BCAS2 is involved in alternative mRNA splicing in spermatogonia and the transition to meiosis

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Breast cancer amplified sequence 2 (BCAS2) is involved in multiple biological processes, including pre-mRNA splicing. However, the physiological roles of BCAS2 are still largely unclear. Here we report that BCAS2 is specifically enriched in spermatogonia of mouse testes. Conditional disruption of Bcas2 in male germ cells impairs spermatogenesis and leads to male mouse infertility. Although the spermatogonia appear grossly normal, spermatocytes in meiosis prophase I and meiosis events (recombination and synapsis) are rarely observed in the BCAS2-depleted testis. In BCAS2 null testis, 245 genes are altered in alternative splicing forms; at least three spermatogenesis-related genes (Dazl, Ehmt2 and Hmga1) can be verified. In addition, disruption of Bcas2 results in a significant decrease of the full-length form and an increase of the short form (lacking exon 8) of DAZL protein. Altogether, our results suggest that BCAS2 regulates alternative splicing in spermatogonia and the transition to meiosis initiation, and male fertility.
Alternative pre-mRNA splicing is critical for post-transcriptional regulation of gene expression, during which particular exons from the same pre-mRNA might be excluded, included or modified to produce multiple mature mRNAs, often in an organ-, tissue- or cell-type-specific manner. Thus, alternative splicing significantly expands the form and function of the genome of organisms with limited gene number and is especially important for highly complex organisms and tissues. Highly complex tissues, such as the testis and brain, have more gene splicing variants than any other tissues. In mouse testis, spermatogenesis is a complex process involving mictotic cell division, meiosis and spermiogenesis to give rise to haploid spermatozoa. Alternative splicing variants, especially exon-skipping forms, are enriched in several stages of mouse spermatogenesis. In addition, a number of trans-acting regulators of pre-mRNA splicing are primarily or exclusively expressed in the testis.

Substantial evidence suggests that pre-mRNA splicing is an important regulator of mouse spermatogenesis. Although the roles of most spliced forms of specific genes during this process are unclear, several genes important for spermatogenesis have specific splice variants in different developmental stages. For example, c-kit is specifically expressed in differentiating spermatogonia and is essential for the survival and proliferation of pre-meiotic germ cells. However, the truncated form of c-kit (tr-kit) is expressed in the post-meiotic stages of spermatogenesis and enriched in spermatozoa, and could trigger parthenogenesis activation when injected into the cytoplasm of MII oocytes. In addition, numerous trans-acting regulators of pre-mRNA splicing are important for spermatogenesis. For example, Ptbp2, a key alternative-splicing regulator in the nervous system, is critical for male germ cell survival and male fertility through regulating the proper alternative splicing of germ cell messenger RNAs (mRNA) in the testis. Rbm5, a male germ cell splicing factor, is essential for the appropriate alternative splicing of pre-mRNAs involved in spermatid differentiation. Ranbp9 (Ran-binding protein 9) is also involved in regulating the proper splicing pattern of some spermatogenic mRNAs by interacting with several essential splicing factors (e.g., SF3B3 and HNRNPM) and poly (A) binding proteins (PABPs). Despite protracted effort, deciphering how alternative pre-mRNA splicing functions during spermatogenesis remains a great challenge for the field.

Breast carcinoma amplified sequence 2 (BCAS2) is preferentially expressed in the testis. Subsequent studies reveal that BCAS2 is a core component of the CDC5L/Prp19 complex. The Prp19 complex is highly conserved and is involved in the assembly and conformation of the spliceosome, especially important for the catalytic activation of the spliceosome. Mutation of the yeast BCAS2 ortholog Cwf7 or Snt309 results in the accumulation of pre-mRNA. In Drosophila melanogaster, BCAS2 is essential for viability and may function in pre-mRNA splicing. BCAS2 has also been shown to be involved in DNA repair through the replication protein A (RPA) complex in various cell lines. Recently, we found that maternal BCAS2 responds to endogenous and exogenous DNA damage in mouse zygotes and maintains the genomic integrity of mouse early embryos through RPA. Thus, the roles of BCAS2 are context-specific depending on the model system. Currently, the physiological function of BCAS2 is still largely unclear.

In this study, we found that BCAS2 was comparatively enriched in spermatogonia of the mouse testis. Disruption of BCAS2 in germ cells with Vasa-Cre led to male infertility, but has little effect on spermatogonia. Although the spermatogonia were grossly normal, spermatocytes in meiosis prophase I were scarce and meiosis events did not occur in the BCAS2-depleted testis. We further showed that BCAS2 was involved in pre-mRNA splicing in spermatogonia in the mouse testis. Our data reveal a critical role of BCAS2 involving in pre-mRNA splicing of spermatogonia and the transition to meiosis, and male fertility.

**Results**

**The expression of BCAS2 in mouse testes.** To explore the potential function of BCAS2 in mouse spermatogenesis, we first examined the expression of BCAS2 in the testis by immunostaining with rabbit anti-BCAS2 antibody. BCAS2 was expressed in the nucleus of both germ cells and somatic cells during tests development (Fig. 1a). Interestingly, in embryonic day 15.5 (E15.5) and newborn mouse testes, BCAS2 expression was relatively high in the prospermatogonia located in the centre of the seminiferous tubules of the testes. At postnatal day 5 and 14 (P5 and P14), BCAS2 was enriched in certain cells located in the basement membrane (Fig. 1a).

**Figure 1** | Expression of BCAS2 in male mouse germ cells. (a) Immunofluorescence (IF) staining of BCAS2 in the paraffin sections of testes from E15.5 to P14 mice. The DNA was stained with Hoechst 33342. Scale bar, 50 μm. (b) Real-time PCR analysis of Bcas2 expression in the fraction of spermatogenic cells (FSPCs) and the fraction of somatic cells (FSCs) enriched from P9 testes. Gapdh was used as the internal control for normalization (n = 4). Error bars represent s.e.m. (c) Western blotting analysis of BCAS2 expression in the fraction of spermatogenic cells (FSPCs) and the fraction of somatic cells (FSCs) enriched from P9 testes. Germ cell markers (DAZL and MVH) were used as the indicator of the enrichment efficiency and α-tubulin was used as the loading control. (d) Paraffin sections of P8 testes were co-stained with rabbit anti-BCAS2 and mouse anti-PLZF antibodies. The DNA was stained with Hoechst 33342. Scale bar, 20 μm.
BCAS2 is essential for male fertility and spermatogenesis.

To investigate the function of Bcas2 in spermatogenesis, Bcas2 was specifically deleted from mouse germ cells by crossing Bcas2Fl/Fl (Bcas2F/F) mice30 with Vasa-Cre transgenic mice in which the recombinase is specifically active in germ cells as early as P8 in the spermatogonia. Immunofluorescence results revealed that the number of germ cells marked by MVH was dramatically lower in Bcas2F/F;Vasa-Cre male mice. (Fig. 2d). Thus, we successfully established male germ cell-specific knockout mice for Bcas2, as early as P8 in the spermatogonia.

BCas2F/F;Vasa-Cre male mice developed to form grossly normal adults. Although copulatory plugs were routinely observed, no pups were obtained when adult Bcas2F/F;Vasa-Cre males mated with normal fertile females (Table 1). Compared with controls, the testes of adult (more than two-month-old) and one-month-old Bcas2F/F;Vasa-Cre males were much smaller (Fig. 2c; Supplementary Fig. 2a) and the testis weight was significantly lower (Fig. 2f; Supplementary Fig. 2b). We next analysed the histology of the testes from the adult and one-month-old males by hematoxylin and eosin (H&E) staining. The seminiferous tubules in the control testes contained a basal population of spermatogonia, several types of spermatocytes and spermatids. However, germ cells were severely reduced in Bcas2F/F;Vasa-Cre testes, with only a few MVH-positive cells around the basement membrane. The number of both Ki67−/Vasa-Cre male mice developed to form grossly normal adults. Although copulatory plugs were routinely observed, no pups were obtained when adult Bcas2F/F;Vasa-Cre males mated with normal fertile females (Table 1). Compared with controls, the testes of adult (more than two-month-old) and one-month-old Bcas2F/F;Vasa-Cre males were much smaller (Fig. 2c; Supplementary Fig. 2a) and the testis weight was significantly lower (Fig. 2f; Supplementary Fig. 2b). We next analysed the histology of the testes from the adult and one-month-old males by hematoxylin and eosin (H&E) staining. The seminiferous tubules in the control testes contained a basal population of spermatogonia, several types of spermatocytes and spermatids. However, germ cells were severely reduced in Bcas2F/F;Vasa-Cre testes, with only a few MVH-positive cells around the basement membrane. The number of both Ki67−/Vasa-Cre male mice developed to form grossly normal adults. Although copulatory plugs were routinely observed, no pups were obtained when adult Bcas2F/F;Vasa-Cre males mated with normal fertile females (Table 1). Compared with controls, the testes of adult (more than two-month-old) and one-month-old Bcas2F/F;Vasa-Cre males were much smaller (Fig. 2c; Supplementary Fig. 2a) and the testis weight was significantly lower (Fig. 2f; Supplementary Fig. 2b). We next analysed the histology of the testes from the adult and one-month-old males by hematoxylin and eosin (H&E) staining. The seminiferous tubules in the control testes contained a basal population of spermatogonia, several types of spermatocytes and spermatids. However, germ cells were severely reduced in Bcas2F/F;Vasa-Cre testes, with only a few MVH-positive cells around the seminiferous tubules in the testes of P9 mice based on that somatic cells are mainly attached to the bottom of the culture plate, while spermatogenic cells are enriched in the suspended cells31. Real-time RT-PCR and flow cytometry analysis using germ cell and somatic cell markers showed that the enrichment was efficient (Supplementary Fig. 1a,b). Next, we examined the expression of Bcas2 in these two types of cells using real-time RT-PCR and Western blotting. Our results showed that both mRNA and protein levels of Bcas2 were relatively high in the fraction of spermatogenic cells (FSPCs) (Fig. 1b,c). To further investigate the identity of the BCAS2-enriched cells, we co-stained BCAS2 with the spermatogonia-specific transcription factor PLZF (Promyelocytic leukemia zinc finger) in P8 mouse testes. The expression of BCAS2 was comparatively decreased in PLZF-positive cells (Fig. 1d).

Spermatogonia appear grossly normal in Bcas2 null males.

The MVH positive cells in the basement membrane persisted in the P15 Bcas2F/F;Vasa-Cre testes, suggesting that the spermatogonia might be normally persistent in these mice (Fig. 3b). To reduce the possibility of an indirect defect of BCAS2 loss on spermatogonia, we stained PLZF with a specific antibody in sections from early developmental testes at P12. The distribution of PLZF-positive cells was similar in Bcas2F/F;Vasa-Cre testes and the control (Fig. 4a). Furthermore, the average number of PLZF-positive cells per seminiferous tubule cross-section in the Bcas2F/F;Vasa-Cre testes was not significantly different from the control testes (Fig. 4b). These data suggest that the location and number of spermatogonia are not obviously affected in Bcas2F/F;Vasa-Cre testes.
positive cells in the basement membrane were observed in the BCAS2 null spermatogonia. PLZF was co-stained to indicate the location of spermatogonia. The DNA was stained with Hoechst 33,342. Scale bar, and Bcas2F/

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normal in (Fig. 4c,d), suggesting that the proliferation of spermatogonia was

of BCAS2 protein in control and Bcas2F/–;Vasa-Cre testes of P8 mice. MVH and α-tubulin was used as a germ cell marker and loading control, respectively. (c) Relative abundances of BCAS2 in the control and Bcas2F/–;Vasa-Cre testes of P8 mice were determined by Western blotting analyses from 4 independent experiments. Error bars represent s.e.m. (d) IF staining of BCAS2 in the control and Bcas2F/–;Vasa-Cre testes of P8 mice. White circles denote the BCAS2 null spermatagonia. PLZF was co-stained to indicate the location of spermatogonia. The DNA was stained with Hoechst 33,342. Scale bar, 20 μm. (e) Morphological analysis of adult testes showed that the Bcas2F/–;Vasa-Cre testes were smaller than the control. (f) Testes weight of adult control and Bcas2F/–;Vasa-Cre mice (**P<0.001, n = 5). Error bars represent s.e.m. (g) Hematoxylin and eosin (H&E) staining of adult testes in control and Bcas2F/–;Vasa-Cre mice. Spermatocytes and spermatids were almost absent in the seminiferous tubules of the Bcas2F/–;Vasa-Cre mice. Scale bar, 100 μm. (h) IF staining of MVH (a germ cell marker) in adult testes in control and Bcas2F/–;Vasa-Cre mice. Compared with the control, only a few MVH positive cells in the basement membrane were observed in Bcas2F/–;Vasa-Cre testes. The DNA was stained with Hoechst 33342. Scale bar, 50 μm.

Table 1 | The fertility of Bcas2F/–;Vasa-Cre males.

| Genotype          | No. of male mice | No. of plugged female mice | No. of litters | No. of pups per litter |
|-------------------|------------------|----------------------------|----------------|------------------------|
| Male              | Female           |                            |                |                        |
| Control           | Wt               | 5                          | 24             | 23                     | 11.92 ± 0.5                |
| Bcas2F/–;V-Cre   | Wt               | 5                          | 23             | 0                      | 0                        |

(Fig. 4c,d), suggesting that the proliferation of spermatogonia was normal in Bcas2F/–;Vasa-Cre testes. To analyse the apoptosis of spermatogonia, we performed double staining of PLZF and cleaved caspase 3 (CAP3) in Bcas2F/–;Vasa-Cre testes. Compared with the control, the number of CAP3+ PLZF+ spermatogonia was not significantly higher in Bcas2F/–;Vasa-Cre testes (Fig. 4e,f), implying that the spermatogonia did not undergo apoptosis.

Antibodies to the MVH protein label all stages of spermatogonia and spermatocytes38, and PLZF is specifically expressed in the undifferentiated spermatogonia37. In the literature, spermatogonia expressing only MVH but not PLZF (MVH+PLZF−) around the basement membrane of seminiferous tubules in the testis are regarded as differentiating spermatogonia38. Thus, we simultaneously determined the expression of PLZF and MVH in the cells around the basement membrane of seminiferous tubules in P10 testes of control and Bcas2F/–;Vasa-Cre mice. Compared with the control, the number of all stages of germ cells (MVH+) around the basement membrane and differentiating spermatogonia (MVH+PLZF−) was not significantly different in Bcas2F/–;Vasa-Cre testes (Supplementary Fig. 4a,b). Taken together, these data suggest that the spermatogonia are almost unaffected in Bcas2F/–;Vasa-Cre mice.

BCAS2 modulates pre-mRNA splicing in spermatogenesis. To investigate the molecular consequences of BCAS2 depletion in germ cells, we isolated mRNA from control and Bcas2F/–;Vasa-Cre testes at P9 and performed RNA sequencing. A total of


**Figure 3 | Spermatogenesis could not progress into meiosis in Bcas2f⁻/⁻;Vasa-Cre testes.** (a) Western blotting analysis of MVH in control and Bcas2f⁻/⁻;Vasa-Cre testes at P10, P12 and P15. *α*-tubulin was used as the loading control. (b) IF staining of MVH in control and Bcas2f⁻/⁻;Vasa-Cre testes at P10, P12 and P15. The DNA was stained with Hoechst 33342. Scale bar, 50 μm. (c) Western blotting analyses of SCP3 and γH2AX in control and Bcas2f⁻/⁻;Vasa-Cre testes at P10 and P12. *α*-tubulin was used as the loading control. (d-f) Hematoxylin and eosin (H&E) staining of control (d-f) at P10, P12 and P15. Spermatogenic cells were shown in cross-sections of seminiferous tubules from control and Bcas2f⁻/⁻;Vasa-Cre testes. Scale bar, 15 μm. Black arrows indicate the representative stages of the spermatocytes. L, leptotene; eP, early pachytene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; PL, pre-leptotene spermatocytes; red arrows, apoptotic cells. (*P<0.05; **P<0.01; ***P<0.001, n = 5). Error bars represent s.e.m.
BCAS2 regulates mRNA splicing of functional genes. From the 279 significantly affected AS events, 245 genes were identified and further analysed for Gene ontology (GO) term enrichment using the DAVID (Database for Annotation, Visualization and Integrated Discovery) software program (Supplementary Table 4). This analysis revealed that 11 genes were involved in sexual reproduction, including 6 genes (Hsf1, Dazl, Cit, Ehmt2, Hmga1 and Bcl2l11) related to spermatogenesis (Fig. 5d). We analysed AS changes in these six genes according to the splicing site predicted by ASD software using Integrative Genomics Viewer (IGV), a visualization tool for efficient and flexible exploration of the large and complicated data sets obtained from sequencing. Dazl, Hsf1 and Ehmt2 were within the top statistically significant events for the skipped exon category. Hmga1 and Bcl2l11 belonged to the alternative first exon category, and Cit was categorized to alternative 5’ splice sites. In addition, intron 7 of Dazl was significantly retained in Bcas2F−/−;Vasa-Cre testes (Fig. 6a).

We next verified the aberrant splicing of these six genes by semi-quantitative and real-time RT-PCR with specific primers of target genes. We successfully verified the aberrant splicing patterns in Dazl, Ehmt2 and Hmga1 in Bcas2F−/−;Vasa-Cre testes (Fig. 6b,c). These data suggest that BCAS2 is involved in pre-mRNA splicing of functional genes during mouse spermatogenesis.

Depletion of BCAS2 results in decreases in DAZL protein. Dazl, encoding a RNA binding protein, is an essential gene for germ cell survival and serves as the intrinsic meiosis-promoting factor during meiosis initiation. Strikingly, Bcas2 depletion led to an obvious switch from the Dazl-FL to the Dazl-Δ8 isoform and retained intron 7 in Bcas2F−/−;Vasa-Cre testes (Fig. 6b,c). The dramatically altered splicing of Dazl and the similar phenotypes...
between Bcas2F/–;Vasa-Cre and Dazl null mice prompted us to investigate the expression of DAZL protein in Bcas2F/–;Vasa-Cre testes. Consistent with their mRNA, the proteins of the two isoforms were present in normal testes. The expression of DAZL-FL was much stronger than the expression of the shorter Dazl-D8. Compared with the controls, the expression of DAZL-FL was dramatically reduced in Bcas2F/–;Vasa-Cre testes, while DAZL-D8 expression was upregulated in Bcas2F/–;Vasa-Cre testes (Fig. 6d). The DAZL-FL protein was reduced to B90% in Bcas2F/–;Vasa-Cre testes at P9 and P12 (Fig. 6e). Meanwhile, DAZL total protein (including DAZL-FL and DAZL-D8) was much lower in the Bcas2F/–;Vasa-Cre testes at P9 and P12 mice compared with the control (Fig. 6f). Thus, these results suggest that BCAS2 may be involved in the splicing of Dazl, a germ cell intrinsic factor promoting meiosis initiation.

Discussion

The alternative pre-mRNA splicing process is implemented by a synergism of the spliceosome, cis-acting RNA elements and specific trans-acting factors47,48. The spliceosome is a large and dynamic RNA–protein complex that consists of five small nuclear ribonucleoprotein particles (snRNPs) (U1, U2, U4, U5 and U6) and several hundred proteins that are critical for the recognition of splice sites, assembly of specific stages of the spliceosome and catalytic activity of the splicing reaction49. During the mitotic-to-meiotic transition in mouse spermatogenesis, the expression of many key alternative splicing regulators is stage-specific9. In addition, proteomic analyses have shown that 58 RNA splicing proteins, including spliceosome components U1–U6 snRNPs-related proteins and many splicing factors, were more highly expressed in type A spermatogonia and pachytene spermatocyte cell clusters50. Consistently, the patterns of alternative splicing are

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Figure 5 | Global analysis of Bcas2-mediated alternative splicing (AS). (a) Scatter plot of significantly differentially expressed transcripts in Bcas2F/–;Vasa-Cre testes compared with the controls. Blue dots represent significantly down-regulated transcripts, while red dots show significantly up-regulated transcripts (P < 0.05, fold-change of RPKM > 1.5). Grey dots illustrated unchanged transcripts. (b) Real-time RT-PCR analysis of Pre-mRNA and mature (m) mRNA expression level of Tuba3a, Tuba3b and Tubb4b in control and Bcas2F/–;Vasa-Cre testes of P9 mice with Gapdh as the internal control (*P < 0.05, n = 5). Error bars represent s.e.m. The pre-mRNA primers were designed in one of the exons and the adjacent intron and the mature mRNA primers were designed to span an exon-exon junction. (c) Seven AS events significantly affected by depletion of BCAS2 in the testes at P9. The simple diagrams of seven AS events recognized by ASD software and splicing events affected by depletion of BCAS2 analysing the RNA-seq data using ASD software (P < 0.05). (d) GO term enrichment analysis of genes with significantly affected AS events.
substantially changed in a stage-specific manner during this process. The stage-enriched expression of splicing proteins and the substantial changes in alternative splicing patterns around meiosis suggest that alternative splicing might be critical for the mitosis-to-meiotic transition during mouse spermatogenesis.

As a core component of the splicing-related Prp19 complex, direct interaction of BCAS2 with PRP19 and the spliceosome is important for spliceosome assembly in yeast. BCAS2 is universally expressed in organisms, and conventional disruption of BCAS2 results in early embryonic lethality. The early embryonic lethality and the complexity of cell types at this stage impede the investigation of BCAS2 in detail. In this study, BCAS2 was comparatively enriched in mouse spermatogonia, prompting us to investigate the role of BCAS2 in mouse spermatogenesis. Using a conditional knockout mouse model, we demonstrated that specific deletion of BCAS2 in male germ cells leads to a failure of spermatogenesis and male infertility. In BCAS2-depleted testes, meiosis failed to initiate and very few meiotic prophase spermatoocytes were observed. However, spermatogonia appeared to be grossly normal in BCAS2-depleted male mice. These results suggest that the major defect is most likely in the mitosis-to-meiosis transition during spermatogenesis in BCAS2-depleted male mice. Thus, BCAS2 is a model for investigating the role of BCAS2 involved in splicing in the mitosis-to-meiosis transition of mouse spermatogonia.
The switch from mitosis to meiosis is a critical step of germ cell development that requires Dazl, the ‘meiosis promoting factor’. RNA-binding protein DAZL can target many crucial mRNAs (such as Mvh, Syce3, Tpx-1 and Tdx19.1) and regulate the translation of these target genes during germ cell development. Although the role of its variants is still unclear, Dazl-FL and Dazl-A8 variants have been identified in ES cells. Here, we found that the level of DAZL-FL protein is much higher than DAZL-A8 in normal mouse testes, suggesting that DAZL-FL might be the main functional form during spermatogenesis. Deletion of Bcas2 leads to dramatic skipping of exon 8 and significant retardation of intron 7 in Dazl, suggesting a critical role for the precise control of Dazl splicing during normal spermatogenesis. Although the roles of Dazl-A8 with skipped exon 8 and retention of intron 7 are unknown, in fact, we observed a dramatic decrease in DAZL-FL protein and a significant reduction in total DAZL protein, suggesting that deletion of Bcas2 leads to a significant degree of loss-of-function of DAZL. Although spermatogonia remain in the seminiferous tubules of Bcas2−/−Vasa-Cre testes, only a few spermatocytes are observed that do not move over the pachytene stage, which is consistent with observations in Dazl null testes. In addition, the protein levels of SCP3, MVH and STRA8, which are targets of DAZL, are dramatically lower in the testes of Bcas2−/−Vasa-Cre males. Consistently, the meiotic events (recombination and synapsis) do not occur in Bcas2−/−Vasa-Cre testes and the expression of marker genes of meiotic prophase is significantly reduced as early as P9. These observations suggest that Bcas2 might be critical for meiotic initiation via regulating Dazl splicing. We also find that Bcas2 depletion affects splicing of several hundred genes. The affected genes include genes of the functional class ‘RNA processing’, ‘chromosome organization’ and ‘regulation of transcription’. Importantly, several verified genes are grouped in the functional class ‘sexual reproduction’. Many of these genes play important roles in mouse spermatogenesis. Ehmt2 (also known as G9a) is an important mammalian H3K9 methyltransferase and is essential for mouse embryogenesis by transcriptional silencing. G9a deficient male germ cells are arrested at the early pachytene stage of meiosis prophase with disordered progression of synaptonemal complex formation. Hmgal (the high mobility group A1) is critical for determining chromatin structure and is involved in transcriptional regulation. The tubules of Hmgal−/− male mice are already apparently devoid of spermatocytes and no other stages of spermatooza and spermatids. Thus, abnormalities in Ehmt2 and Hmgal splicing regulated by Bcas2 may play a role in both male fertility and growth retardation of the germ cells. In this study, we also found that the abundance of mature tuba3a, tuba3b and tubb4b mRNAs was lower in the Bcas2−/−;Vasa-Cre testes. Previous studies showed that loss-of-function of CDC5 (core component of Prp19 complex) leads to cell cycle arrest at the G2/M phase because of inefficient splicing of the tubulin-encoding TUB1 mRNA in Saccharomyces cerevisiae. Moreover, microtubule dynamics function in determining Sertoli cell shape, and mitotic and meiotic spindle and sperm flagella development and are thus essential for male fertility. Taken together, these abnormal splicing events resulting from Bcas2 depletion may also account for the loss of spermatocytes and failure of meiosis initiation.

BCAS2 has multiple functions in several pathways, including RNA splicing and DNA damage repair. However, we did not observe an accumulation of γH2AX expression in spermatogonia in Bcas2−/−;Vasa-Cre testes during the meiosis initiation phase (Supplementary Fig. 5). Furthermore, the expression of most genes is not affected in Bcas2−/−;Vasa-Cre testes at P9. These data suggest that BCAS2 mainly functions through RNA splicing but not DNA damage in mouse spermatogonia.

In conclusion, our results suggest that BCAS2 is involved in pre-mRNA splicing in spermatogonia and is essential for mouse spermatogenesis and male fertility. This research provides the first evidence for alternative splicing machinery regulating meiosis initiation in germ cells during mouse spermatogenesis.

**Methods**

Mice maintenance and generation of gene-targeted mice. All mice were maintained under specific-pathogen-free (SPF) conditions in compliance with the guidelines of the Animal Care and Use Committee of the Chinese Academy of Sciences (CAS). The generation of the Bcas2Flox/Flox (Bcas2F/F) mouse line was described previously. Male Bcas2−/− mice were crossed with Vasa-Cre transgenic mice to obtain mice with Bcas2 specifically ablated in the male germ line. The Bcas2−/−(Vasa-Cre) mouse line was maintained on a mixed background (129S/C57BL/6). Genotyping of Bcas2 was performed by PCR of mice tail genomic DNA. Forward primer (F1, 5′-ATTCAGACGTGTGGTGG-3′) and a reverse primer (R, 5′-CATCTGTCAGACAAAGTTGAG-3′) were used to detect the wild-type allele (402 bp) and the floxed allele (522 bp), and forward primer (F2, 5′-AGGTGTATGAATGCCTGAACAAG-3′) and reverse primer (R, 5′-CGAGGAAAGGCTGTAAGACG-3′) were used to remove the background (129/C57BL/6). Genotyping of Dazl was genotyped with the forward primer (F-V, 5′-CACCTGACCGCTTAAATAGCG-3′) and a reverse primer (R-V, 5′-TCTTCCATTCTTAAACACACACTGGG-3′) to produce a 240 bp product. Bcas2F/F−;Vasa-Cre male mice were used as controls and Bcas2−/−;Vasa-Cre male mice were used as mutants.

**Histological analysis, immunostaining and imaging.** Testes from control and Bcas2−/−;Vasa-Cre male mice were isolated and fixed in Bouin’s solution. The testes were embedded in paraffin and sectioned (5 μm). The sections were dehydrated stepwise through an ethanol series (30, 50, 70, 80, 90 and 100% ethanol), embedded in paraffin, and sectioned (5 μm). After dewaxing and hydration, the sections were stained with hematoxylin and 1% eosin and imaged with a Nikon ECLIPSE Ti microscope.

For immunostaining, following dewaxing and hydration, the sections were boiled in Buffer TE (10 mM Tris, 1 mM EDTA, pH 9.0) for 20 min using a microwave oven for antigen retrieval. After washing with PBS 3 times, the sections were permeated in 0.2% Triton X-100 for 15 min, blocked with 5% BSA in PBS for 1 h at room temperature and incubated overnight at 4°C with primary antibodies diluted in 1% BSA. Following three washes with PBS, secondary antibodies were added to the sections and incubated for 1 h at room temperature. The sections were then washed in PBS three times, incubated in 2 μg/ml of Hoechst 33342 (Invitrogen, H21492) for 10 min at room temperature, and mounted with Fluorount-G medium (Southern Biotech, 1000-01). The primary antibodies were as follows: rabbit anti-BCAS2 polyclonal antibody (10414-1-AP, Proteintech, 1:200); mouse anti-PLZF monoclonal antibody (sc28319, Santa Cruz, 1:200); rabbit anti-DDX4/MVH polyclonal antibody (ab13840, Abcam, 1:250); mouse phosphor-Histone H2A.X (ser139/Tyr142) antibody (#5438, CST, 1:500); mouse anti-SCP3 monoclonal antibody (sc57459, Santa Cruz, 1:200); rabbit anti-Stra8 polyclonal antibody (ab94054, Abcam, 1:250); rabbit anti-Cleaved Caspase-3 (Asp175) antibody (#9661, Cell Signaling, 1:200); and rabbit anti-Ki67 antibody (ab92742, Abcam, 1:400). The secondary antibodies were as follows: For immunohistochemistry images of PLZF were obtained under the bright-field of a Nikon ECLIPSE Ti microscope. The immunofluorescence staining was imaged with a laser scanning confocal microscope LSM780 (Carl Zeiss).

**RNA extraction and real-time RT-PCR.** Total RNA was extracted from whole testes using TRIzol reagent (Invitrogen, 15596-026) following the manufacturer’s instructions. After removing the residual genomic DNA with the DNase I Kit (Promega, M6101), 500 ng of total RNA was reverse-transcribed into cDNAs (Superscript PrimeScript RT Forgent Kit (TaKaRa, RR042A) and 5 μL) or according to the manufacturer’s protocol. Real-time RT-PCR was performed using a SYBR Premix Ex Taq kit (TakaRa, DRR420A) on a LightCycler 480 instrument (Roche). Relative gene expression was analysed based on the 2−ΔΔCt method with Hprt and Gapdh as internal controls.

Primer sequences used to determine the amount of Tuba3a, Tuba3b and Tubb4b mRNA were designed to span an exon-exon junction, with Tuba3a-mRNA primers annealed to the 3′ end of exon3 and 5′ end of exon5, Tuba3b-mRNA primers annealed to the 3′ end of exon3 and 5′ end of exon5 and Tubb4b-mRNA primers annealed to the 3′ end of exon2 and 5′ end of exon3. The information of all primers was listed in Supplementary Table 5.
Western blotting. Protein samples were prepared using RIPA lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM NaVO₃, 5–10 mM NaF) containing a protease inhibitor cocktail (Roche, 04693132010) and quantified using a BCA reagent kit (Beyotime, P0012-1). Equal amounts of total protein were separated in a 10% SDS–PAGE gel and transferred onto PVDF membranes. After blocking with 5% non-fat milk for half an hour at room temperature, the membranes were incubated in diluted primary antibodies at 4 °C overnight. After three washes with TBST, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (1:3,000, Jackson ImmunolResearch) at room temperature for 1 h. The signals were developed with Pierce ECL Substrate (Thermo Fisher Scientific, #34080). Films were exposed to X-ray film and scanned using a X-ray film scanner. The band intensity was analyzed using ImageJ (NIH, USA).

Statistical analysis. The results of all quantitative experiments were based on at least three independent biological samples. Statistical analysis was performed by using at least five samples and expressed as the mean ± s.e.m. The results of all statistical analyses were similar as previously reported17,18,30. Experiments were not randomized.

Data availability. Data for RNA sequencing of P9 testes from control and Bcas2−/−;Vasa-Cre males have been deposited in the Gene Expression Omnibus database under accession code GSE89801. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author on reasonable request.

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Author contributions
W.L. designed and performed the major experiments and data analysis and wrote the manuscript. F.W. established the Bcas2Floxed/Floxed mice. Q.X., J.S., X.Z., X.L., Z.Z., Z.G. and H.M. contributed to mouse maintenance and technical assistance. E.D. and F.G. contributed to data analysis. L.L., Z.Y. and S.G. initiated and organized the study, analysed the data and wrote the manuscript. All authors commented on the manuscript.

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