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BNC1 is required for maintaining mouse spermatogenesis

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Abstract

Basonuclin (BNC1) is a zinc finger protein expressed primarily in gametogenic cells and proliferative keratinocytes. Our previous work suggested that BNC1 is present in spermatogonia, spermatocytes and spermatids, but absent in the Sertoli cells. BNC1's role in spermatogenesis is unknown. Here we show that BNC1 is required for the maintenance of spermatogenesis. *Bnc1*-null male mice were sub-fertile, losing germ cells progressively with age. The *Bnc1*-null seminiferous epithelia began to degenerate before 8 weeks of age and eventually became Sertoli cell-only. Sperm count and motility also declined with age. Furthermore, *Bnc1* heterozygotes, although fertile, showed a significant drop in sperm count and in testis weight by 24 weeks of age, suggesting a dosage effect of *Bnc1* on testis development. In conclusion, our data demonstrate for the first time BNC1's essential role in maintaining mouse spermatogenesis.

Keywords

testis; male sub-fertility; basonuclin-knock out

Introduction

The testis contains somatic and germ cells. The principal somatic elements (endothelial cells, Leydig cells, Sertoli cells, and myoid cells) provide supportive or regulatory functions to enable germ cell viability, replenishment, and differentiation (McLaren, 2000; de Rooij, 2009). The Sertoli cells, in particular, form the somatic component of the seminiferous epithelium, which is established during embryonic development as solid cords and later reorganized into tubules with a central lumen. At day 13 p.c., the germ cells arrive in the genital ridges, and are called gonocytes at this stage. During the next several days, the gonocytes actively migrate to and establish residence on the basement membrane, where they become known as spermatogonia (de Rooij, 1998; de Rooij and de Boer, 2003). There is evidence indicating that the gonocytes that have moved to the basement membrane have two options for further differentiation (Yoshida et al., 2004). In one scenario, gonocytes

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directly give rise to *Kit*-positive differentiating spermatogonia (cells that lack neurogenin 3 or NGN3). In the second and parallel situation, NGN3-positive undifferentiated spermatogonia are produced from the gonocytes and function as the stem cells. As a result, the first wave of spermatogenesis is driven directly by the gonocytes (NGN3-negative) whereas the subsequent waves are sustained by gonocyte-derived adult stem cells (NGN3-positive). Therefore, defects affecting the second pathway (NGN3+, undifferentiated spermatogonia) would lead to a depletion of stem cells and, consequently, a loss of germ cells over time. The rate of depletion would then depend on the severity of the defect. Although the pathway of differentiation from the NGN3+ spermatogonia to later stages of spermatogenesis remains to be elucidated (Yoshida et al., 2004), several genes are identified as being involved in this process, including *Gfra1* (Hofmann et al., 2005), *Plzf* (Buaas et al., 2004; Costoya et al., 2004), *Taf4b* (Falender et al., 2005), *Ret* (Kubota et al., 2004), and *Kit* (Shinohara et al., 2000).

BNC1 is a zinc finger protein expressed primarily in gametogenic cells and proliferative keratinocytes (Green and Tseng, 2005). BNC1 is a transcription factor with multiple functions. A number of studies suggested that BNC1 functions as a transcription regulator for both RNA polymerase I and II (Pol I & II)(Ma et al., 2006; Tian et al., 2001; Tseng et al., 1999; Wang et al., 2006; Zhang et al., 2007; Zhang and Tseng, 2007). BNC1's function as a Pol I transcription factor is particularly intriguing, because it differs from the known dedicated Pol I transcription factors (e.g., UBF) in two important ways: (i) BNC1 is the first cell-type specific Pol I transcription regulator identified and; (ii) BNC1 appears to regulate a subset of rDNA (Tian et al., 2001; Tseng, 2006; Tseng et al., 2008; Zhang et al., 2007; Zhang and Tseng, 2007). These properties of BNC1 make understanding its role in gametogenesis extremely interesting.

A role of BNC1 in reproduction was first demonstrated by knocking down BNC1 exclusively in the oocytes via a transgenic RNAi technique (Ma et al., 2006). BNC1-deficient oocytes could be fertilized but embryonic development was disrupted at the 2-cell stage. Because BNC1 is not present in the developing embryo, these data indicate that *Bnc1* is a maternal effect gene, of which there are several other mammal examples (Burns et al., 2003; Christians et al., 2000; Payer et al., 2003; Tong et al., 2000). To understand the role of BNC1 in spermatogenesis, we previously investigated its expression in mouse testis (Mahoney et al., 1998). *Bnc1* mRNA is present at high levels in total RNA from both human and mouse testes (Mahoney et al., 1998; Yang et al., 1997). Immunohistochemistry showed that BNC1 protein was present in the nucleus of a small number of cells of postnatal day 4 (P4) mouse seminiferous tubules. The number of BNC1-containing cells increased during neonatal testis development, as differentiated spermatogenic cells appeared. Eventually, BNC1-containing cells were distributed over the entire seminiferous epithelium, encompassing the compartments of spermatogonia, spermatocytes and spermatids. BNC1 was translocated from nucleus to cytoplasm during spermiogenesis and eventually localized in the mid-piece of mature spermatozoa. BNC1 was apparently absent in the Sertoli cells, because BNC1 immunofluorescence did not overlap with that of GATA1 (Mahoney et al., 1998).

The extensive presence of BNC1 in spermatogenic cells raises the question of its function in male fertility. Here we describe a characterization of *Bnc1*-null male mice and we present evidence that BNC1 is critical for maintaining adult spermatogenesis.

Results

Bnc1-null mice are sub-fertile

We tested the fertility of *Bnc1*-knockout (*Bnc1*-null) mice. Nullizygous males and females at 8 weeks of age were paired respectively with wild-type females and males with normal fertility, and the *Bnc1* heterozygous littermates were used as control. During the 3-month testing period the heterozygous control males and females produced a total of 120 pups, 68 from the males and 52 from the females. In contrast, the nullizygous males and females produced 3 and 0 pups, respectively (Table 1). These results demonstrated the degree of sub-fertility of *Bnc1*-null male mice. They also showed that the heterozygotes were fertile, at least in their early puberty. During the production of *Bnc1*-null mice by mating *Bnc1* heterozygotes, we noted a decrease in the number of *Bnc1*-null pups, resulting in a skewed Mendelian ratio of approximately 1.0:1.85:0.56 (Table 2). Thus, BNC1 is critically required for mouse reproduction. The female infertility of *Bnc1*-null mutant mice confirmed our previous study of a BNC1-knock-down model (Ma et al., 2006). Here we focus on characterization of the sub-fertility of *Bnc1*-null male mice.

Bnc1-null testes are smaller due to seminiferous epithelium degeneration

We noted that pubertal testes of *Bnc1*-null males were one-half to one-quarter the size and weight of testes of their normal littermates (Fig. 1A, B). The lower testis weight of *Bnc1*-null male was not due to the loss of overall body weight, which was similar between the wild type littermates and the mutants (Fig. 1C). The perinatal *Bnc1*-null testes were normal in weight and did grow in size, albeit at a slower rate than that of the wild type. This slow growth was halted at around 8 weeks of age and a decline in weight then ensued (Fig. 1B). Significantly, in the *Bnc1* heterozygous males, testis weight never reached the level found in wild type animals, and the weight difference became significant at 24 weeks and beyond (Fig. 1B), suggesting a dosage-effect of BNC1 on testis development. Histological examination revealed a massive cell loss in the *Bnc1*-null mutant testes starting at around 8 weeks of age (Fig. 1D, indicated 22 weeks). Surprisingly, during the course of tubule degeneration, apparently normal tubules with terminally differentiated spermatozoa co-existed with the affected tubules, indicating heterogeneity of seminiferous tubules in response to the *Bnc1*-null mutation. The number of degenerated tubules increased with age and at 1 year of age, virtually all tubules were affected (Fig. 1E). While the testis weight was severely affected by the *Bnc1*-null mutation, the mutant seminal vesicles grew at a near normal rate (Fig. 1F), indicating normal androgen production. We conclude that spermatogenesis, but not steroidogenesis, is affected by *Bnc1*-null mutation.

Bnc1-null adult testes lose germ cells progressively while retaining Sertoli cells

To verify that *Bnc1*-null mutation induced loss of germ cells but not that of Sertoli cells, we examined the control and mutant testes by GCNA and GATA4 immunohistochemistry. GCNA is a marker of mammalian germ cells (spermatogonia, spermatocytes and round spermatids) (Enders and May, 1994) while GATA4 identifies Sertoli cells (Imai et al., 2004). Immunostaining showed that the loss of germ cells could account for the degeneration of seminiferous tubules (Fig. 2A, B) and in the tubules devoid of germ cells, Sertoli cells were still present (Fig. 2C). These results suggest that the *Bnc1*-null mutation impairs the survival of germ cells but not that of Sertoli cells.

Bnc1-null mutation reduces germ cell proliferation

Previously, we showed that *Bnc1*-null mutation impairs keratinocyte proliferation (Zhang and Tseng, 2007). To investigate if *Bnc1*-null mutation similarly affects germ cell proliferation, we pulse labeled proliferative germ cells with BrdU via intraperitoneal

injection and sacrificed the mice 120 min later. Scoring of testicular BrdU-positive cells at various perinatal and pubertal time points (Fig. 3) showed a significant reduction in germ cell proliferation in *Bnc1*-null testes at as early as 2 weeks of age, when histology was normal in the mutant testes. This result confirms our previous observation made in a stratified epithelium (keratinocytes) and further supports the notion that *Bnc1*-null mutation impaired germ cell function.

Sperm count and function decline rapidly in adult *Bnc1*-null mice

The heterogeneity of seminiferous tubules in *Bnc1*-null testes led us to investigate if the apparently normal tubules could still produce normal sperm. At 8 weeks of age, the sperm count and motility of *Bnc1*-null mutants and their control littermates were similar, but thereafter, the mutant sperm count and motility declined rapidly (Fig. 4A, B, C). At 24 weeks of age, the null mutant testes produced virtually no motile sperm. The apparently normal seminal vesicles in the mutant mice (Fig. 1F) suggested that the loss of sperm motility was not due to the alteration of the maturation environment, though this conclusion needs to be verified. Interestingly, *Bnc1* heterozygous males showed a significant decline in sperm count at 24 weeks of age but sperm motility (as measured by computer-assisted semen analysis) was not significantly affected. This observation again suggests a dosage effect of BNC1, with sperm quantity appearing to be more sensitive to the dose change than sperm motility was.

Discussion

Our analysis of *Bnc1*-null mutant demonstrates that BNC1 is critically required for mouse spermatogenesis, because *Bnc1*-null male mice have extremely low fertility. The most striking phenotype of *Bnc1*-null testis is the massive loss of germ cells. We show that in older *Bnc1*-null mutants, the testes have a Sertoli cell-only phenotype (i.e., germ cell lineage is lost), and that *Bnc1*-null mutation reduces germ cell proliferation at a younger age. Adult *Bnc1*-null mutants also have both low sperm count and low sperm motility.

An interesting issue is the relationship of BNC1, PLZF and TAF4b. The three proteins are transcriptional regulators in multiple tissues and the testis phenotypes of their individual null mutation are highly similar (Buaas et al. 2004; Costoya et al. 2004; Falender et al. 2005; this work). A notable similarity is that each individual null mutation affects only adult spermatogonia stem cell (SSC) maintenance and does not appear to perturb the first wave of spermatogenesis. In *Bnc1*-null testes, no histologically discernable change could be detected before the age of 4 weeks and the sperm number and motility also appeared normal. Similar observations were made in null mutants of PLZF and TAF4b. These findings support the notion that the requirements of neonatal SSC development and SSC maintenance in adults are different (Yoshida et al. 2006). The results of others and ours indicated clearly that BNC1, PLZF and TAF4b are required for maintaining adult spermatogenesis. However, mechanistically, their actions in testis appear to differ. BNC1 appears to function as either a positive or negative regulator of Pol II transcription (Wang et al. 2006). Our unpublished results suggest that in testis BNC1 is not a transcription regulator of PLZF or TAF4b, because *Bnc1*-null mutation did not affect the level of PLZF and TAF4b mRNAs. On the other hand, PLZF is a transcription repressor and thought to repress the expression of SCF/*kit* (Filipponi et al. 2007). Loss of PLZF therefore leads to more stem cell differentiation. In testis, the null mutation of TAF4b lowers expression of PLZF and genes involved in retinoic acid signaling and meiosis. The mutation also affects mRNA level of GDNF and GFRA1 (Falender et al. 2005). The testis phenotype of TAF4b null mutation may be partially mediated through impaired PLZF function and TAF4b could be an up-stream regulator of PLZF. This analysis suggests that BNC1's role in spermatogenesis is not upstream to that of PLZF and TAF4b, and may be entirely independent of these two proteins.

A notable phenotype of *Bnc1*-heterozygote (+/-) testes is a drop in sperm counts to about one half the number of the wild-type by 24 weeks of age (Fig. 4A). The sperm count in *Bnc1* +/- was then maintained at this level. This phenotype of fewer sperm in *Bnc1* +/- at 24 weeks of age appears to be consistent with the slight but significantly lower testis weight in this genotype observed at the same time and beyond (Fig. 1B). Interestingly, neither apparent seminiferous tubule degeneration (Fig. 1E), nor deterioration in sperm motility (Fig. 4B, C) was observed in *Bnc1* (+/-) testes at this age, and the *Bnc1* heterozygote mice remain fertile as compared to wild type up to 6 months of age (we do not have data beyond that age). These observations suggest that BNC1's role in some processes may be more dose-sensitive than others. It is possible that later in life, *Bnc1* +/- mice have an accelerated aging phenotype as reflected in an earlier decline of male fertility. This possibility warrants further investigation.

BNC1 is unique in that it has been shown to be a cell-type specific regulator of rRNA synthesis. Furthermore, BNC1 appears to regulate a subset of rDNA (Tian et al., 2001; Tseng et al., 2008; Zhang et al., 2007; Zhang and Tseng, 2007). This BNC1 function and its critical requirement for spermatogenesis suggest a yet unrecognized specific role of rRNA in mammalian testis physiology. This notion is supported by at least two pieces of circumstantial evidence. First, it has been shown that mitochondria rRNAs are a common component of germinal granules in Drosophila and Xenopus (Kobayashi et al., 1998). In Drosophila, large and small mitochondria rRNAs were shown to be transported out of mitochondria to the polar granules. These mitochondrial rRNAs were identified as an inducing factor for pole cell development (Kobayashi and Okada, 1989) and inhibition of mitochondria rRNA in germ plasm by anti-mitochondria rRNA ribozymes led to failure of formation of pole cells (Iida and Kobayashi, 1998). These observations suggested a special requirement of rRNA or ribosomes in germ cell development. Second, we showed that mouse rDNA contains multiple subtypes of variants (Tseng et al., 2008). We identified, by single nucleotide polymorphism, seven rDNA variants (v-rDNA). Most tissues expressed two to three of these variants. However, in the testis, as well as in the brain, two additional variants were expressed. Future study should focus on investigating the role of the additional v-rDNAs in spermatogenesis and if *Bnc1*-null mutation affects their expression.

To summarize, we present evidence to support the notion that BNC1 plays an essential role in maintaining mouse spermatogenesis. *Bnc1* deletion leads to male sub-fertility, which is due to germ cell loss. BNC1 may have additional roles in spermatogenesis, and these roles have different *Bnc1* dose requirement.

Materials and Methods

Animals

Bnc1-null mice were generated as previously described (Zhang and Tseng, 2007). Protocols for the use of mice in these experiments were approved by the University of Pennsylvania Animal Care and Use Committee and in accordance with National Institutes of Health standards established by the Guidelines for the Care and Use of Experimental Animals. Mice were housed in a standard animal facility with free access to food and water. Body weight was recorded just before euthanizing each mouse. Weights of seminal vesicles and testes were measured right after tissue collection.

Fertility assays

Five *Bnc1*-null females were mated with the same two C57BL/6J males in a random sequence. The same males were also used to cross with the seven *Bnc1* (+/-) females. During the same period, six *Bnc1*-null males were mated with the same seven C57BL/6J

females in a random sequence. The same females were also used to cross with the four *Bnc1* (+/-) males. The numbers of pups delivered per litter were recorded to assess the fertility.

Histology, immunohistochemistry, and BrdU assays

Mice testes were fixed in Bouin's fixative immediately after euthanasia. Five-micron paraffin sections were stained with hematoxylin and eosin (H&E) for histological analysis. For immunohistochemistry, paraffin sections were de-paraffinized, re-hydrated and then washed in PBS. After antigen retrieval treatment, sections were blocked with 5% normal goat serum and incubated with primary antibody at 4°C overnight. The antibodies used are as follows: anti-GATA4 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GCNA1 (1:50, kindly provided by G.C. Enders, University of Kansas, Kansas City, Kansas). The next day, slides were incubated with a biotinylated secondary antibody, ABC complex, DAB substrate (Vector Laboratories, Burlingame, CA) sequentially. Slides were photographed using a Nikon Eclipse TE2000-U microscope and a Nikon D1 camera. Pictures from a whole section were merged into a single image by Adobe Photoshop. The BrdU assay was performed as previously described (Zhang and Tseng, 2007). The number of positive cells per seminiferous tubule or positive tubules per section was counted according to (Buaas et al., 2004; Costoya et al., 2004).

Sperm counts and motility

Caudal epididymal sperm were collected into Modified Whitten's medium. After 5 minutes, adequate dispersion of the sperm was determined visually, and an aliquot was diluted 1:20 in the same medium, which was placed on a pre-warmed counting chamber slide (100 um in depth) (Cell Vision, Hopedale, MA) and analyzed by computer-assisted semen analysis (CASA) on a Hamilton-Thorne IVOS V12.2L analyzer (Hamilton Thorne Research, Danvers, MA). Five randomly chosen fields were analyzed. Motility (%) and curvilinear velocity (VCL, um/sec) were recorded. For total sperm counts, two aliquots of sperm were diluted 1:200 in water for immobilization and put into a hemocytometer for counting. Afterwards, the average of sperm concentration was calculated.

Statistics

Standard *t*-tests (Excel, Microsoft, Redmond, WA) were used and standard error of means were calculated as appropriate. Statistical significance was defined as $p<0.05$.

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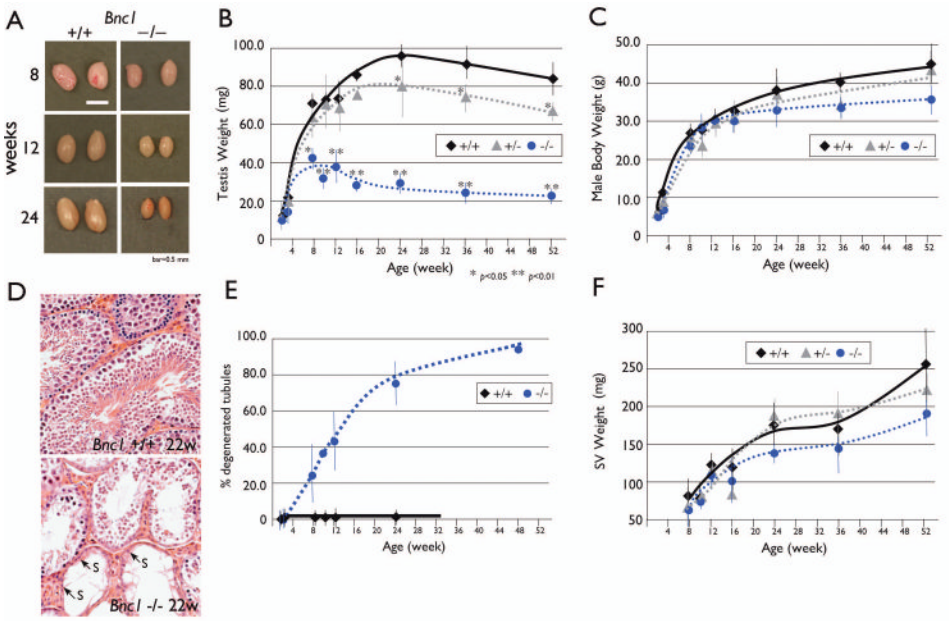

Figure 1.

Bnc1-null males have smaller testis and degenerating seminiferous tubules. **A**, *Bnc1*-null testes appeared smaller than that of the control. Testes were collected and photographed at the specified ages. A ruler (not shown) was photographed with each testis sample and used to maintain a uniform magnification during construction of the figure. **B**, *Bnc1*-null testes failed to increase in weight compared to the control during neonatal period and never reached the weight of the control testis. **C**, *Bnc1*-null mice and their normal litter mates were weighed before sampling testis. No significant body weight difference was seen between the *Bnc1*-null mice and the normal litter mates. **D**, *Bnc1*-null testes contained degenerating seminiferous tubules. Histology (H&E) of mutant testis showed Sertoli cells were still present (S, arrows) when all the germ cells appeared to be lost. **E**, In *Bnc1*-null testes, the number of degenerating tubules, defined as tubules that lacked spermatogonia, increased steadily after birth. **F**, The weight of seminal vesicle of three *Bnc1* genotypes (+/+, +/-, -/-) were measured, no significant difference was observed.

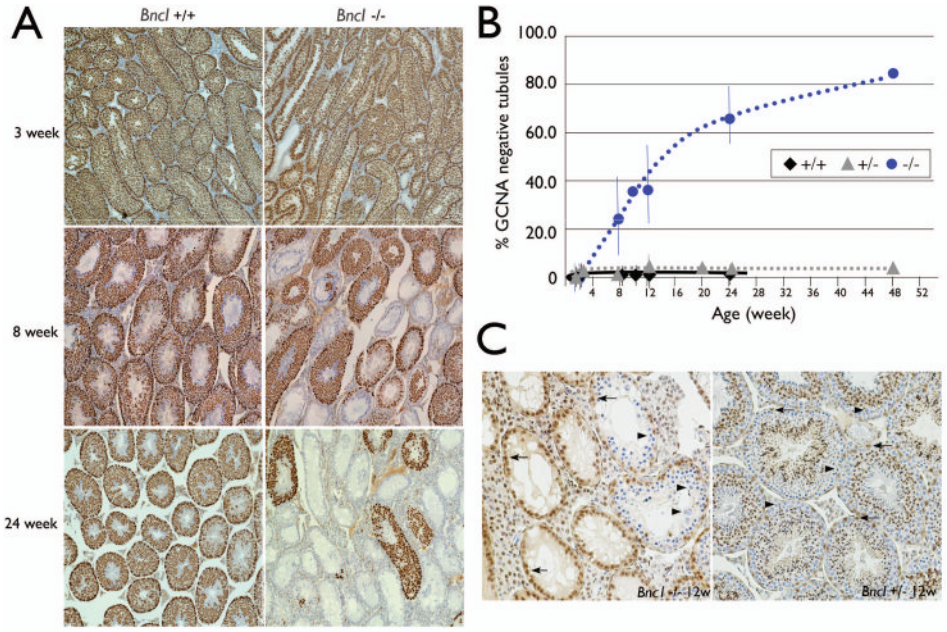

Figure 2.

Bnc1-null germ cells are lost at a rapid rate in adult testes. **A**, Germ cells were monitored by GCNA antigen immunohistochemistry at the indicated time in littermate control (left panels) and *Bnc1*-null testes (right panels). **B**, The number of GCNA-negative tubules per section was scored and the average percentage was plotted against the age of the mice. GCNA-positive tubules were classified as containing one or more GCNA-positive cells within the section. n=2 for 2-week, 3-week and 24-week; n=3 for 8-week, and 12-week; n=1 for 52-week. **C**, Sertoli cells were still present in *Bnc1*-null testis. Sertoli cells were identified by the presence of GATA4 antigen immunohistochemistry (arrows show examples of GATA4 positive cells and arrow heads that of the residual germ cells).

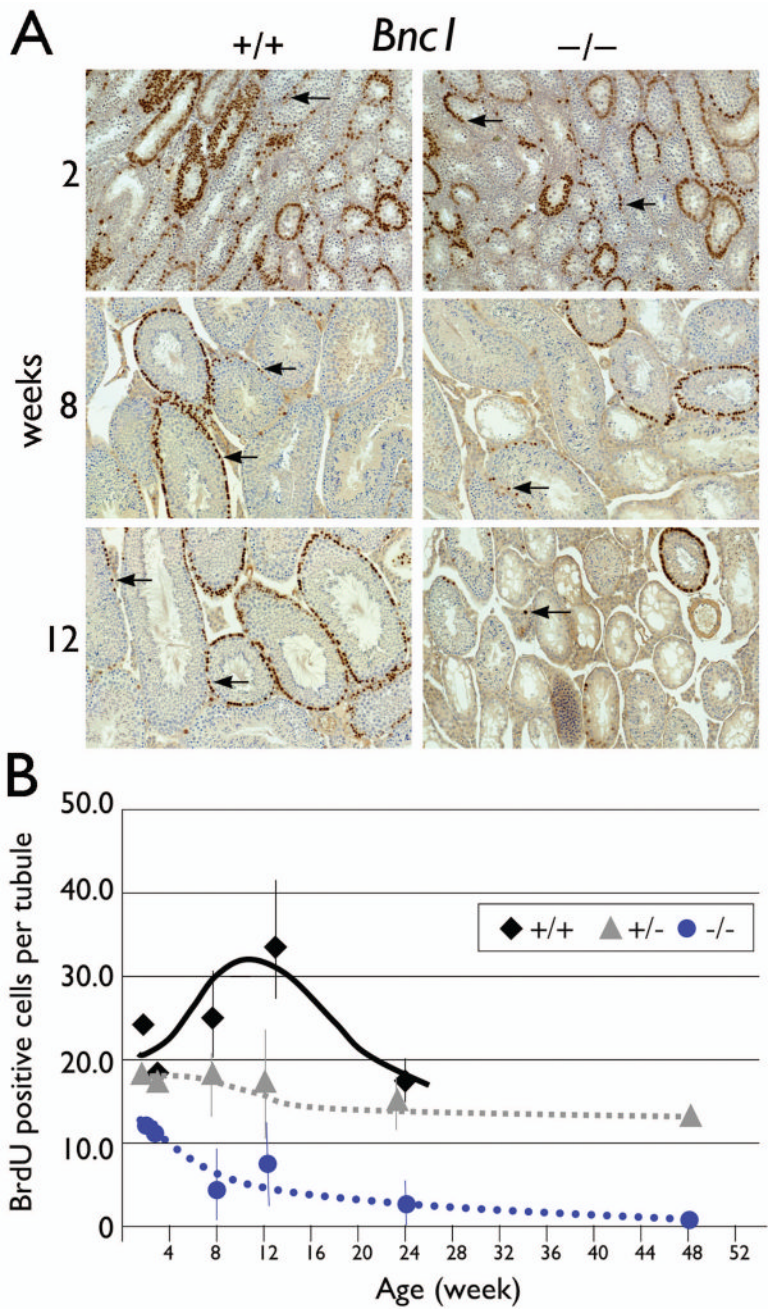

Figure 3.

Bnc1-null mutation impairs germ cell proliferation. **A**, Germ cell proliferation was assessed by BrdU labeling at the indicated ages (listed to the left of the panels). Shown are the littermates (left panels) and *Bnc1*-null mutant (right panels) at the same age. Arrows show examples of BrdU-positive cells. **B**, The number of BrdU-positive cells was scored for the control and the mutant and expressed as the average of BrdU-positive cell per tubule. At least 40 tubules were scored on each section. n=1 for 2- week, 3-week and 48-week; n=2 for 8-week, 12-week and 24-week.

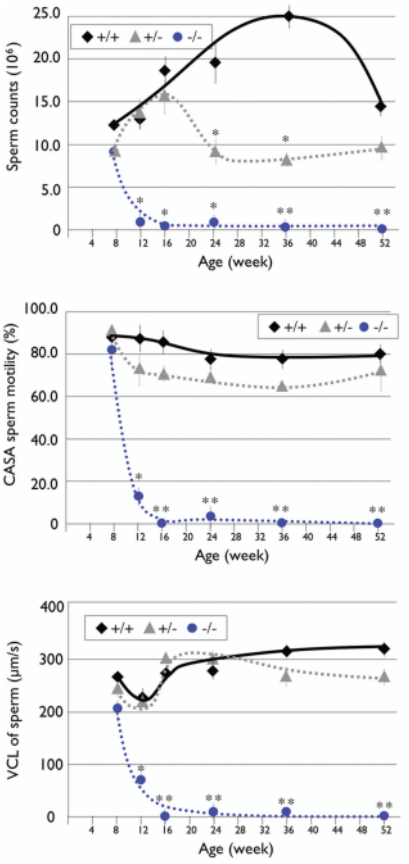

Figure 4.

Bnc1-null mutation impairs spermatogenesis both in quantity and in quality. **A**, Sperm were produced in similar quantity in *Bnc1*-null male and the littermate control at the puberty. Sperm numbers reduced precipitously in *Bnc1*-null mice older than 8 weeks. **B**, The motility of *Bnc1*-null sperm appeared similar to the control at 8 weeks of age, but deteriorated rapidly thereafter. **C**, VCL of sperm of *Bnc1*-null mice also dropped quickly beginning at 12-week-old. n≥3 for all time points.

Table 1

Fertility of *Bnc1*-null male and female.

Genotype	Gender	Total litters	Total pups	pups/mouse
+/-	4 males	10	68	17
	7 females	7	52	7
-/-	6 males	1	3	0.5
	5 females	0	0	0

Mice of various *Bnc1* genotypes on a mixed 129Sv/C57BL/6J were mated with wild type mice (C57BL/6J) of normal fertility during a 3-month period (age 8-12 weeks for both genotype).

Table 2

Genotype distribution of progeny of *Bnc1* heterozygote parents.

	Total litters	**Total pups**	**+/+ (1.0)**	**+/- (1.85)**	**-/- (0.56)**
mixed	52	344	101	189	54
C57BL6	8	62	18	31	13

+/+, +/- and -/- are *Bnc1* genotypes.