METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

***Ggnbp2*-Null Mutation in Mice Leads to Male Infertility due to a Defect at the Spermiogenesis Stage**

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Accepted for publication
July 20, 2017.

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Gametogenetin binding protein 2 (GGNBP2) is an evolutionarily conserved zinc finger protein. Although *Ggnbp2*-null embryos in the B6 background died because of a defective placenta, 6.8% of *Ggnbp2*-null mice in the B6/129 mixed background were viable and continued to adulthood. Adult *Ggnbp2*-null males were sterile, with smaller testes and an azoospermic phenotype, whereas mutant females were fertile. Histopathological analysis of 2-month-old *Ggnbp2*-null testes revealed absence of mature spermatozoa in the seminiferous tubules and epididymides and reduction of the number of spermatids. Ultrastructural analysis indicated dramatic morphological defects of developing spermatids in the *Ggnbp2*-null testes, including irregularly shaped acrosomes, acrosome detachment, cytoplasmic remnant, ectopic manchette, and ill-formed head shape in both elongating and elongated spermatids. However, the numbers of spermatogonia, spermatocytes, Leydig cells, and Sertoli cells in *Ggnbp2*-null testes did not significantly differ from the wild-type siblings. Gonadotropins, testosterone, and the blood-testis barrier were essentially unaffected. Western blot analyses showed increases in α -E-catenin, β -catenin, and N-cadherin, decreases in E-cadherin, afadin, and nectin-3, and no changes in vinculin, nectin-2, focal adhesion kinase, and integrin- β 1 protein levels in *Ggnbp2*-null testes compared to wild-type siblings. Together, this study demonstrates that GGNBP2 is critically required for maintenance of the adhesion integrity of the adluminal germ epithelium and is indispensable for normal spermatid transformation into mature spermatozoa in mice. (*Am J Pathol* 2017, 187: 2508–2519; <http://dx.doi.org/10.1016/j.ajpath.2017.07.016>)

Mammalian male fertility requires sufficient numbers of morphological normal and motile sperm that is generated through a highly organized cyclic process known as spermatogenesis. Spermatogenesis originated from diploid spermatogonial multiplication, followed by two successive meiotic reductive divisions to produce haploid round spermatids; round spermatids finally undergo a complex cyto-differentiation process called spermiogenesis to become motile spermatozoa.^{1,2} During the spermiogenic phase, drastic morphological changes take place to transform circular spermatids into spermatozoa, which include progres-sive elongation, acrosomal and flagellar formation, nuclear condensation, and dramatic cytoplasmic remodeling.

Extensive studies suggest that this remarkable cellular dif-ferentiation process is elaborately regulated by a battery of male germ cell-specific genes estimated to be approxi-mately 600 to 1000.^{3,4} Mouse gene-knockout studies in the past two decades have demonstrated that >40 genes are essential for normal spermiogenesis.^{5,6} These represent a few well-established genes that participate in spermiogen-esis; many more need to be identified.

Supported in part by NIH grants R01-HD057501 (Z.L.), 5P20RR017702-10, and 8P20 GM103453-10, and project I (Z.-J.L.).

Disclosures: None declared.

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Gametogenetin binding protein 2 (GGNBP2) is encoded by a gene consisting of 15 exons. It is highly conserved across humans, rats, and mice, sharing 87% homology in nucleotide sequence and 96% homology in amino acid sequence between humans and mice. The full-length murine GGNBP2 protein is composed of 740 amino acids. It contains a Cys2His2 zinc finger motif at the N terminus and is also named zinc finger protein 403 (ZFP403 or ZNF403).^{7–10} A shorter form of GGNBP2, previously called laryngeal carcinoma-related protein 1, was reported to be dramatically down-regulated in primary laryngeal carcinomas, drug-resistant ovarian cancers, and several human cancer cell lines.^{11–14} It has been speculated that GGNBP2 might play a role in regulation of tumorigenesis. Overexpression of human GGNBP2 in a hepatic cancer cell line inhibited cell growth, proliferation, and migration.¹² GGNBP2 knockdown, on the other hand, resulted in G₂/M cell cycle arrest.¹⁵ Recent studies demonstrated that GGNBP2 acted as a tumor suppressor in human estrogen receptor α -positive breast cancer cell lines by its activity in the impediment of tumorigenicity in nude mice.¹⁶

Ggnbp2 was initially identified as a dioxin-induced factor 3 in mouse embryonic stem cells in 2001.⁷ The murine *Ggnbp2* is in chromosome 11, which is known to contain several important reproductive-related genetic loci. For example, mutation of the *11A2-3* locus and targeted disruptions of *Ube2b* or *Ace-T* led to male infertility.¹⁷ *Ggnbp2* is abundantly expressed in the testis during spermatogenesis in adult testes, and the protein was predominately localized in spermatocytes and round spermatids.⁷ Further study revealed that GGNBP2 was one of the several proteins that interact with a testicular protein, gametogenetin (GGN).⁸ GGNBP2 is hence postulated to play a role in spermatogenesis. To elucidate the function of GGNBP2 *in vivo*, we generated a *Ggnbp2* knockout mouse line. *Ggnbp2*-null embryos in the B6 background died because of a defective placenta.¹⁰ A portion of *Ggnbp2*-null mice on the B6/129 mixed background were viable and continued to adulthood. Adult *Ggnbp2*-null males were sterile, with smaller testes and an azoospermic phenotype, whereas mutant females were fertile. Loss of *Ggnbp2* resulted in a severe impairment of spermiogenesis that eventually led to male infertility. This study, for the first time, demonstrates that GGNBP2 is critically required for maintenance of the adhesion integrity of the adluminal germ epithelium and is indispensable for normal spermatid transformation into mature spermatozoa in mice.

Materials and Methods

Animals

Ggnbp2 knockout (*Ggnbp2KO*) heterozygous female mice with the C57BL/6 background were crossed with wild-type (WT) male mice with the 129/Sv background, and then these *Ggnbp2* heterozygous males and females with the mixed background were bred. Animal genotyping was

performed by PCR, as described previously, with genomic DNA isolated from tail clips of mice.¹⁸ The PCR primers are listed in Table 1. For a fertility test, six 2-month-old *Ggnbp2*-null and WT male mice were mated with proven fertile females (one male/one female per cage) for 6 months. The number of pups produced from each female was recorded, as described previously.¹⁸ All of the mice were raised under standard laboratory conditions, with a 12-hour light/dark cycle and free access to food and water. Mice were euthanized under ketamine anesthesia for tissue and blood collections, and efforts were made to minimize their discomfort. The studies have been approved by the Animal Care and Use Committee of the University of Louisville (Louisville, KY).

RT-PCR and Real-Time Quantitative PCR

Total RNA was extracted from the testes using Trizol reagent (Invitrogen, Waltham, MA), according to the manufacturer's instructions. Total RNA (2 μ g) was reverse transcribed into cDNA with random primers (Invitrogen) and Avian Myeloblastosis Virus Reverse Transcriptase (Promega Corp., Madison, WI). Testicular *Ggnbp2* expression during postnatal development was detected by real-time quantitative PCR, performed with SYBR Green reagents kits (Invitrogen) using the StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA). The real-time PCR steps included holding at 50°C for 2 minutes and 95°C for 2 minutes, followed by repeated 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 60 seconds. The relative expression levels of *Ggnbp2* were calculated by the comparative $\Delta\Delta$ Ct method¹⁹ and normalized to a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).

For RT-PCR, the cDNA was amplified with *Ggnbp2* and *Gapdh* primers. Each PCR cycle consisted of denaturation for 45 seconds at 94°C, annealing for 1 minute at 57°C, and extension for 1 minute at 72°C. The amplified products were separated by electrophoresis in agarose gels and stained by ethidium bromide.

Real-time quantitative PCR and RT-PCR primers, as listed in Table 1, were designed according to the sequences obtained from GenBank and synthesized by Operon Technologies (Alameda, CA).

mRNA *in Situ* Hybridization

Formalin-fixed, paraffin-embedded testicular tissue sections (5 μ m thick) were processed for *Ggnbp2* mRNA *in situ* detection using the RNAscope 2.0 formalin-fixed, paraffin-embedded fast red detection kit (Advanced Cell Diagnostics, Hayward, CA), according to the manufacturer's recommended procedures. Briefly, the sections were deparaffinized and boiled with a pretreatment buffer for 15 minutes. After being rinsed with distilled water, the sections were incubated at 40°C with murine *Ggnbp2* RNAscope

Table 1 Primer Sequences for Genotyping and Semiquantitative RT-PCR

Gene	Primer sequence	PCR cycles
<i>Ggnbp2</i> (genotyping)	F: 5'-AGTGCCATTTACCCACCAAG-3' R: 5'-GAAAGGAGGAGGAAAGGAA-3'	30
<i>Ggnbp2</i> (qPCR)	F: 5'-CCTTCGAGAGGAGGCAGATCC-3' R: 5'-AACTGCTTTAGTTGTGCACCATTAT-3'	40
<i>Gapdh</i> (qPCR)	F: 5'-CTGGAGAAACCTGCCAAGTA-3' R: 5'-TGTTGCTGTAGCCGTATCA-3'	40
<i>Ggnbp2</i> (RT-PCR)	F: 5'-CTCATTTGGTGAACCTTGACTGC-3' R: 5'-TCACTGCTTTCTCGTCTGCGGTG-3'	30
<i>Gapdh</i> (RT-PCR)	F: 5'-TGTTCAGAGACGGCCGCAT-3' R: 5'-CGGCCTCACCCCTTTGATGT-3'	30

F, forward primer; qPCR, real-time quantitative PCR; R, reverse primer.

probes, followed by a colorimetric reaction with fast red to visualize the hybridized signal. Then, sections were counterstained with hematoxylin, dehydrated, and mounted. To control for background, we used a negative control probe against the *Bacillus subtilis* dihydrodipicolinate reductase, *dapB* (Advanced Cell Diagnostics). All sections were observed and imaged with a bright-field Olympus microscope (model IX71/DP72; Olympus, Tokyo, Japan).

Immunohistochemical Staining

The testes were fixed with 10% formalin and embedded in paraffin. The immunostaining procedure was performed by an avidin-biotin immunoperoxidase method. Briefly, deparaffinized sections were rehydrated and then incubated with hydrogen peroxide. The sections were then incubated with the primary antibodies, as listed in Table 2, at 4°C overnight and then incubated with biotinylated secondary antibody (1:100; Vector Laboratories, Burlingame, CA).

After rinsing with phosphate-buffered saline (PBS), sections were incubated with an avidin–biotin–horseradish peroxidase complex using a Vectastain ABC kit (Vector Laboratories). Immunostaining was detected by incubation of the sections with the substrate 3,3'-diaminobenzidine. All sections were counterstained with hematoxylin. Replacement of the primary antibody with irrelevant rabbit or mouse IgG was used as a procedure control.

Periodic Acid–Schiff Staining

The testes were fixed with 10% formalin and embedded in paraffin. Deparaffinized and rehydrated sections were incubated with periodic acid solution (Sigma-Aldrich, St. Louis, MO) for 5 minutes at room temperature and rinsed in distilled water. Sections were immersed in Schiff's reagent for 15 minutes at room temperature. After washing with distilled water for 5 minutes, the sections were counterstained with hematoxylin solution for 90 seconds.

Table 2 Antibodies Used in This Report

Antibody	Host species	Vendor	Catalog no.	Application	Dilution
GGNBP2	Rabbit	Pacific Immunology Corp. (Ramona, CA)	PAC-139	IHC	1:1500
Cldu11-11	Mouse	Santa Cruz Biotechnology (Dallas, TX)	SC-9412	IF	1:400
SYCP3	Rabbit	Sigma-Aldrich (St. Louis, MO)	L9393	IF	1:200
SALL4	Mouse	Sigma-Aldrich	B8434	IF	1:400
GATA-4	Goat	Santa Cruz Biotechnology	sc-69269	IF	1:400
CYP17	Mouse	Cell Signaling Technology (Danvers, MA)	2107	IHC	1:1000
α -Tubulin	Mouse	Cell Signaling Technology	2102	IF	1:5000
α -E-catenin	Rabbit	Cell Signaling Technology	2123	WB	1:1000
β -Catenin	Rabbit	Cell Signaling Technology	9145	WB	1:1000
E-cadherin	Rabbit	Cell Signaling Technology	9134	WB	1:1000
N-cadherin	Rabbit	Santa Cruz Biotechnology	sc-7179	WB	1:1000
Afadin	Rabbit	Cell Signaling Technology	9171	WB	1:800
Vinculin	Rabbit	Cell Signaling Technology	4441	WB	1:1000
Nectin-2	Rabbit	Cell Signaling Technology	9359	WB	1:1000
Nectin-3	Rabbit	Cell Signaling Technology	9361	WB	1:1000
FAK	Rabbit	Cell Signaling Technology	3285	WB	1:1000
Integrin- β 1	Rabbit	Sigma-Aldrich	SAB4501583	WB	1:800
GAPDH	Rabbit	Sigma-Aldrich	3331	WB	1:1000

CYP17, 17 α -hydroxylase; FAK, focal adhesion kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GATA, GATA motif binding factor; GGNBP, gametogenetin binding protein; IF, immunofluorescence; IHC, immunohistochemistry; SALL, spalt like transcription factor; SYCP, synaptonemal complex protein; WB, Western blotting.

Hormone Assays

Mice were anesthetized and exsanguinated by cardiac puncture. Sera were separated and stored at -80°C until assayed. Luteinizing hormone, follicle-stimulating hormone, and testosterone levels were determined by the Ligand Assay and Analysis Core, Center for Research in Reproduction, the University of Virginia (Charlottesville, VA). Mouse luteinizing hormone was detected by immunoradiometric assays. Radioimmunoassays were used for mouse follicle-stimulating hormone and testosterone measurements. The limits of sensitivities of these assays were 0.07 ng/mL for luteinizing hormone, 2 ng/mL for follicle-stimulating hormone, and 5 ng/dL for testosterone.

Stages of Seminiferous Tubule Cycle

The stages of spermatogenesis were quantitatively evaluated using formalin-fixed, paraffin-embedded testicular sections stained by periodic acid–Schiff. Each stage of seminiferous tubule was determined on the basis of the shape of acrosome of spermatid and overall seminiferous epithelium composition, as described previously,^{20,21} using a bright-field Olympus microscope. The numbers of each stage of tubules in three different cross sections per testis per mouse were counted. A total of three 2-month-old mice in each genotype were analyzed.

The testicular sections were also stained with tetramethylrhodamine-conjugated lectin peanut agglutinin (PNA; Molecular Probes, Waltham, MA) and DAPI (Santa Cruz Biotechnology, Dallas, TX). Each stage (I to XII) of spermatogenesis was identified and imaged with an Olympus fluorescence microscope.

Immunofluorescence Staining

Testes were fixed in 4% paraformaldehyde, and embedded in paraffin. Deparaffinized sections were microwaved with 0.1 mol/L citrate buffer (pH 6.0) for 12 minutes, washed in PBS, blocked with 5% normal serum containing 0.3% Triton X-100, and then incubated with primary antibodies, as listed in Table 2, at 4°C overnight. Subsequently, α -tubulin was detected by tetramethylrhodamine-conjugated donkey anti-mouse IgG (1:100; Jackson ImmunoResearch Lab, West Grove, PA). Cldu11, Spalt-like transcription factor 4, synaptonemal complex protein 3, and GATA motif binding factor 4 were detected by fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:100; Jackson ImmunoResearch Lab), and acrosomes were stained by tetramethylrhodamine-conjugated lectin PNA in the same slides.

Dispersed spermatogenic cells were prepared by incubation of the seminiferous tubules in PBS supplemented with 0.5% bovine serum albumin, 2 mg/mL collagenase, 0.1 $\mu\text{g/mL}$ DNase I, and 0.5% trypsin (Sigma-Aldrich) for 10 minutes at 33°C . The cell suspension was filtered through a nylon mesh with 50- μm pores. The dissociated cells were then

Figure 1 *Ggnbp2* mRNA and protein expression in the mouse testes. **A:** Testicular expression of *Ggnbp2* during postnatal development by real-time quantitative PCR analyses. *Ggnbp2* mRNA levels are normalized by *Gapdh* mRNA in each sample. **B–E:** *In situ* hybridization (**B** and **C**) and immunohistochemistry (**D** and **E**) are performed to determine the localization of *Ggnbp2* in 2-month-old testes. *Ggnbp2* mRNA (red; **B** and **C**) and protein (brown; **D** and **E**) are primarily expressed in spermatocytes and spermatids. **E:** Weak GGNBP2 immunostaining was also observed in Sertoli cells (arrows). The testicular sections are counterstained with hematoxylin. Boxed areas in **B** and **D** are enlarged in **C** and **E**, respectively. Representative images are from one of three mice examined. Data are expressed as means $\pm$ SEM (**A**). * $P < 0.05$ versus postnatal day 5; ** $P < 0.01$ versus postnatal day 12. Scale bars = 50 μm (**B–E**).

resuspended in 4% paraformaldehyde, washed with PBS, spread on slides, and air dried. α -tubulin was detected by tetramethylrhodamine-conjugated donkey anti-mouse IgG, and acrosomes were stained by fluorescein isothiocyanate-conjugated lectin PNA in the same slides.

All sections were covered with a DAPI-containing mounting medium to visualize the nuclei. Fluorescent signals on testicular sections were visualized and imaged using an Olympus fluorescence microscope. Replacement of the primary antibody with irrelevant mouse or rabbit IgG was used as a procedure control.

Biotin Tracer Assay

The procedure was performed as described by Meng et al²² previously. Two-month-old WT and *Ggnbp2*^{KO} mice were anesthetized. Testes were exposed by a small incision through the scrotal layer, and 40 μL of freshly prepared 10 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Thermo-Fisher Scientific,

Figure 2 *Ggnbp2*-null mutation and testicular phenotype. **A**: RT-PCR analysis of total RNA from 2-month-old adult wild-type (+/+), *Ggnbp2*KO heterozygous (+/-), and *Ggnbp2*KO homozygous (-/-) testes shows the absence of *Ggnbp2* mRNA in null mutant testes. The housekeeping gene *Gapdh* serves as an internal control. **B** and **C**: In contrast to wild-type (WT; **B**), *in situ* hybridization detects no *Ggnbp2* mRNA in seminiferous tubules of null mutant testes (KO; **C**). **D** and **E**: Compared to 2-month-old WT mice, the sizes of *Ggnbp2*-null mutant (KO) testis (Tes) and epididymis (Epi) are noticeably smaller (**D**), whereas the seminal vesicles are indistinguishable at the same age (**E**). **F** and **G**: Immunohistochemical staining of 17 α -hydroxylase (a Leydig cell marker) reveals no noticeable difference in the interstitial space between *Ggnbp2*-null (KO) and WT testes. **H**–**K**: Phase-contrast (**H** and **I**) and periodic acid–Schiff (PAS)–hematoxylin–stained (**J** and **K**) images show abundance of mature spermatozoa in 2-month-old WT epididymis (**H** and **J**), whereas *Ggnbp2*-null mutant (KO) epididymis contains a few exfoliated germ cells (**I** and **K**). **L**–**O**: PAS-hematoxylin–stained testicular sections of 2-month-old WT (**L** and **N**) and *Ggnbp2*-null mutant (KO; **M** and **O**) mice show that the seminiferous tubules contain disorganized and malformed elongated spermatids. **N** and **O**: Higher-magnification views of stage XI seminiferous tubules. Representative images are from one of three WT and three KO mice that gave similar results. Scale bars = 50 μ m (**B**–**O**).

Waltham, MA) was gently injected into the interstitium of the testis. Thirty minutes later, the testes were removed and fixed in 10% formalin and processed for paraffin embedding. The testicular sections were deparaffinized, rehydrated, incubated with 0.02 mg/mL fluorescein isothiocyanate–conjugated streptavidin (Sigma-Aldrich) at room temperature for 60 minutes, rinsed with PBS, and then mounted with a medium containing DAPI. The slides were examined and imaged with a fluorescence Olympus microscope.

Transmission Electron Microscopy

Testicular tissues were fixed in 3% glutaraldehyde and 1.5% paraformaldehyde (Sigma-Aldrich) for 4 hours at 4°C. Post-fixation was performed for 1.5 hours at room temperature in 1% osmium tetroxide and 1.5% potassium ferrocyanide (Electron Microscopy Sciences, Hatfield, PA). After several rinses in PBS, samples were dehydrated in a graded series of ethanol-acetone and embedded in epoxy resin 618.

Table 3 Effects of *Ggnbp2*KO on Other Testicular Characteristics of Adult Mice

Testicular characteristics	Wild-type mice	<i>Ggnbp2</i> KO mice	<i>P</i> value
Ratio of testis/body weight, g	0.56 $\pm$ 0.1	0.41 $\pm$ 0.07	<0.05 (<i>n</i> = 10)
Diameter of seminiferous tubule, μ m	106.6 $\pm$ 0.8	95.1 $\pm$ 0.6	<0.05 (<i>n</i> = 3)
Spermatocytes/tubule, <i>n</i>	62 $\pm$ 11	68 $\pm$ 12	>0.05 (<i>n</i> = 3)
Spermatogonia/tubule, <i>n</i>	4.9 $\pm$ 0.2	4.4 $\pm$ 0.2	>0.05 (<i>n</i> = 3)
Sertoli cells/tubule, <i>n</i>	18.7 $\pm$ 0.2	18 $\pm$ 0.7	>0.05 (<i>n</i> = 3)
Apoptotic cells/tubules, <i>n</i>	5.3 $\pm$ 0.6	5.7 $\pm$ 0.4	>0.05 (<i>n</i> = 3)

Data are expressed as means $\pm$ SEM, unless indicated otherwise.

Table 4 Serum Hormone Levels of Adult Wild-Type and *Ggnbp2*KO Mice

Hormone	Wild-type mice	<i>Ggnbp2</i> KO mice	<i>P</i> value
Testosterone, ng/dL	30.8 ± 7.45	31.68 ± 7.72	>0.05
LH, ng/mL	0.28 ± 0.08	0.3 ± 0.16	>0.05
FSH, ng/mL	34.5 ± 2.01	36.62 ± 1.78	>0.05

Data are expressed as means ± SEM, unless indicated otherwise. *n* = 10 in each group.

FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Ultrathin (100 nm thick) sections were then cut, counterstained with uranyl acetate and lead citrate (Electron Microscopy Sciences), and examined and imaged with a Philips EM 208 transmission electron microscope (Philips, Eindhoven, the Netherlands).

Western Blotting

Western blotting was performed as described previously.¹⁰ Briefly, the testes of 1-month-old mice were homogenized using a Tissue-Tearor (RPI Corp., Mount Prospect, IL) in a lysis buffer. Protein aliquots were separated in SDS-PAGE gels, transferred to Immobilon-polyvinylidene difluoride membranes (Millipore, Billerica, MA), and then incubated overnight with antibodies, as listed in Table 2. Peroxidase-conjugated secondary antibody (1:2000; Vector Laboratories)

was used as the secondary antibody. Immunoblotting signals were detected by the enhanced chemiluminescence Western blotting detection system (GE Healthcare Biosciences, Pittsburgh, PA). All membranes were reblotted with GAPDH antibody as the loading control. The intensity of specific bands was scanned using image analysis software, TotalLab version 2.00 (Nonlinear USA Inc., Durham, NC). The results were presented as the ratio of target protein/GAPDH.

Statistical Analysis

The data presented are the means ± SEM. The results were analyzed by one-way analysis of variance and paired *t*-test using a version 17.0 SPSS program (SPSS Inc., Chicago, IL). *P* < 0.05 was considered statistically significant.

Results

Expression and Localization of *Ggnbp2* in the Postnatal Mouse Testes

To determine the expression of *Ggnbp2* during postnatal development, real-time quantitative RT-PCR was performed to analyze RNA isolated from mouse testes at postnatal days 5, 9, 12, 14, 21, 28, and 56. Low levels of *Ggnbp2* mRNA were detected in the testes of 5- to 12-day-old mice

Figure 3 The integrity of the blood-testis barrier (A–D), spermatogonia (E and F), spermatocytes (G and H), and Sertoli cells (I and J) in the seminiferous tubules of 2-month-old wild-type (WT) and *Ggnbp2*-null mutant (KO) testes. A and B: No biotin tracer (green) was observed in the lumen in either WT or KO seminiferous tubules. C–J: Immunofluorescence staining (green) of claudin-11 (a tight junction protein; C and D), SALL4 (a spermatogonial marker; E and F), synaptonemal complex protein 3 (a spermatocyte marker; G and H), and GATA motif binding factor 4 (a Sertoli cell marker; I and J) does not reveal any differences between WT and KO testes. The cell nuclei are stained by DAPI (blue). Also costaining with peanut agglutinin–lectin (red; E–J). Representative images are from one of three WT and three KO mice that gave similar results. Scale bars = 50 μm (A–J).

Figure 4 Analyses of spermatogenic stages of 2-month-old wild-type (WT) and *Ggnbp2*-null mutant (KO) testes. **A–V:** Fluorescent images of peanut agglutinin–lectin (red) and DAPI (blue) stained seminiferous epithelium enlarged from boxed areas of lower-magnification micrograph (insets) display different stages of spermatogenesis (I to XII) of WT and KO testes. **A and B:** Stage I. **C and D:** Stage II to III. **E and F:** Stage IV. **G and H:** Stage V. **I and J:** Stage VI. **K and L:** Stage VII. **M and N:** Stage VIII. **O and P:** Stage IX. **Q and R:** Stage X. **S and T:** Stage XI. **U and V:** Stage XII. **W:** The proportion of the stage of seminiferous epithelium in WT and KO testes shows no significant difference. **X and Y:** The numbers of spermatids at different stages (I to XII) of seminiferous tubules (**X**) and the developmental steps (1 to 16) of spermatids per tubules (**Y**) are significantly reduced in *Ggnbp2*KO mice. Representative images are from one of three WT and three KO mice that gave similar results. Data are expressed as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ versus WT. Scale bars = 50 μ m (**A–V**).

(Figure 1A). The expression significantly increased from postnatal day 14 and remained constant in juvenile mice. The highest levels were found in sex mature adults (Figure 1A).

The localization of *Ggnbp2* mRNA and its protein in the adult mouse testes was examined by mRNA *in situ* hybridization (Figure 1, B and C) and immunohistochemical staining (Figure 1, D and E). The results showed that *Ggnbp2* mRNA was primarily expressed in spermatocytes and spermatids in seminiferous tubules. A weak expression was seen in Sertoli cells but not in spermatozoa and interstitial Leydig cells. The control probe *dapB* did not detect any signals above the background levels (data not shown). Although no *Ggnbp2* mRNA hybridization signal was found in spermatozoa (Figure 1C), immunohistochemistry showed that GGNBP2 presented not only in spermatocytes and spermatids but also in spermatozoa. Moreover, the immunostaining is observed in the nuclei of spermatids and

spermatozoa (Figure 1E). Compared to the adult testis, intermediate levels of *Ggnbp2* mRNA and protein were also seen in the adult mouse ovarian tissues (data not shown).

Ggnbp2-Null Mutation Results in an Azoospermic Phenotype

*Ggnbp2*KO heterozygous mice on a C57BL/6 background were generated in our laboratory previously. *Ggnbp2*-null mutant fetuses died in utero because of a defective placenta.¹⁰ In this study, we established *Ggnbp2*KO heterozygous colonies on a mixed C57BL/6/129 background. By breeding this mixed background of *Ggnbp2*KO heterozygous mice, 6.8% instead of 25% (the mendelian ratio) of *Ggnbp2*-null mutant males and females were born alive and grew to adulthood. There were no *Ggnbp2* transcripts detectable in 2-month-old null mutant testes by RT-PCR

Figure 5 Immunofluorescence staining of manchette by α -tubulin (red) of 2-month-old wild-type (WT) and *Ggnbp2*-null mutant (KO) testicular sections (A and B) and dispersed elongating spermatids (C–F). The nuclei of spermatogenic cells are stained by DAPI (blue), and the acrosomes of dispersed spermatids are stained by peanut agglutinin–lectin (green; C–F). B: Dislocated and dispersed α -tubulin staining in the null mutant testicular seminiferous epithelium is observed. D and F: The microtubule bundles of the manchette skirt in null mutant elongating spermatids are unevenly distributed, with a shaggy appearance and distorted acrosomes. Representative images are from one of three WT and three KO mice that gave similar results. Scale bars = 20 μ m (A–F).

(Figure 2) and *in situ* hybridization (Figure 2). *Ggnbp2*-null mutant mice appeared to be healthy, with normal extra-genital development and sexual behavior. However, despite repeated mating with proven fertile females for 6 months, *Ggnbp2*-null mutant males did not sire any offspring (data not shown). In contrast to males, null mutant females produce progeny normally, indicating that deletion of *Ggnbp2* that led to infertility is sex specific.

Although the seminal vesicles of *Ggnbp2*-null mutant males were developed well, the size of testes of 2-month-old mice were smaller than those of WT siblings (Figure 2) and testicular weight was significantly reduced (Table 3). Interestingly, serum luteinizing hormone, follicle-stimulating hormone, and testosterone levels were not statistically different from WT siblings (Table 4). Moreover, no noticeable difference in interstitial space of the seminiferous tubules was noticed between null mutant (Figure 2) and WT (Figure 2) testes, as revealed by immunostaining of a Leydig cell marker, 17 α -hydroxylase (Figure 2). This suggests that reduced testicular size is not attributable to the endocrine dysfunction of the hypothalamus-pituitary-gonadal axis in *Ggnbp2*-null mutant mice.

Light microscopic analyses of the epididymis and testes revealed no spermatozoa presence in either organ of

2-month-old *Ggnbp2*-null mice (Figure 2). The lumens of epididymal ducts of *Ggnbp2*-null mice only contained a few sloughed germ cells (Figure 2). In contrast, the lumens of epididymal ducts from WT siblings filled with mature spermatozoa (Figure 2). The diameters of the seminiferous tubules were significantly reduced in 2-month-old null mutant testes (Table 3). There was no obvious difference in histological appearances in 1-month-old testes between WT and null mutants (data not shown). The seminiferous epithelium in 2-month-old *Ggnbp2*-null testes consisted of spermatogonia, spermatocytes, round spermatids, and Sertoli cells. Although elongated spermatids were present, they were disorganized, with deformed nuclei. The lumens of the seminiferous tubules contained sloughed late steps of abnormal spermatids (Figure 2). Together, these data indicate that disruption of *Ggnbp2* results in an azoospermic phenotype.

To test whether the integrity of the blood-testis barrier was impaired in *Ggnbp2*-null testes, surface biotinylated tracer was injected into the testicular interstitium. In both WT and null mutant testes, biotin tracer was limited to the testicular interstitium and the basal compartment of the seminiferous tubules. No biotin tracer was detected in the lumens of the seminiferous tubules (Figure 3, A and B). These results suggest that defective spermatogenesis in *Ggnbp2*-null mutant testes is not associated with an impaired blood-testis barrier. Consistent with this interpretation, a blood-testis barrier tight junction component, claudin-11 immunofluorescence staining, showed no difference in the tubular basal compartment in WT and null mutant testes (Figure 3, C and D).

Defective Spermiogenesis Leads to Azoospermia in the Absence of *Ggnbp2*

By examining cross sections of the seminiferous tubules of 2-month-old testes stained with Spalt-like transcription factor 4 (a spermatogonial marker) (Figure 3, E and F), synaptonemal complex protein 3 (a spermatocyte marker) (Figure 3, G and H), and GATA motif binding factor 4 (a Sertoli cell marker) (Figure 3, I and J) and quantitatively analyzing the numbers, the results showed that the mean numbers of spermatogonia, spermatocytes, and Sertoli cells per cross section of the seminiferous tubule were not significantly different between *Ggnbp2*-null mutants and WT siblings (Table 3). In addition to the observation of spermatogonia, spermatocytes, and Sertoli cells in the seminiferous tubules, staging analyses of cross sections of the seminiferous tubules were performed in periodic acid–Schiff–hematoxylin– and lectin–PNA–stained 2-month-old WT and *Ggnbp2*-null testes using the staging criteria described by Russell et al.²⁰ and Nakata et al.²¹ Twelve stages of lectin–PNA–stained cross sections of the seminiferous tubules of WT and *Ggnbp2*-null testes were presented in Figure 4. Quantitative data demonstrated that *Ggnbp2*-null mutants have a complete continuous

Figure 6 Ultrastructural abnormalities of spermatids in the *Ggnbp2*-null testes. Transmission electron microscopic images show elongating (A–D) and elongated (E) spermatids in 2-month-old wild-type (WT) testes. In the absence of *Ggnbp2* (KO), the mutant elongating (F–H) and elongated (I–N) spermatids frequently contain ill-formed nuclei, cytoplasmic remnant (asterisks), ectopic manchette (open arrow), and distorted or detached acrosomes (closed arrows). Scale bars = 1 μ m (A–N).

process of spermatogenesis, similar to WT siblings (Figure 4). However, there was a marked decline in quantity of spermatids in each stage of the seminiferous tubules in the null mutant mice (Figure 4). On the basis of the morphology of the acrosome formation and shape of spermatids, as described by Russell et al²⁰ and Nakata et al,²¹ 16 steps of spermatid development were analyzed. As shown in Figure 4, the spermatid numbers per tubule between WT and *Ggnbp2*-null mutants were noticeably lower in the null mutant testes than WT siblings. A more dramatic decrease was observed at later steps of spermatid development. These observations indicate that defective spermiogenesis leads to azoospermia in the absence of *Ggnbp2* in the testis.

Loss of *Ggnbp2* Causes Abnormal Transformation of Spermatids

Immunofluorescence analysis using α -tubulin as a microtubule marker revealed that *Ggnbp2*-null spermatids contained abnormal manchette. Unlike WT spermatids, normal bell-shaped manchettes covering typical ovoid-shaped nuclei in elongating spermatids (Figure 5A), the manchette lost its special perinuclear symmetrical distribution in most

spermatids (Figure 5B). Dislocated and dispersed α -tubulin staining, with misshapen spermatid nuclei, was observed. Costaining of α -tubulin and acrosome by lectin-PNA showed that the microtubule bundles of the manchette skirt were unevenly distributed, with a shaggy appearance and distorted acrosomes (Figure 5, D and F) compared to WT cells (Figure 5, C and E).

Transmission electron microscopic analyses of testes of 2-month-old *Ggnbp2*-null mutant mice were performed to further interrogate the pathological changes of spermatid development. Ultrastructural evidence revealed dramatic morphological defects of developing spermatids in the *Ggnbp2*-null testes, including irregularly shaped acrosomes, acrosome detachment, cytoplasmic remnant, ectopic manchette, and ill-formed bizarre head shape in both elongating and elongated spermatids (Figure 6, F–N). In contrast, normal acrosome, nucleus, and manchette were found in a well-organized arrangement in WT testes (Figure 6, A–E).

Altered Adhesion Protein Expression in 1-Month-Old *Ggnbp2*-Null Testes

Pronounced spermatid deformation in the *Ggnbp2*-null testes prompted us to investigate whether *Ggnbp2* ablation

Figure 7 Altered adhesion protein expression in 1-month-old *Ggnbp2*-null testes. Western blot results show that the protein levels of α -E-catenin, β -catenin, and N-cadherin are significantly elevated, E-cadherin, afadin, and nectin-3 are markedly reduced, and vinculin, nectin-2, focal adhesion kinase (FAK), and integrin- β 1 are not changed in *Ggnbp2*-deficient testes compared to wild type (WT). Representative images of three WT (1 to 3) and knockout (KO; 4 to 6) mice are shown. * $P < 0.05$, ** $P < 0.01$ versus WT. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

had an impact on the interactions of spermatids and Sertoli cells in the adluminal compartment. Western blots were performed to examine a range of adhesion and associated protein levels in 1-month-old testes. The reason for examining 1-month-old testes was that round spermatids have been formed and spermiogenesis begins by this time, whereas the testes were morphologically comparable between WT and *Ggnbp2*-null mutants at this age. The results showed that α -E-catenin, β -catenin, and N-cadherin increased, E-cadherin, afadin, and nectin-3 decreased, and vinculin, nectin-2, focal adhesion kinase, and integrin- β 1 were unchanged in *Ggnbp2*-null testes compared to WT siblings (Figure 7).

Discussion

In this report, we demonstrated that GGNBP2 is an important regulator of spermatid differentiation in mice. Our laboratory previously reported that *Ggnbp2*-null mutant on a C57BL/6 background was embryonic lethal.¹⁰ Chen et al²³ recently reported that only three null mutant males (4 of 179) were viable on the B6 background, and they were infertile, suggesting that most *Ggnbp2*-null fetuses die in utero. On a mixed B6/129 genetic background, 6.8% of viable *Ggnbp2*-null mutant mice are obtained, suggesting an incompletely penetrant phenotype. Interestingly, a recent high-throughput study of 1751 unique gene knockouts in

mice reveals that incomplete penetrance and variable expressivity are common, even on a defined genetic background.²⁴

Histological examination reveals that the most dramatic impact of the loss of *Ggnbp2* causes failure of round spermatids from developing into elongated spermatids. As a result, there was complete lack of mature spermatozoa. Ultrastructural analysis shows the presence of multiple malformed elongating and elongated spermatids. Improper formation and detachment of the acrosomes, misshapen nuclei, abnormal tails, ectopic manchettes, and cytoplasmic retention all point to a failure to proceed through the complicated spermiogenic process.

Cells of the seminiferous adluminal compartment are located between elongating/elongated spermatids and Sertoli cells and anchor the spermatids until they complete their differentiation. Several adhesion and associated proteins, such as nectin/afadin, cadherin/catenin, and β -1 integrin/focal adhesion kinase/vinculin, are known to constitute anchoring junction complexes and are crucial for normal spermiogenesis.^{25–28} For example, nectin-2 is known to bind to nectin-3 heterotypically in the plasma membrane of spermatid–Sertoli cells.^{29,30} Alteration of the nectin-2/nectin-3 ratio results in aberrant morphogenesis and positioning of spermatids.^{31–34} In the *Ggnbp2*-null testes, we observed that disfigured spermatids are populated in disorganized seminiferous adluminal epithelium. The seminiferous lumen contains spermatids, likely being sloughed off seminiferous epithelium prematurely. Western blot analyses show that several adhesion and associated protein expression levels in the testes are altered in the absence of *Ggnbp2*, which include increased α -E-catenin, β -catenin, and N-cadherin and decreased afadin, N-cadherin, and nectin-2 protein levels. These data suggest that dysregulation of these adhesion proteins and associated molecules caused by *Ggnbp2* deletion may lead to an imbalance of these anchoring junction complexes and perturb the interactions among spermatid–spermatid and spermatid–Sertoli cells that eventually contribute to the failure of spermatid morphogenesis and differentiation. In this context, future detailed studies of the adhesion and associated protein recycling and turnover in the WT and *Ggnbp2*KO testes, as spermatids differentiate to spermatozoa, should provide a better understanding of GGNBP2-regulated processes during spermatogenesis.

Ggnbp2 is predominately expressed germ cells coinciding with the appearance of spermatocytes during postnatal development. The adhesion and associated proteins that are mainly in Sertoli cells, such as nectin-2, vinculin, integrin- β 1, and focal adhesion kinase,^{29,30,35,36} are not significantly altered by *Ggnbp2* deletion. Moreover, using GATA motif binding factor 4 as a Sertoli cell maker, the number of GATA motif binding factor 4–positive cells appears to be comparable to WT testes. Although the abnormalities of spermatid transformation

observed in *Ggnbp2*-null mutant testes appears to arise from defects in germ cells, we could not completely exclude the possibility of malfunctioned Sertoli cells. Recently, Chen et al²³ reported that sex-determining region Y-box 9 was not detectable by immunofluorescence staining in adult *Ggnbp2*-null testes. SOX9 is known to be necessary for testicular development and Sertoli cell differentiation.³⁷ Therefore, further studies of selective Sertoli and germ cell *Ggnbp2* knockout *in vivo* as well as Sertoli-Sertoli and Sertoli-germ cell interactions *in vitro* should provide a better understanding of the biochemical and cell biological roles of GGNBP2 in regulation of spermiogenesis.

It is likely that a lack of GGNBP2 in germ cells disrupts the integrity of the seminiferous epithelium, which consequently causes defective spermatid differentiation. There is evidence showing that GGNBP2 physically interacts with GGN. GGN is a germ cell-specific protein highly expressed in the testes.^{13,38} Complete deletion of *Ggn* leads to embryonic lethality before implantation. Haploinsufficiency of *Ggn* in mouse pachytene spermatocytes results in an increased incidence of unrepaired DNA double-strand breaks.³⁹ Whether GGNBP2 may affect germ cell meiosis through the interaction with GGN is warranted for further studies.

A spermiogenesis defect is one of the common causes of male infertility in humans, but the etiology is not completely understood.^{6,40,41} Highly conserved GGNBP2 between mice and humans¹⁰ and the specific loss of GGNBP2 function that leads to spermiogenic failure in mice raise the possibility that GGNBP2 may have a similar role in human spermiogenesis. Thus, the *Ggnbp2KO* mouse model provides a basis for further studies to explore the biological processes that are regulated by this protein during the spermiogenic stage of spermatogenesis, and to help us understand the molecular defects that are associated with male infertility. Moreover, *Ggnbp2KO* leads to male-specific infertility without affecting the hypothalamus-pituitary-gonadal axis. The integrity of testicular blood-testis barrier and the numbers of spermatogonia, spermatocytes, and Sertoli cells appear to be unaffected. A selective effect of GGNBP2 on haploid spermatid differentiation without disturbance of the entire spermatogenic processes makes GGNBP2 a candidate for future development of a male nonhormonal contraceptive target.

In summary, we discover that GGNBP2, possibly through its role in regulation of adluminal integrity of germ epithelium, is essential for spermiogenesis. However, the molecular actions of GGNBP2 in the testis remain unclear. Further studies will help us gain more insight into the genetic regulation of spermatogenesis.

Acknowledgment

We thank Robin J. Lawrence for helpful advice in the preparation and editing of the manuscript.

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