected in the presence of 30 μM SPINK2 protein.

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To investigate the expression patterns of SPINK2 at the protein level, we generated a polyclonal antibody against a synthetic peptide (residues 70–86) and performed immunoblot analyses on testes from WT and W/Wv (germ cell-lacking) mutant mice. As shown in Fig. 3D, two bands with molecular sizes of 10 and 8 kDa were detected in WT testes, but no bands were detected in testes lacking germ cells, confirming the germ cell-specific expression pattern found in the RT-PCR analysis (Fig. 3B). Because SPINK2 has a signal peptide (Fig. 1B), it is highly likely that the 10-kDa band represents the intact precursor SPINK2 protein containing the signal peptide, whereas the 8-kDa protein corresponds to a processed form. Further immunoblot analyses, carried out using mouse testes obtained on different postnatal days, showed a developmentally regulated expression pattern (Fig. 3E), similar to the RT-PCR results (Fig. 3C). Finally, we examined the expression pattern of SPINK2 protein in germ cells during spermatogenesis. Immunoblot analyses were performed on cells from different stages during sperm development, such as testicular spermatogenic cells and testicular sperm, and mature sperm from the epididymis. The population of testicular cells includes spermatogenic cells corresponding to spermatogonia, spermatocytes, and round spermatids. Testicular sperm are a small portion of elongating and condensing spermatids and a larger fraction of fully developed sperm. Sperm represent posttesticular, mature sperm from the cauda epididymis and vas deferens (Fig. 3F). The result showed that SPINK2 was expressed in all cell types (Fig. 3G). Notably, the 10-kDa band present in testicular cells was absent from testicular sperm and mature sperm, suggesting that the signal peptide of SPINK2 is removed during spermatogenesis.

To establish the cellular localization of SPINK2 further, we carried out immunohistochemical analysis using paraffin sections of adult mouse testis. As a result, we observed the SPINK2 signal in the cytoplasmic region of round spermatids, but not in spermatogonia and spermatocytes (Fig. 4). In addition, as round spermatids differentiate into spermatozoa, the signal was detected in acrosomal regions. Our results collectively demonstrate that mouse SPINK2 protein is specific to germ cells in the testis and is developmentally regulated during spermatogenesis.

Generation of Spink2 mutant Mice—The trypsin-inhibitory activity and germ cell- and stage-specific expression of SPINK2 suggest that SPINK2 plays an important role in male reproduction. To investigate the in vivo role of Spink2 in the reproductive process, we generated Spink2 mutant mice by means of gene trap mutagenesis. Gene trapping is a high-throughput approach that randomly generates loss-of-function mutations by introducing insertional mutations across the mouse genome. Insertion of a gene trap vector with an upstream 3′ splice site and a downstream transcriptional termination sequence into an intron of an expressed gene results in a fusion transcript that encodes a truncated protein (23). We screened public gene trap databases for an ES cell line with insertion of the gene trap vector in the Spink2 gene, which generates an abnormal fusion transcript. Such an ES cell line was obtained, and the precise location of the genomic insertion was mapped by splinkerette PCR and DNA sequencing analyses. The integration site of the gene trap vector mapped to a position upstream of exon 1, producing a fusion transcript lacking exon 1 (Fig. 5A). Chimeric mice and heterozygous mice carrying the
gene trap allele (Spink2\(^{+/gr}\)) were generated using standard procedures. Homozygous (Spink2\(^{gr/gr}\)) males were obtained by crossing with heterozygous mice. Spink2\(^{+/gr}\) and Spink2\(^{gr/gr}\) mice were verified by PCR genotyping based on sequence information (Fig. 5B). Spink2\(^{gr/gr}\) mice were born live with the predicted Mendelian pattern of inheritance. The weights and growth rates of Spink2\(^{gr/gr}\) newborn pups were not significantly different from those of WT (Spink2\(^{+/+}\)) mice. To investigate
SPINK2 protein expression in Spink2gt/gt mice, we performed immunoblot analyses on testes using the anti-SPINK2 antibody described above. This analysis revealed a reduction in SPINK2 levels in the testes from Spink2gt/gt mice compared with that in WT mice (Fig. 5C). The presence of SPINK2 protein in Spink2gt/gt mice is likely due to the production of a WT transcript by alternative splicing, resulting in hypomorphic mutations, a situation that often occurs in gene trap mutagenesis. It should be noted that the degree of reduction was variable among Spink2gt/gt mice (Fig. 5D). This could be because there is variable penetrance of the effect of the gene trap mutation on the expression of the Spink2 gene among mice. In subsequent phenotypic investigations of Spink2gt/gt mice, only mutant mice with >50% reduction in SPINK2 levels were examined.

Phenotypic Analyses of Spink2gt/gt Mice—To determine whether the reduction in SPINK2 levels affected reproductive functions, we performed a fertility test. Adult WT and Spink2gt/gt males were mated with WT females. The average litter size of pups produced over a breeding period was significantly reduced in Spink2gt/gt males compared with WT males, despite frequent observations of vaginal plugs in females. Overall, the in vivo fertility rate of Spink2gt/gt males was reduced by about 40% relative to WT males (Table 1). Adult Spink2gt/gt female mice exhibited normal fertility (data not shown). Next, we examined testes from adult Spink2gt/gt mice. Testes from mutant males were smaller than those from WT littermates (Fig. 5A). In fact, the testis-body-weight ratio was significantly lower in Spink2gt/gt mice than in WT mice (Fig. 6B), suggesting an abnormality in testicular function and/or spermatogenesis. To determine whether the observed testicular changes led to altered sperm production, we evaluated mature sperm collected from the cauda epididymis and vas deferens of 2-month-old Spink2gt/gt mice and WT litters. It should be noted that the appearance and weight of epididymis from Spink2gt/gt mice were normal compared with those from WT mice (Fig. 5, C and D). An analysis of sperm revealed a significant reduction in sperm counts (48% of WT) (Fig. 6E). In addition, we found that Spink2gt/gt mice produced more morphologically abnormal sperm than did WT mice (Fig. 6F). The most frequently observed abnormalities were sperm in which the flagellum was bent at a region between the principal piece and the mid-piece (Fig. 6G). These results demonstrate that a decrease in the amount of SPINK2 disrupts testicular integrity and normal sperm production, leading to reduced fertility.

Abnormalities and Apoptosis in Spink2gt/gt Germ Cells—To identify the underlying cause of abnormalities in testicular size and sperm production in Spink2gt/gt mice, we analyzed the histological appearance of seminiferous tubules in the adult testis. Spink2gt/gt testes contained seminiferous tubules that were significantly reduced in size compared with those of WT testes (Fig. 7, A and B) and exhibited vacuolization and germ cell loss; in contrast, spermatogenesis was robust in testes from WT adult males (Fig. 7C). In severe cases, most germ cells were degenerated, and only a few spermatogenic cells were detected in the seminiferous tubules of Spink2gt/gt mice (Fig. 7A). To determine whether the loss of germ cells in Spink2 mutant testes was related to apoptosis, we performed TUNEL assays. TUNEL staining of histological sections of WT and Spink2gt/gt testes revealed a significant increase in the number of apoptotic cells in Spink2gt/gt mice compared with WT mice (Fig. 8, A and B). These results indicate that SPINK2 is important for the survival and development of male germ cells and may be involved in regulating apoptosis in these cells.

Increased Serine Protease Activity in Spink2gt/gt Testes—Germ cell apoptosis in Spink2gt/gt testes might be directly related to excessive activity of serine proteases. We measured protease activity in the testis lysates of WT and Spink2gt/gt mice using the synthetic substrate (CBZ-Ile-Pro-Arg)2-Rhodamine110. This analysis revealed that serine protease activity is significantly higher in Spink2gt/gt testes compared with WT testes (Fig. 8C), indicating dysregulated protease activity in the mutant testes. Finally, we investigated whether caspase-3, an executioner of caspase-dependent apoptosis, is activated dur-

**Figure 3.** Distribution of Spink2 transcripts and protein. A, tissue distribution of Spink2, showing predominant transcription in testes. Complementary DNAs from various mouse tissues were amplified by PCR. Gapdh was included as a loading control. T, testis; E, epididymis; O, ovary; B, brain; H, heart; K, kidney; L1, liver; L2, lung; Sp, spleen; Gapdh, glyceraldehyde-3-phosphate dehydrogenase. B, germ cell-specific expression of Spink2. RT-PCR was performed using testes and epididymis from WT and germ-cell-lacking W/W" mice and mature sperm. Protamine 1 (Pmn1) was used as a control. C, developmental expression pattern of Spink2. Juvenile spermatogenesis consists of mitotic, meiotic, and postmeiotic phases. Stage-specific expression of Spink2 was determined from mouse testes on different days after birth (days 8, 10, 12, 14, 16, 20, 30, and 84). PL, preleptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene; MI, meiotic division I; MI, meiotic division II. D, SPINK2 protein in WT and W/W" testes. Total lysates from WT testes and germ-cell-lacking testes from W/W" mutant mice were immunoblotted with the anti-SPIK2 antibody. SPINK2 was not found in normal mouse testes. An anti-α-tubulin antibody was used as a control. E, stage-specific expression of SPINK2 during spermatogenesis examined by immunoblotting using total lysates obtained from prepubertal and adult male mice (days 7, 14, 21, 28, 35, 42, 49, and 56). SPINK2 was present from day 21, and the processed form was detected beginning on day 35. F, diagram of sperm development and maturation. Three populations of cells analyzed in the present study are shown: testicular cells (TC), testicular sperm (TS), and mature sperm (S). G, protein samples from TC, TS, and S immunoblotted with the anti-SPINK2 antibody. The 8-kDa protein is considered a processed form that lacks the signal peptide present in the 10-kDa protein. SPINK2 was present in all stages of germ cells. ADAM2 protein was included as a reference protein. 

**Figure 4.** Immunohistochemical analysis of SPINK2. Paraffin sections of mouse testes were stained with anti-SPINK2 antibody and normal rabbit serum. The nucleus was stained by Hoechst 33342 dye. A, specific antibody; B, positive control; C, negative control; D, merged images between antibody and Hoechst. Scale bar, 5 μm.
ing apoptosis caused by SPINK2 deficiency. In general, upon caspase-dependent apoptotic stimulation, caspase-3 is formed from a 32-kDa pro-caspase-3 that is cleaved into 17- and 12-kDa subunits. Our immunoblot analysis showed that 17- and 12-kDa subunits of caspase-3 were absent from both WT and Spink2\(^{gt/gt}\) testes samples (Fig. 8D). Thus, our results indicate that SPINK2 indeed functions as a serine protease inhibitor and the deficiency of SPINK2 causes apoptosis independent on caspase.

**DISCUSSION**

Genes expressed specifically or predominantly in male germ cells are critical for spermatogenesis. The present study provides new information on the Kazal-type serine protease inhibitor, Spink2, at transcriptional, protein, and functional levels in mice. We found that the Spink2 gene is transcribed predominantly in the testis, where its transcription is limited to germ cells. Protein analyses demonstrated the presence of SPINK2 in spermatogenic cells and mature spermatids, and our results indicate that SPINK2 indeed functions as a serine protease inhibitor and the deficiency of SPINK2 causes apoptosis independent on caspase.
sperm. Importantly, we generated mutant mice expressing SPINK2 at reduced levels and found that fertility was significantly decreased in mutant male mice. Further phenotypic analyses revealed that testicular integrity was disrupted, leading to significant changes in sperm number and morphology. Finally, we found that tests from mutant mice exhibited abnormal spermatogenesis and germ cell apoptosis.

Cell death occurs through several processes, namely necrosis, autophagy, and apoptosis. Apoptosis is a type of cell death that does not cause cell lysis and therefore does not initiate immune responses. Among the distinctive features of apoptotic cells are nuclear and cytoplasmic condensation, membrane blebbing, and internucleosomal DNA fragmentation, which produces a characteristic laddering pattern on agarose gels (24). Caspases have traditionally been presumed to play a dominant role as the primary mediator of apoptosis. However, several lines of evidence indicate that serine proteases also play crucial roles in mediating and promoting apoptosis through both
Apoptosis and increased serine protease activity in Spink2 mutant testes. A, germ cell apoptosis in Spink2+/+ and Spink2mutant testes. Sections of testes were TUNEL-stained; positive cells are indicated by arrows. Scale bar, 50 μm. B, comparison of TUNEL-positive germ cells in Spink2+/+ and Spink2mutant testes. The percentage of apoptotic germ cells per seminiferous tubule in Spink2+/+ testes is shown in the y-axis. Values are means ± S.E.M. Statistical significance (*, p < 0.05; n = 4) was assessed using Student’s t test. The average percentage of apoptotic germ cells in Spink2mutant testes was 529.88% ± 77.28% of that in WT testes. C, increased serine protease activity in Spink2mutant testes. Testis extracts from Spink2+/+ and Spink2mutant males were incubated with the rhodamine-conjugated substrate, and serine protease activity was measured. Values are means ± S.D. (n = 3). Statistical significance (*, p < 0.01) was assessed using Student’s t test. Each experiment was repeated at least three times. Increased activity in Spink2mutant testes was 133.36 ± 2.09% of that in WT testes. D, caspase-3 activation in the testes. Immunoblot of whole cell lysates of Spink2+/+ and Spink2mutant testes was performed with anti-caspase-3 antibody. An anti-α-tubulin antibody was used as a control for sample loading.

In conclusion, we comprehensively investigated Spink2 in mice, providing key information on testicular distribution, expression patterns in germ cells, and reproductive defects in Spink2 mutant mice. Notably, our study suggests that SPINK2 is required for maintaining normal spermatogenesis, potentially acting as a regulator of proteolytic events and apoptosis in germ cells. This is the first study highlighting the role of a Kazal-type protease inhibitor in reproduction. Our discovery provides a framework for future studies designed to address the detailed mechanisms during male germ cell development.

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