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Expression of a Testis-Specific Form of *Gal3st1* (*CST*), a Gene Essential for Spermatogenesis, Is Regulated by the *CTCF* Paralogous Gene *BORIS*▽†

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Previously, it was shown that the *CTCF* paralogous gene, *BORIS* (brother of the regulator of imprinted sites) is expressed in male germ cells, but its function in spermatogenesis has not been defined. To develop an understanding of the functional activities of BORIS, we generated *BORIS* knockout (KO) mice. Mice homozygous for the null allele had a defect in spermatogenesis that resulted in small testes associated with increased cell death. The defect was evident as early as postnatal day 21 and was manifested by delayed production of haploid cells. By gene expression profiling, we found that transcript levels for Gal3st1 (also known as cerebroside sulfotransferase [CST]), known to play a crucial role in meiosis, were dramatically reduced in *BORIS* KO testes. We found that *CST* is expressed in testis as a novel testis-specific isoform, *CST* form F_{TS}, that has a short exon 1f. We showed that BORIS bound to and activated the promoter of *CST* form F_{TS}. Mutation of the BORIS binding site in the promoter reduced the ability of BORIS to activate the promoter. These findings define transcriptional regulation of CST expression as a critical role for BORIS in spermatogenesis.

CTCF is a ubiquitously expressed chromatin factor containing an 11-zinc-finger (11-ZF) DNA binding domain that is highly conserved from *Drosophila melanogaster* to humans (39, 43, 61). Identities among vertebrate orthologs of 90 to 99% imply critical functions for the protein now known to include regulation of gene expression and chromatin organization (10, 28, 41). CTCF was originally identified as a repressor of *Myc* but was later shown to function in regulation of transcription when bound to promoters of specific genes such as *APP*, *Rb*, *p53*, *hTERT*, and *ARF* (9, 11, 12, 29, 35, 46, 49, 54, 57). In addition, CTCF is also known to act as a chromatin insulator, which blocks inappropriate activation or inactivation of genes by flanking regulatory elements (39). A constitutive insulator function of CTCF was originally identified in a reporter construct based on the HS4 element cloned from the chicken β-globin locus (3, 21), while later studies revealed that CTCF also mediate methylated CpG-sensitive insulation of imprinted genes (24). CTCF was found to bind unmethylated sequences in the maternal allele of the *H19* imprinting control region (*H19* ICR) as a part of a complex three-dimensional loop structure that partitions the enhancer from *IGF2*, resulting in inhibition of expression. CTCF could not bind methylated *H19* ICR sequences of paternal origin, resulting in loss of enhancer blocking activity and activation of *IGF2* expression (14, 33). More recently, CTCF sites have been found to maintain epigenomic integrity of unstable repeats (34) and latency control of herpes simplex virus (HSV), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (2, 5, 50).

The gene *BORIS* (*b*rother *o*f the *r*egulator of *i*mprinted *s*ites), also known as *CTCFL* (CTCF-like) and *CTCF-T* (CTCF testis specific), has been identified as a mammalian paralog of *CTCF* (28, 37). Recent studies showed that a *BORIS* ortholog is present in the platypus, reptiles, and higher organisms (20). Evolutionarily, expression of *BORIS* became progressively restricted to the testis, whereas CTCF is ubiquitously expressed in all species tested. This implies that BORIS has acquired testis-specific functions in mammalian organisms.

In addition to its normal expression in the testis, recent studies revealed that various tumors and cancer cell lines are also BORIS positive, with frequent coexpression of cancer testis antigens (CTA) (7, 17, 48, 52, 55). In addition, it was shown that conditional expression of BORIS induced expression of a series of CTA genes, including MAGE-A1, NY-ESO-1 (17, 52), and SPANX (30). This suggests that BORIS may normally act to regulate expression of CTA genes, although some data suggest otherwise (25).

Recent genome-wide analyses of CTCF binding sites by chromatin immunoprecipitation (ChIP)-chip or ChIP-sequencing analysis identified approximately 14,000 or more CTCF binding sites throughout the genome (6, 27). Kim et al. (27) documented that almost 80% of the binding sites shared

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consensus recognition sequences with evolutionary conservation. It is important to recognize that in contrast to the 11-ZF domain, the N- and C-terminal regions of BORIS share no homology with similarly placed CTCF sequences. This suggests that BORIS may exhibit functions distinct from CTCF based upon partner interactions that are dependent on these unique domains. In fact, BAT3 and PRMT7 were found to interact with BORIS through the unique N-terminal domain, implying that BORIS is functionally distinct from CTCF despite sharing DNA recognition sequences (23, 40).

Production of seminolipid, a primary glycolipid in the testis, is catalyzed by cerebroside sulfotransferase (CST), which is encoded by *Gal3st1* through sulfation of galactosylalkylacylglycerol (19, 53). The importance of *CST* and its product in spermatogenesis was revealed through analyses of *CST* knockout (KO) mice, which displayed male-specific sterility due to a spermatogenesis defect apparent before the first meiotic division (18, 60). Mouse *CST* has eight splicing variants, all of which have the same coding region with different 5′-untranslated region (5′-UTR) sequences (16). Although different tissues express various combinations of *CST* splicing variants, testis expresses only *CST* form F, implying that there are testis-specific transcription factors that activate the promoter of this unique isoform. In view of the crucial function of CST in spermatogenesis, regulation of the *CST* form F promoter should be a critical determinant for proper spermatogenesis.

In this study, we analyzed *BORIS* KO mice to develop a firm understanding of the physiological functions of BORIS. We found that *BORIS*$^{-/-}$ mice exhibited a spermatogenesis defect at meiosis. We identified a novel testis-specific *CST* splicing variant, *CST* form F_{TS}, for which expression is dramatically suppressed by the loss of *BORIS*. Our findings suggest that BORIS plays an important function in spermatogenesis through regulation of *CST* expression.

MATERIALS AND METHODS

Antibodies. A glutathione *S*-transferase (GST)-tagged mouse BORIS C-terminal fragment containing amino acid residues 538 to 636 was prepared from *Escherichia coli* and used to immunize two rabbits. Serum antibodies were affinity purified on a GST-BORIS C-terminal fragment column, and then GST-reactive antibodies were depleted with a GST column. Antibodies were produced and purified by GenScript (Piscataway, NJ).

Targeted disruption of the *BORIS* gene. *BORIS* knockout mice were generated at Ozgene (Ozgene Pty. Ltd., Bentley, Western Australia, Australia). The targeting vector was designed to replace the *BORIS* gene from the first methionine to exon 8 with an *eGFP* reporter casette flanked by *LoxP*/*Lox511* sequences and a *PGK*-neomycin cassette flanked by FLP recombinase target (FRT) sequences. Primers used for targeting vector construction, genotyping, and generation of probes for Southern blotting are listed in Table S1 of the supplemental material. The targeting vector was linearized and electroporated into 129S1/Sv-derived W9.5 embryonic stem (ES) cells. Targeted ES cells were injected into blastocysts from C57BL/6 (B6) mice. Chimeric male mice were crossed with C57BL/6 females, and the *BORIS*$^{+/-}$ mice obtained were mated to obtain *BORIS* null mice. The *PGK*-neomycin cassette was deleted by crossing with B6.Cg-Tg(ACTFLPe)9205Dym/J mice (The Jackson Laboratory, Bar Harbor, ME). The use of mice in this study was approved by the NIAID Animal Care and Use Committee under protocol LIP-5.

Histochemistry. Testes were fixed with 10% formalin and embedded in paraffin. Five-micrometer sections were prepared for histological analysis. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed using the DeadEnd colorimetric TUNEL system (Promega, Madison, WI) according to the manufacturer's protocol. TUNEL-positive cells in a field, which corresponded to approximately 0.6 mm^2, were counted to quantify apoptotic cells in testes. Three mice were examined for each genotype.

DNA flow cytometry. DNA flow cytometry was performed as described previously with slight modifications (31). Briefly, testicular cells that were fixed with 70% ethanol at least overnight were treated with 0.5% pepsin solution for 75 min at 37°C followed by treatment with phosphate-buffered saline (PBS) supplemented with 80 μg/ml RNase and 1% fetal bovine serum (FBS) for 20 min at room temperature. Cells were then stained with PBS containing 5 μg/ml propidium iodide (PI) and 1% FBS for 30 min on ice. Stained cells were analyzed with a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Purification of spermatocytes and round spermatids. Spermatocytes and round spermatids were purified by centrifugal elutriation (38) followed by flow cytometric sorting of cells stained with Vybrant DyeCycle Green (Invitrogen, Carlsbad, CA) to obtain cell fractions with high purity. Briefly, decapsulated testes were treated with collagenase followed by treatment with trypsin, and the dissociated cells were used for centrifugal elutriation. Partially purified spermatocytes and the round spermatids fractions were incubated with 10 μM Vybrant DyeCycle Green for 30 min at 32°C followed by 4,6-diamidino-2-phenylindole (DAPI) staining. Cells were then sorted on a FACSAria (Becton Dickinson) to purify spermatocytes and round spermatids. DAPI-positive dead cells were eliminated. The purity of cells was confirmed by flow cytometry analysis of DNA content using sorted cells fixed with ethanol (spermatocytes, 95.7% ± 2.7%; round spermatids, 92.2% ± 2.5% [means ± standard deviations]).

Surface spread staining. Surface spreads of spermatocytes were prepared as described previously (42). Samples were stained with anti-SCP3 (Novus Biologicals, Littleton, CO) and anti-γH2AX antibodies (Millipore, Billerica, MA) for 1 h at room temperature followed by staining with Alexa Fluor 488- or Cy3-conjugated secondary antibody for 1 h at room temperature. Samples were counterstained with DAPI.

Microarray analysis. Microarray chips printed by the NIAID Microarray Research Facility comprised approximately 18,000 genes represented by 70-mer oligonucleotides. We studied five sets of wild-type and *BORIS*$^{-/-}$ testes prepared from postnatal day 14 (P14) mice. After the raw data were normalized with the Lowess smoothing function, the significant genes were identified with significance analysis of microarrays (51) with a false discovery rate of 3%, followed by a selection of genes with changes greater than twofold. Expression of genes with significant changes by microarray analyses was validated by quantitative PCR (qPCR). Microarray data were deposited to the NCBI Gene Expression Omnibus database (accession number GSE19162).

RT-PCR and qPCR. Total RNA was prepared using an RNeasy minikit (Qiagen, Valencia, CA). cDNA was prepared using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's protocol. Quantiative PCR (qPCR) was performed using the SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and the 7900HT sequence detection system (Applied Biosystems). Primers used for conventional reverse transcription-PCR (RT-PCR) and quantitative PCR are listed in Table S1 in the supplemental material. Primers for qPCR were designed using the Primer Express software (Applied Biosystems). Expression levels were normalized against the housekeeping gene *Gapdh*. For ΔC_t calculations, a C_t value of 40 was used for samples that had C_t values over 40. Absolute quantification was performed to analyze the transcription level of *CST* form F_{TS} and F_{SS} in *CST* form F_{Total} by using a plasmid that contained the partial cDNA sequence of *CST* form F_{SS} cloned from stomach cDNA as a standard. The numbers of samples prepared from individual mice for qPCR are described in the figure legends. Each sample subjected to qPCR was analyzed in triplicate. Student's *t* test was performed to evaluate statistical significance. Data shown are means ± standard deviations.

5′-RACE. Total RNA prepared from 3-month-old mouse testes was subjected to 5′ rapid amplification of cDNA ends (5′-RACE) analysis utilizing the GeneRacer kit (Invitrogen), which ensures amplification of only full-length transcripts by eliminating truncated messages from the amplification process, according to the manufacturer's instructions. Reverse transcription was performed using a *CST*-specific reverse primer, 5′-CCATTGGGGAAAGCGAACTTGAG-3′, followed by nested PCR using a reverse primer specific for *CST*, 5′-AGTGTGCT GCTGGCGGTCTTGTG-3′. Both *CST*-specific primers were designed on the open reading frame of *CST*, which is common for all isoforms of *CST*. Purified PCR fragments were cloned in a pGEM-T vector (Promega), sequenced (Genomics Research Facility, Rocky Mountain Laboratories, NIAID, NIH, MT), and analyzed.

Bisulfite sequencing. Bisulfite modification of genomic DNA prepared from kidney or spermatocytes was performed using an Imprint DNA modification kit (Sigma-Aldrich, St. Louis, MO). A 314-bp sequence was amplified using 5′-GG GAAAGTTTTTGTATTATTGTATGAT-3′ and 5′-AACTAACTCTCTACTA AACCTAAAAACC-3′ as primers with PCR Platinum *Taq* polymerase under the following conditions: 94°C for 2 min; 35 cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. The amplicon was ligated into the

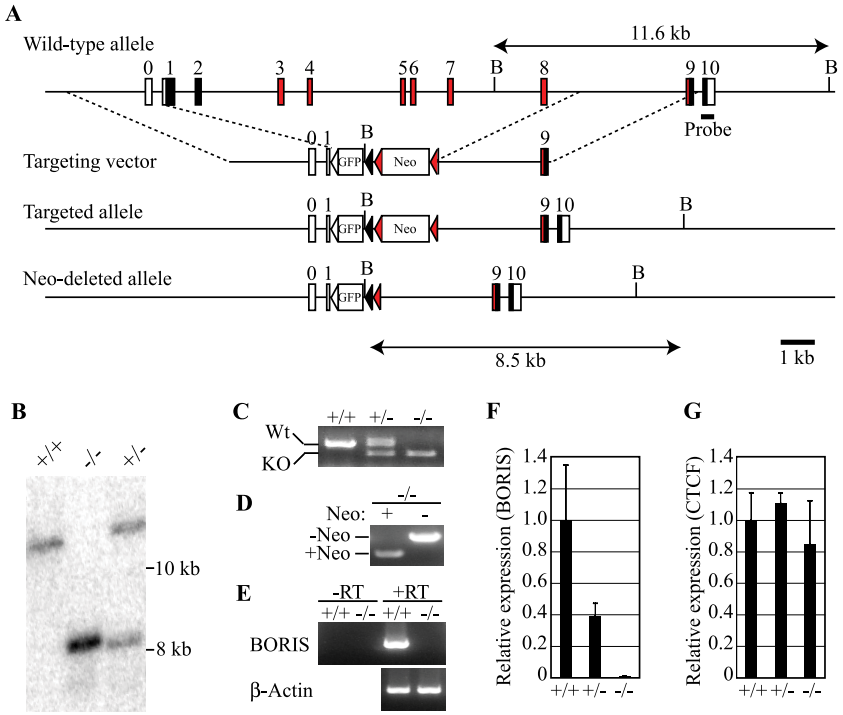

FIG. 1. (A) Targeting scheme for generation of *BORIS* (*Ctcfl*) KO mice. Deletion of the *BORIS* gene proceeds from the first methionine to exon 8, which includes ZF-1 to -10, and the N-terminal half of ZF-11 is replaced with *GFP* and a neomycin cassette. The probe used for Southern blotting is indicated. Filled boxes indicate open reading frames; red boxes indicate the coding regions of the 11-ZF domain; open boxes indicate untranslated regions; the open triangle indicates a LoxP site; the filled triangle indicates a Lox511 site. FRT sequences are shown with red triangles. B, BglII. (B) Southern blotting. Genomic DNA was digested with BglII and hybridized with a probe flanking the targeting construct at the 3′ end as diagramed in panel A. (C) Genomic PCR typing of *BORIS*$^{-/-}$ mice. (D) Genomic PCR typing of *BORIS*$^{-/-}$ (*-neo*) mice. (E) cDNAs prepared from testes of 3-month-old mice were subjected to RT-PCR. (F and G) cDNAs prepared from testes of 3-month-old mice were quantified by qPCR for expression of transcripts for *BORIS* (F) and *CTCF* (G) ($n = 3$). Expression levels are shown as the ratio to wild type.

pGEM-T vector. Following transformation, plasmids from individual bacterial colonies were isolated and subjected to sequencing.

EMSA and methylation interference assay. An electrophoretic mobility shift assay (EMSA) and methylation interference assay were performed as described previously (13, 44). Briefly, proteins for the assays were synthesized using plasmid pET-16b (Novagen, Madison, WI) with inserts of BORIS, CTCF, CTCF zinc-finger domain (11-ZF) sequences, or luciferase T7 control DNA with the TnT *in vitro* transcription-translation system (Promega). One primer for each DNA fragment was labeled with [γ-^{32}P]ATP and used for PCR to prepare DNA fragments. Amplicons were gel purified and used for the assays. For supershift assays, proteins and DNA fragments were preincubated for 30 min at room temperature for complex formation followed by incubation with antibodies for 20 min. A mixture of two rabbit anti-mouse BORIS antibodies and a mixture of mouse monoclonal antibodies against CTCF (44) were used for supershift assays. Samples were loaded onto 5% native polyacrylamide gels in 0.5×Tris-borate-EDTA. The supershifted band of BORIS was found at the top of the gel due to the polyclonality of the anti-mouse BORIS antibodies.

Luciferase assay, cells, and transfection. Fragments containing the entire 5′-UTR sequences of *CST* form F_{TS} and its promoter sequences starting from promoter position −179, −359, or −515 were cloned into pGL3-basic vector (Promega). NIH 3T3 cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and penicillin-streptomycin were transfected using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol with luciferase constructs together with internal control vector and BORIS or CTCF expression vectors cloned into pCIneo where indicated. Cells were cultured for 48 h at 37°C with 5% CO_2. Luciferase assays were performed using a Dual-Luciferase reporter assay system kit (Promega) according to the manufacturer's protocol. Three independent samples were analyzed for each experiment, and the numbers of experiments are described in the figure legends. Student's *t* test was performed to evaluate statistical significance. Luciferase activity data shown are the means ± standard errors of the means.

ChIP assay. ChIP assays were performed based on the Upstate Biotechnology protocol. Briefly, decapsulated testes were washed with PBS and minced with a scalpel. Samples were fixed with 1% formaldehyde for 15 min with rocking followed by two washes with PBS. Fixed samples were homogenized with a Dounce homogenizer, and pelleted samples were suspended in SDS lysis buffer (20 mM Tris-HCl [pH 8], 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) supplemented with a protease inhibitor cocktail. Lysates corresponding to 30 mg of tissue were used for a ChIP assay. A mixture of rabbit anti-mouse BORIS antibodies was used for ChIP. Preimmune sera were used as a control. Primers used for quantitative PCR are listed in Table S1 of the supplemental material.

RESULTS

Targeted disruption of the *BORIS* gene. To identify the physiological function of BORIS and its target genes, we generated *BORIS* knockout mice. The *BORIS* gene, from the first methionine to exon 8, which includes ZF 1 to 10 and the N-terminal half of ZF11, was replaced with *GFP* and a neomycin cassette (Fig. 1A). We confirmed the recombination by Southern blotting (Fig. 1B), genomic PCR (Fig. 1C), RT-PCR (Fig. 1E), and quantitative RT-PCR (Fig. 1F), as well as the generation of mice with the *PGK*-neomycin cassette removed [*BORIS*$^{-/-}$ (*-neo*)] (Fig. 1D). Expression of *CTCF* did not change with the deletion of *BORIS* (Fig. 1G). By RT-PCR, we detected expression of *GFP* only in testis (Fig. 2), consistent with the normal expression pattern of *BORIS* transcripts; how-

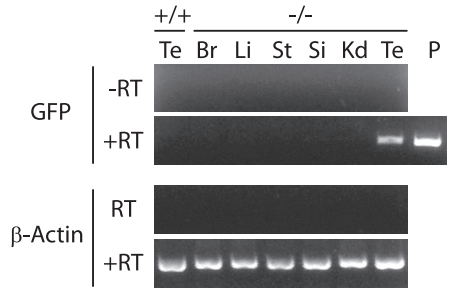

FIG. 2. Expression of GFP was analyzed by RT-PCR. cDNAs prepared from each tissue are indicated: Te, testis; Br, brain; Li, liver; St, stomach; Si, small intestine; Kd, kidney; P, positive control.

ever, we could not detect GFP expression by fluorescence microscopy or flow cytometry (data not shown), probably due to low expression of GFP.

Spermatogenesis defect in *BORIS*$^{-/-}$ mice. Matings of *BORIS*$^{+/-}$ mice yielded progeny at the expected Mendelian ratios (data not shown). As *BORIS* is exclusively expressed in testis (37) (Fig. 2), we focused our studies on testicular development and function. Although the *BORIS*$^{-/-}$ male mice were fertile (Table 1), we found that *BORIS*$^{-/-}$ testes were significantly smaller than those of their wild-type counterparts (Fig. 3A and B), indicating that *BORIS* had important functions in testicular development. A reduction in testicular size for *BORIS*$^{-/-}$ mice could be detected as early as P28 (Fig. 3B). To determine the basis for this difference, we performed histologic studies of testes from P28 *BORIS*$^{-/-}$ mice. Testes from *BORIS*$^{-/-}$ mice had reduced cellularity compared to testes of wild-type mice (Fig. 3A). Furthermore, testes from *BORIS*$^{-/-}$ mice had multinucleated cells (Fig. 3A, arrows), a feature found in a number of spermatogenesis-defective mice. We confirmed that *BORIS*$^{-/-}$ (*-neo*) mice also displayed a similar defect (Fig. 3A, right column, lower panel) and that they were fertile as well (data not shown). These results indicate that the spermatogenesis defect was due specifically to the loss of *BORIS* and was not affected by the presence of the *PGK*-neomycin cassette. To analyze whether the reduced cellularity of *BORIS*$^{-/-}$ testis might be due to increased cell death, we performed TUNEL staining and found that the frequency of apoptotic cells was greatly increased in *BORIS*$^{-/-}$ testis compared to wild-type testis (Fig. 3C). This result suggests that the smaller testes of *BORIS*$^{-/-}$ mice could be ascribed at least in part to increased cell death with effects on spermatogenesis.

To further analyze the effects of BORIS deficiency on the progression of spermatogenesis, we performed DNA flow cytometry on testicular cells. This allowed us to quantify subpopulations of testicular cells: tetraploid cells (primarily spermatocytes), diploid cells (a mixture of spermatogonia, secondary spermatocytes, and somatic cells), and haploid cells (spermatids). We found a deficiency in production of haploid cells in *BORIS*$^{-/-}$ testis compared to wild-type testis as early as P21 (17.8% ± 5.2% [wild type] versus 9.5% ± 5.2% [*BORIS*$^{-/-}$]; $P < 0.05$) as well as at P28 (48.8% ± 2.3% [wild type] versus 28.9% ± 6.6% [*BORIS*$^{-/-}$]; $P < 0.05$) (Fig. 3D). The fact that there was no significant difference in testicular weight at P21 was probably due to the scarcity of haploid cells at this stage of development, the beginning step in haploid cell production. To analyze the effect of BORIS on synapsis, we performed surface spread staining of spermatocytes. Although XY bodies positive for γH2AX were formed in spermatocytes of both *BORIS*$^{-/-}$ and wild-type mice, around 20% of *BORIS*$^{-/-}$ spermatocytes showed abnormal aggregation of the synaptonemal complex protein, SCP3, which does not coincide with γH2AX (Fig. 3E). This finding is similar to that of the abnormal structure reported in *Dnmt3L*-deficient spermatocytes (4). These results indicate that abnormalities in spermatogenesis in *BORIS*$^{-/-}$ mice begin around P21 and are evidenced by a significant delay and defect in meiosis.

Loss of *BORIS* is associated with reduced expression of CST. We reasoned that alterations in gene expression responsible for the spermatogenesis defect found in *BORIS*$^{-/-}$ mice would be evident before day 21, when the defect could first be detected. To examine this possibility, we performed gene expression profiling using oligonucleotide microarrays to study testes prepared from P14 mice, an age at which we found no significant changes in the population of spermatogenic cells (wild type 2n, 70.6% ± 4.8%; wild type 4n, 20.7% ± 5.7%; *BORIS*$^{-/-}$ 2n, 74.7% ± 4.2%; *BORIS*$^{-/-}$ 4n, 18.7% ± 3.6%) (Fig. 3D). We identified 24 genes showing statistically significant differences in expression, and 4 of these genes showed more-than-2-fold differences in expression levels (see Fig. S2 in the supplemental material). To validate the differential expression, we employed qRT-PCR analyses and found three out of the four genes are truly differentially expressed between wild-type and *BORIS*$^{-/-}$ testis (Table 2). 1700019B21Rik is a gene with no known function that has been identified in mice and rats but not in humans. TSP50 is a testis-specific protease which is known to be a cancer testis antigen (47, 59). The expression is regulated in part by methylation of the promoter (22), is negatively regulated by p53 (56), and is abnormally activated in a high proportion of breast cancers (59). The third gene, *Gal3st1*, which encodes CST, showed the greatest reduction in transcripts in *BORIS*$^{-/-}$ testis (Table 2). Importantly, *CST*$^{-/-}$ mice exhibit male infertility due to an arrest of spermatogenesis prior to the metaphase of the first meiosis (18), paralleling the defect identified in *BORIS*$^{-/-}$ mice.

There are eight splice variants of *CST* expressed in different tissues (16), but only one, form F, is expressed in testis (Fig. 4A and B). To confirm the microarray analyses, we used qRT-PCR to quantify total *CST* transcript levels and showed that *CST* transcript levels were reduced more than 10-fold in testis of *BORIS*$^{-/-}$ compared to wild-type mice at P14 (Fig. 4C). A

TABLE 1. Fertility analysis[a]

Breeder (female × male) (*n*)	No. of litters (mean ± SD)	Litter size (mean ± SD)
B6 × *BORIS*$^{+/+}$ (4)	2.75 ± 0.50	6.90 ± 3.48
B6 × *BORIS*$^{-/-}$ (4)	2.00 ± 0.82	8.38 ± 0.74
BORIS$^{+/+}$ × B6 (3)	3.67 ± 0.58	8.45 ± 2.16
BORIS$^{-/-}$ × B6 (6)	3.17 ± 0.41	9.74 ± 2.62

[a] Two- to 3-month-old *BORIS*$^{+/+}$ or *BORIS*$^{-/-}$ mice were mated with 2- to 3-month-old B6 mice for 3 months, and mean litter numbers and litter sizes were analyzed. All breeders produced offspring, and no significant difference was found in litter number and litter size between *BORIS*$^{+/+}$ and *BORIS*$^{-/-}$ breedings.

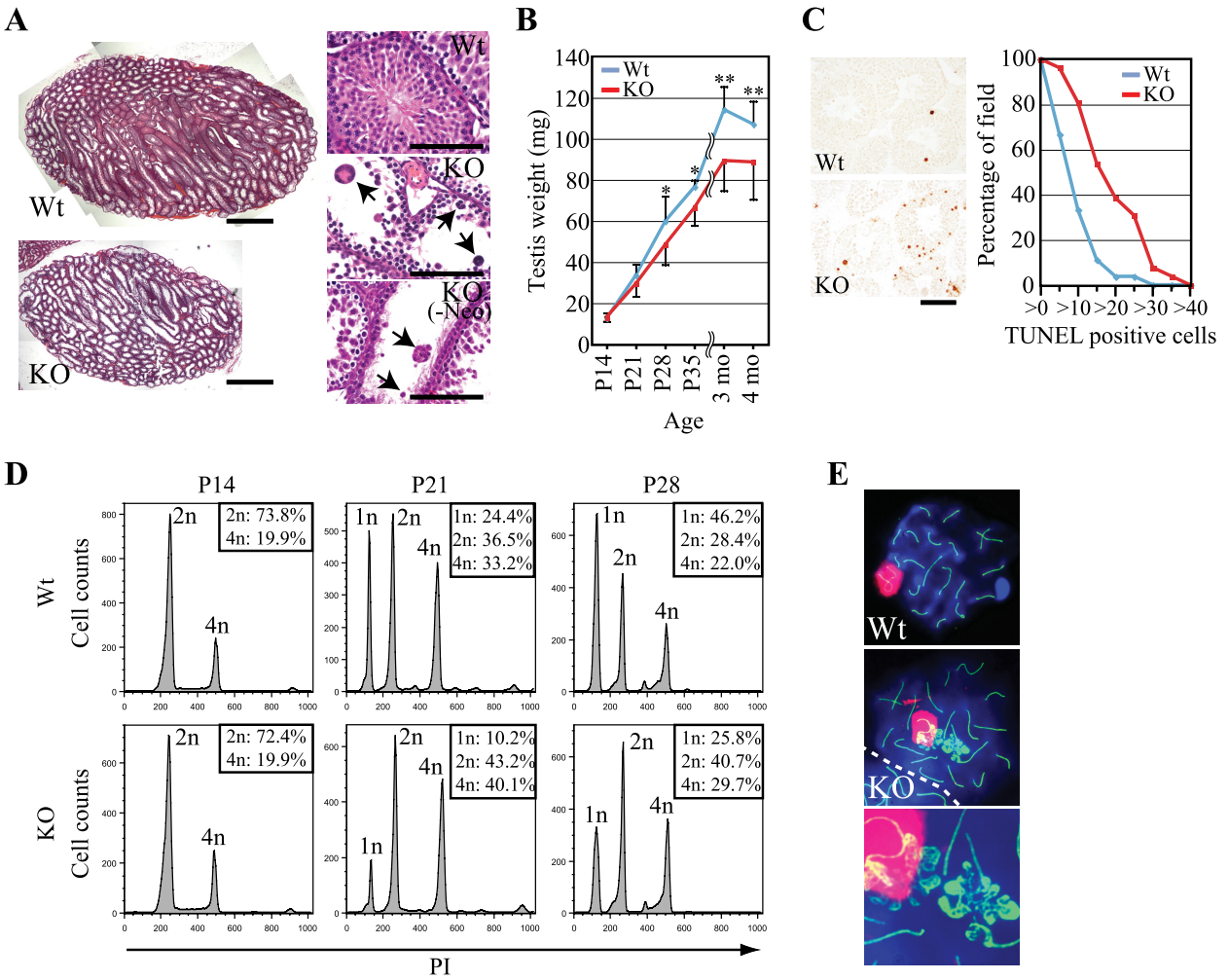

FIG. 3. (A) Hematoxylin and eosin staining of testes prepared from P28 wild-type, *BORIS*$^{-/-}$, and *BORIS*$^{-/-}$ (*-neo*) mice. Arrows indicate multinucleated cells. Bars, 1 mm (left column); 100 μm (right column). (B) Sizes of wild-type (blue) and *BORIS*$^{-/-}$ (red) testes at various stages. *, $P < 0.05$; **, $P < 0.005$. (C) TUNEL staining of testes from P28 wild-type (upper panel) and *BORIS*$^{-/-}$ (lower panel) mice. Bar, 100 μm. TUNEL-positive cells in each field were counted in testes of wild-type (blue) and *BORIS*$^{-/-}$ (red) mice. (D) Representative results of DNA flow cytometry performed on testicular cells prepared from testes of P14, P21, and P28 of wild-type (upper row) and *BORIS*$^{-/-}$ (lower row) mice. The percentage of each population is shown in the inset. At least three mice were analyzed for each analysis. (E) Spermatocytes of wild-type (upper panel) and *BORIS*$^{-/-}$ (middle panel) mice were stained with SCP3 (green), γH2AX (red), and DAPI (blue). A dotted line indicates a boundary of adjacent cells. The lower panel shows a high-magnification image of aberrantly accumulated SCP3 in *BORIS*$^{-/-}$ spermatocytes.

comparable reduction of *CST* expression was also found in testes of mature mice (Fig. 4C). Furthermore, quantitation of transcripts specifically for *CST* form F showed comparable levels of reduction (Fig. 4D). We also confirmed that there was a comparable reduction in the expression of *CST* in *BORIS*$^{-/-}$ (*-neo*) testis (Fig. 4E and F). Analysis of *CST* transcript levels during testicular development showed an abrupt induction of expression at P14, when growing numbers of testicular cells differentiated into spermatocytes, with increased levels of *CST* transcripts being sustained thereafter (Fig. 4G). If BORIS regulates the expression of *CST* in testis, then BORIS should be expressed at or before the time that CST is induced. Indeed, *BORIS* transcripts were detectable from P2 and peaked at P14, with expression sustained at relatively high levels thereafter, consistent with the expression pattern of *CST* (Fig. 4H). Furthermore, we found that *CST* was highly expressed in round spermatids in correlation with the high expression of *BORIS*, while there was no correlation between the levels of expression of *CST* and *CTCF* (Fig. 4I to L). These results demonstrated that expression of *CST* is reduced in testes of *BORIS*$^{-/-}$ mice and suggest that BORIS might be directly involved in its transcriptional regulation.

TABLE 2. Differentially expressed genes in P14 *BORIS*$^{-/-}$ testis

Gene	Accession no.	Fold change[a] based on:	
		Microarray	qRT-PCR
Gal3st1	NM_016922	−7.36	−11.15
1700019B21Rik	XM_001473416	−2.68	−2.41
Tsp50	NM_146227	−2.29	−2.60

[a] cDNAs prepared from P14 mice testes were used for qRT-PCR. qRT-PCR analysis of *Gal3st1* showed a larger difference than microarray results, probably because qRT-PCR analysis has a greater dynamic range and returns more accurate data than microarrays.

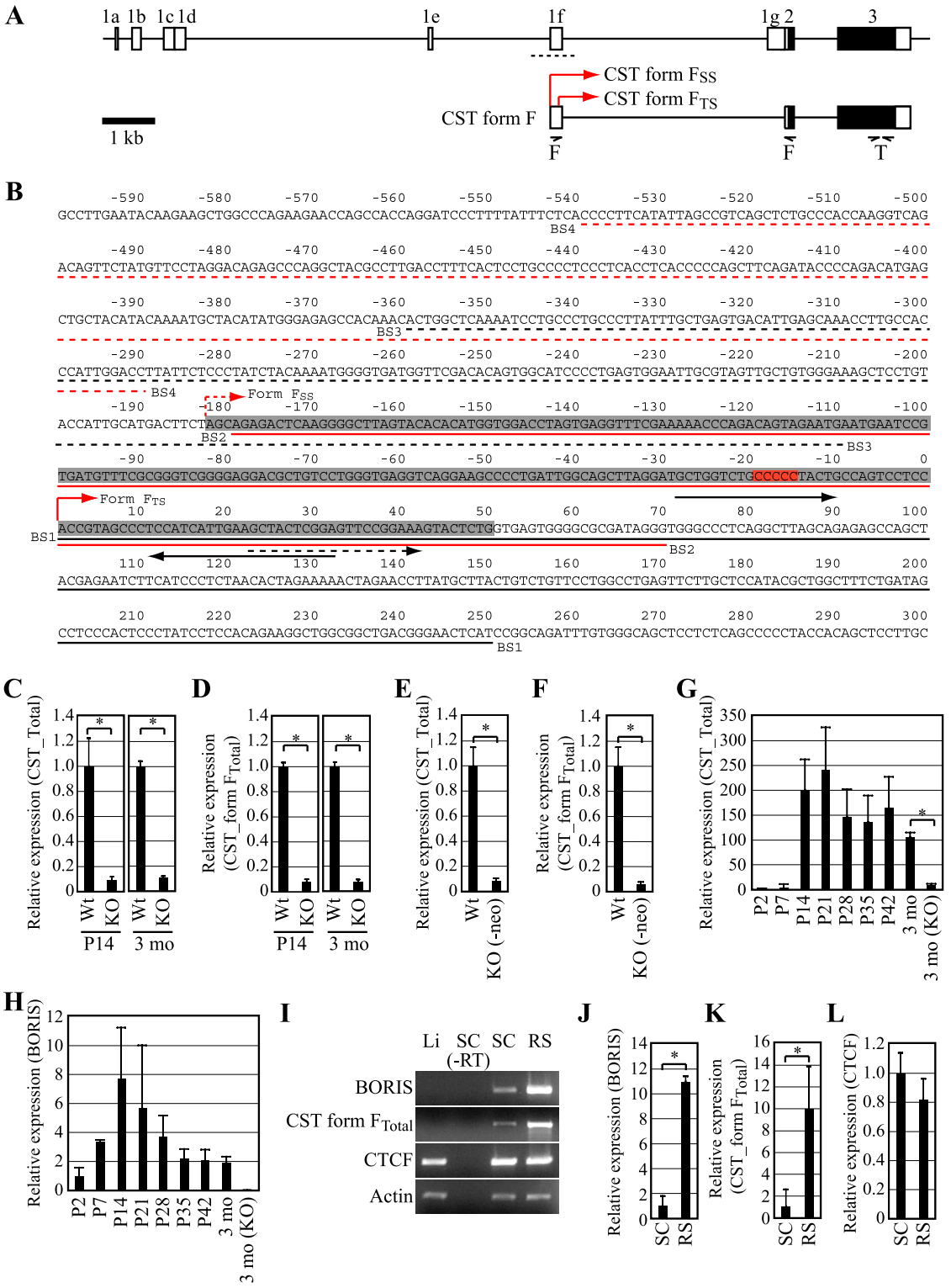

FIG. 4. (A) Genomic structure of the *CST* gene. Open and filled boxes represent UTRs and open reading frames, respectively. It has been reported that *CST* has eight splicing variants, all of which have the same coding region with different 5′-UTR sequences (16). *CST* form F_{TS} is the testis-specific form among splicing variants expressed in other tissues. Arrows denote the positions of primers used to evaluate the expression of *CST* form F_{Total} (F) and total *CST* (T). Red arrows indicate the transcription start site of *CST* form F_{SS} and *CST* form F_{TS}. A dotted line indicates sequences shown in panel B. (B) Sequences surrounding exon 1f. The gray box represents the reported exon 1f sequences. The numbering is relative to the transcription start site of *CST* form F_{TS}, which is marked by a red arrow. The reported transcription start site is marked by a dotted red arrow. The red box shows BORIS/CTCF-contacting residues as determined in a methylation interference assay. Black arrows indicate primers for *CST* form F_{SS}. The dotted black arrow indicates a forward primer for *CST* form F_{Total}. DNA fragments used for EMSA are denoted by solid lines or dotted lines in black or red. (C) cDNAs prepared from wild-type or *BORIS*$^{-/-}$ mouse testis at P14 ($n = 3$) or at 3 months ($n = 3$) were

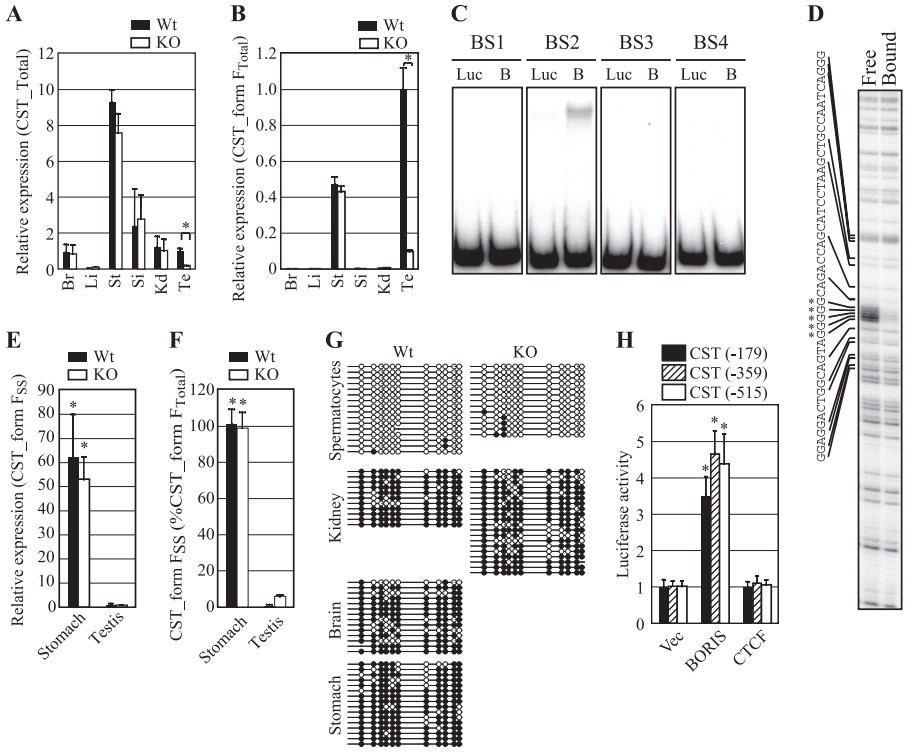

FIG. 5. (A and B) Expression of *CST* and *CST* form F in various tissues of wild-type and *BORIS*$^{-/-}$ mice was analyzed by qPCR ($n = 3$). Primers for *CST* form F_{Total} were used in panel B. Expression levels are shown as the ratio to wild-type testis levels. Asterisks denote statistical significance ($P < 0.005$). (C) BORIS binding to the promoter region of exon 1f was analyzed by EMSA using fragments shown in Fig. 4B. Luciferase protein was used as a negative control. Luc, luciferase; B, BORIS. (D) Methylation interference assay of the BS2 fragment using 11-ZF. Partial sequences of the fragment are shown. Only the bottom strand is shown, as the top strand did not show any differences. Left lane, unbound fragments; right lane, bound fragments. Asterisks indicate contacting guanine residues. (E) Expression of *CST* form F_{SS} in stomach and testis of wild-type and *BORIS*$^{-/-}$ mice was analyzed by qPCR ($n = 3$). Expression levels are shown as the ratio to wild-type testis. Asterisks denote statistical significance versus the testis sample ($P < 0.005$). (F) The ratio of *CST* form F_{SS} in *CST* form F_{Total} was analyzed by qPCR ($n = 3$). Due to the massive and specific reduction of *CST* form F_{TS} expression in *BORIS*$^{-/-}$ testis, the ratio of *CST* form F_{SS} in *CST* form F_{Total} was slightly increased in *BORIS*$^{-/-}$ testis. Asterisks denote statistical significance versus the testis sample ($P < 0.01$). (G) Methylation status around exon 1f was analyzed by bisulfite sequencing. Open and filled circles represent unmethylated and methylated cytosines, respectively. (H) Activity of the *CST* form F_{TS} promoter was analyzed by luciferase assay ($n = 3$). Empty vector (vec), BORIS, or CTCF expression vectors were cotransfected with each construct. Numbers indicate the 5′ end of each construct on the *CST* form F_{TS} promoter region. Luciferase activities are shown as the ratio to the empty vector-transfected sample. Asterisks denote statistical significance versus empty vector-transfected sample ($P < 0.05$).

BORIS regulates expression of the testis-specific *CST* splicing variant *CST* form F_{TS}. To analyze the expression pattern of *CST*, we performed qRT-PCR using cDNA prepared from various tissues. As reported previously, *CST* was widely expressed in multiple tissues including testis although expression of *CST* form F was restricted to stomach and testis (16) (Fig. 5A and B). Consistent with the known expression pattern of *BORIS*, expression of *CST* and *CST* form F was greatly dimin-

subjected to qPCR to evaluate the expression of *CST*. Primers for total *CST* were used for the experiment. Expression levels are shown as the ratio to wild-type level. Asterisks denote statistical significance ($P < 0.005$). (D) cDNAs prepared from wild-type or *BORIS*$^{-/-}$ mouse testes at P14 ($n = 3$) or at 3 months ($n = 3$) of age were subjected to qPCR to evaluate the expression of *CST* form F. Primers for *CST* form F_{Total} were used for the experiment. Expression levels are shown as the ratio to the wild-type level. Asterisks denote statistical significance ($P < 0.005$). (E) cDNAs prepared from wild-type or *BORIS*$^{-/-}$ (*-neo*) mouse testis at P28 were subjected to qPCR to evaluate the expression of *CST* form F ($n = 3$). Primers for total *CST* were used for the experiment. Expression levels are shown as the ratio to wild type. The asterisk denotes statistical significance ($P < 0.005$). (F) cDNAs prepared from wild-type or *BORIS*$^{-/-}$ (*-neo*) mouse testis at P28 were subjected to qPCR to evaluate the expression of *CST* ($n = 3$). Primers for *CST* form F_{Total} were used for the experiment. Expression levels are shown as the ratio to wild type. The asterisk denotes statistical significance ($P < 0.005$). (G) cDNAs prepared from wild-type testes were subjected to qPCR analysis to evaluate the expression of *CST* during testis development. Primers for total *CST* were used for the experiment ($n = 3$). Expression levels are shown as the ratio to the P2 testis level. The asterisk denotes statistical significance ($P < 0.005$). (H) cDNAs prepared from wild-type testes were subjected to qPCR analysis to evaluate the expression of BORIS during testis development ($n = 3$). Expression levels are shown as the ratio to the P2 testis level. (I) Expression levels of *BORIS*, *CST*, and *CTCF* were analyzed by RT-PCR. cDNAs were prepared from liver (Li), spermatocytes (SC), and round spermatids (RS). (J to L) cDNAs prepared from SC and RS were subjected to qPCR analysis to evaluate the expression levels of *BORIS* (J), *CST* (K), and *CTCF* (L) ($n = 3$). Expression levels are shown as the ratio to the level in spermatocytes. Asterisks denote statistical significance ($P < 0.05$).

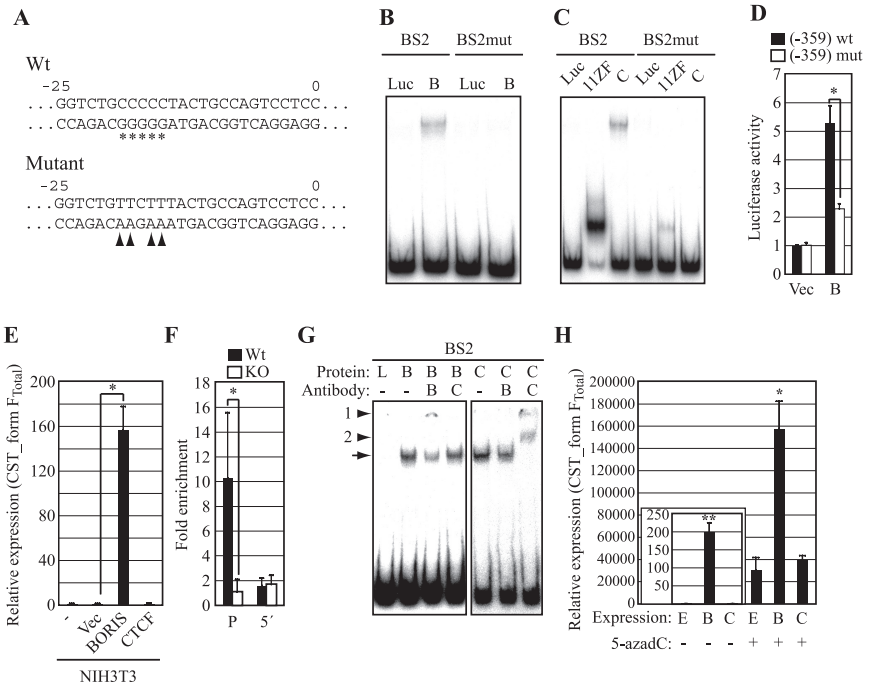

FIG. 6. (A) Sequences of wild-type and mutants of the *CST* form F_{TS} promoter. Asterisks indicate contacting guanine residues. Four contacting guanines were converted into adenines (arrowheads) in mutant constructs. Numbering shows the positions relative to the transcription start site of *CST* form F_{TS}. (B) Binding of BORIS to the mutated BS2 fragment was analyzed by EMSA. Luc, luciferase; B, BORIS. (C) Binding of 11-ZF and CTCF to the mutated BS2 fragment was analyzed by EMSA as for panel B. C, CTCF. (D) Luciferase assays were performed using a mutated promoter of the *CST* form F_{TS} ($n = 3$). Empty vector or BORIS expression vector were cotransfected with each construct. Luciferase activities are shown as the ratio to empty vector-transfected sample. The asterisk denotes statistical significance ($P < 0.05$). Vec, empty vector; B, BORIS. (E) Effect of BORIS on *CST* form F_{Total} expression was analyzed by transient transfection of a *BORIS* expression vector into NIH 3T3 cells. Expression of *CST* form F_{Total} was analyzed by qPCR ($n = 3$). Expression levels are shown as the ratio to mock-transfected sample. The asterisk denotes statistical significance versus empty vector-transfected sample ($P < 0.005$). Vec, empty vector. (F) Binding of BORIS on the *CST* form F_{TS} promoter (P) and 1.6 kb upstream of the transcription start site (5′) in testis was analyzed in a ChIP-qPCR assay using anti-BORIS antibody ($n = 4$). The asterisk denotes statistical significance ($P < 0.05$). (G) Recognition of BORIS/DNA complexes by BORIS antibody was validated by supershift assay. Fragment BS2 was preincubated with BORIS, CTCF, or luciferase followed by incubation with antibody against BORIS or CTCF. The arrow indicates the shifted band. Arrowhead 1 indicates a supershifted band with BORIS antibody, and arrowhead 2 indicates a supershifted band with CTCF antibody. B, BORIS; L, luciferase; C, CTCF. (H) Effects of BORIS and 5-azadC on *CST* form F_{Total} expression were analyzed by transient transfection of a expression vector into NIH 3T3 cells either treated or not with 1 μM 5-azadC. Expression of *CST* form F_{Total} was analyzed by qPCR ($n = 3$). Cells were cultured with or without 5-azadC for 24 h followed by transient transfection. Cells were further cultured for 48 h and harvested for analysis. Expression levels are shown as the ratio to empty vector-transfected cells without 5-azadC. *, statistically significant versus empty vector-transfected cells without 5-azadC ($P < 0.01$); **, statistically significant versus empty vector-transfected cells with 5-azadC ($P < 0.005$). E, empty vector; B, BORIS; C, CTCF.

ished in *BORIS*$^{-/-}$ testis but not in the other tissues, including stomach, suggesting that *CST* form F promoter contains a potential BORIS binding site (Fig. 5A and B).

To determine if BORIS would indeed bind to the promoter region, we performed EMSAs using fragments derived from the promoter region of *CST* form F (Fig. 4B) and found that BORIS bound to fragment BS2 (Fig. 5C). This fragment was also capable of binding CTCF (Fig. 6C). Neither BORIS nor CTCF (data not shown) bound any of the other fragments tested. To identify the BORIS binding site in fragment BS2, we performed methylation interference assays of the fragment using the 11-ZF domain of CTCF, based on its known strong binding to CTCF target sequences *in vitro* and the shared recognition sequence of BORIS and CTCF (Fig. 5D). We found that 11-ZF bound to tandem guanine residues that are located within exon 1f (Fig. 4B).

These results indicate that a BORIS/CTCF target site is located inside exon 1f. Although it was reported that BORIS/CTCF target sites could exist inside exonic sequences (52), most proteins that activate transcription bind upstream of the transcription start site. In view of the finding that the mechanisms governing activation of the *CST* form F promoter seemed to differ between testis and stomach (Fig. 5B), we hypothesized that there is a testis-specific transcription start site downstream of the BORIS/CTCF target site different from that previously described for exon 1f. To analyze this possibility, we performed 5′-RACE using testis RNA and found that all 10 *CST* transcripts analyzed started from exon 1f downstream from the BORIS/CTCF target site (Fig. 4B). Furthermore, we confirmed that *CST* form F transcripts highly expressed in stomach were initiated upstream from the BORIS/CTCF target site (Fig. 5E). Virtually all the *CST* form F transcripts expressed in testis were the short form, while the *CST* form F expressed in stomach was exclusively the longer form (Fig. 5F). Hereafter, we call the short form of the CST form F transcript *CST* form F_{TS}, for testis specific, the origi-

nally reported longer form *CST* form F_{SS}, for stomach specific, and both forms when measured together as *CST* form F_{Total}.

Methylation of CpG sequences in promoter regions is one of the major mechanisms responsible for suppression of gene expression. To analyze the methylation status of the *CST* form F_{TS} promoter, we performed bisulfite sequencing and found that the region was highly methylated in tissues that didn't express *CST* form F_{TS}—kidney, brain, and stomach—but was fully unmethylated in spermatocytes, even in *BORIS*$^{-/-}$ mice (Fig. 5G). This suggested that promoter methylation was responsible for suppression of *CST* form F_{TS} expression, although BORIS being present or absent had no effect on the methylation status of its promoter.

Taken together, these results indicate that transcription of *CST* form F_{TS}, which is initiated downstream from the BORIS/CTCF target site, is regulated by BORIS in testis, while transcription of *CST* form F_{SS} is BORIS independent.

BORIS activates *CST* expression through binding to the BORIS/CTCF target site. To further understand the role of BORIS in regulating the *CST* form F_{TS} promoter, we performed luciferase assays using different fragments from the promoter region (Fig. 5H). All constructs cotransfected with a *BORIS* expression vector showed activation of the promoter, while cotransfection with a *CTCF* expression vector had no appreciable effect. This result suggests that BORIS activates the *CST* form F_{TS} promoter by binding to the BORIS/CTCF target site while CTCF cannot.

As another approach to establishing that activation of the *CST* form F_{TS} promoter was due to binding of BORIS to the BORIS/CTCF target site, we performed EMSA using fragments mutated on the residues recognized by the 11-ZF (Fig. 6A). BORIS did not induce a mobility shift with the mutated BS2 fragments, a finding common to the 11-ZF and full-length CTCF, indicating that BORIS as well as CTCF bound to the sequence (Fig. 6B and C). Furthermore, BORIS-induced activation of the promoter was diminished when the same mutation was introduced in the construct used for luciferase assays (Fig. 6D). We also confirmed that exogenous expression of BORIS, but not CTCF, induced expression of *CST* form F_{Total} in NIH 3T3 cells (Fig. 6E). To analyze binding of BORIS to the *CST* form F_{TS} promoter region, we performed a ChIP-qPCR assay using whole testes derived from wild-type and *BORIS*$^{-/-}$ mice. Consistent with the *in vitro* findings, we found specific enrichment of BORIS on the *CST* form F_{TS} promoter region from wild-type testis but not from *BORIS*$^{-/-}$ testis (Fig. 6F). The validity of using the rabbit anti-mouse BORIS antibodies for ChIP was confirmed by supershift assays (Fig. 6G).

Taken together, these results indicate that BORIS activates expression of *CST* form F_{TS} through binding to the BORIS/CTCF target site.

DISCUSSION

To understand the physiological roles played by BORIS, we generated *BORIS* knockout mice and found that BORIS is critical to normal spermatogenesis (Fig. 3). Although there were no apparent abnormalities in the population of testicular cells at P14, we found delayed spermatogenesis as early as P21, suggesting that BORIS functions in meiosis. We conclude that the reduced size and the hypocellularity of testes in *BORIS*$^{-/-}$ mice are probably due to a failure of meiosis resulting in the apoptotic death found in *BORIS*$^{-/-}$ testis.

Using microarray analyses, we found that BORIS regulated the expression of *CST*, as demonstrated by the greatly reduced levels of transcripts in *BORIS*$^{-/-}$ testis (Table 2). This alteration in *CST* expression was found in the testis but not the somatic tissues of *BORIS*$^{-/-}$ mice, keeping with the testis-specific expression of *BORIS* (Fig. 5A and B). The defect in spermatogenesis seen in BORIS-deficient mice at meiosis is a near replica of that seen in testes of *CST* knockout mice (18). On the other hand, although *CST* knockout mice showed complete sterility, *BORIS* knockout mice were still fertile. Since production of sperm far exceeds the number necessary for fertility, it is likely that residual expression of *CST* found in *BORIS*$^{-/-}$ testis is sufficient to maintain fertility even though it causes significant defect in spermatogenesis.

We found that *CST* was expressed in testis as a novel testis-specific isoform, *CST* form F_{TS}, which has shorter exon 1f sequences (exon $1f_S$) than described previously (16). *CST* form F_{TS} was the dominant form of *CST* expressed in testis even in *BORIS* KO mice (Fig. 5E and F), indicating that BORIS is important for activation of the *CST* form F_{TS} promoter but not for defining the transcription start site of that form. Consistent with the testis-specific expression of *CST* form F_{TS}, the promoter for this isoform was highly methylated in somatic tissues, including stomach, which expressed *CST* form F_{SS}, while it was unmethylated in spermatocytes (Fig. 5G). This suggests that methylation of the promoter is responsible for the repressing expression of *CST* form F_{TS}. The promoter for this isoform was unmethylated in spermatocytes not only in wild-type mice but also in *BORIS*$^{-/-}$ mice. The unmethylated status of the promoter may be responsible for the incomplete repression of *CST* expression in *BORIS*$^{-/-}$ testis, while also indicating that BORIS is not responsible for demethylation of the promoter. We confirmed that ectopic expression of BORIS in somatic cells treated with 5-aza-2′-deoxycytidine (5-azadC), which induces demethylation of CpG dinucleotides, synergistically activates the expression of *CST* form F_{TS} (Fig. 6H), although ectopic expression of BORIS alone or treatment with 5-azadC alone could also induce expression. Taken together, these results suggest that demethylation of the promoter is a prerequisite for BORIS to fully activate *CST* form F_{TS} expression.

We showed that BORIS binds the *CST* form F_{TS} promoter. Methylation interference assays followed by EMSA with mutated fragments revealed that the exact BORIS binding site in the promoter is highly homologous to the TAGGGGG-containing CTCF/BORIS binding sequence mapped by DeJong and coworkers in the testis-specific ALF gene promoter (8, 26), which has been termed the consensus CTCF binding site as identified by genome-wide ChIP analysis (27). Consistent with the finding of a BORIS/CTCF target site in the promoter, ectopic expression of BORIS activated the *CST* form F_{TS} promoter, but not the promoter mutated in the BORIS/CTCF target site. It is interesting that both BORIS and CTCF can recognize the BORIS/CTCF target site, but only BORIS can activate the promoter. The expression pattern of BORIS, CTCF, and CST in spermatogenic cells also suggests a BORIS-specific function in regulation of CST expression. These data provide a clear demonstration that BORIS is functionally different from CTCF, even though they have the same recogni-

tion sequences. Since the amino and carboxy termini of BORIS have no homology with the parallel sequences in CTCF, the distinct molecular functions of BORIS may be attributable to the unique protein-protein interactions specified by these sequences. Our observations on the role of BORIS binding site in activation of the *CST* form F_{TS} transcription are remarkably consistent with results on regulation of testis-specific *TFIIA* α/β-like factor (*ALF*) gene promoter obtained by DeJong and coworkers, who showed that unlike CTCF, expression of BORIS had an activating effect on the ALF promoter-reporter construct (9, 26). However, ALF only starts to express at the time point we performed microarray analysis, and that may be why we missed it as a target (15).

Although orthologs of *CST* can be found in species from zebrafish to humans, the sequence of exon $1f_S$ is found only in mammals. This suggests that the testis-specific regulation of *CST* expression by BORIS evolved after mammalians diverged. Interestingly, the sequence of exon $1f_S$ is not well conserved among mammals, while the BORIS/CTCF target site as well as the splice site flanking exon $1f_S$ are highly conserved (see Fig. S3 in the supplemental material). This suggests that the regulation of *CST* form F_{TS} expression by BORIS, which depends on the BORIS/CTCF site, is important in mammalian species, although the 5′-UTR sequence of exon $1f_S$ itself is not. It is noteworthy that *BORIS* is expressed in a variety of tissues in lower vertebrates, while expression is restricted to the testis in mammals (20). This suggests that the acquisition of BORIS-specific regulatory mechanisms developed in conjunction with the functional specialization of *BORIS* to testicular germ cells.

Aside from *CST*, we found a few other genes that are differentially expressed in testes of wild-type and BORIS-deficient mice. This is probably due in part to the fact that we analyzed testes from P14 mice to identify BORIS target genes that were responsible for initiating the spermatogenesis defect. This is the earliest stage of testicular development at which we can expect to identify differentially expressed genes. The fact that spermatogenesis is disturbed in *BORIS* KO mice makes the identification of potential BORIS target genes that function later in spermatogenesis difficult. Transcriptional profiles of purified testicular cell subsets would be certain to yield additional targets. However, we also found several genomic targets for BORIS binding that could not be correlated with transcriptional regulation, suggesting other BORIS functions. Further studies are required to examine these possibilities.

Although we found that BORIS is a major determinant of normal spermatogenesis, *BORIS*$^{-/-}$ male mice were still fertile, indicating that there are mechanisms that can compensate for the loss of BORIS. Since *CTCF* is the only known paralog for *BORIS*, *CTCF* may partially compensate for the loss of *BORIS*, enabling *BORIS*$^{-/-}$ male mice to remain fertile. To determine if this model is correct, it would be necessary to analyze BORIS/CTCF double mutant mice. In addition, we cannot rule out the possibility that there is a protein other than CTCF that can compensate for the loss of *BORIS*. Finally, the phenotypes associated with different gene knockout mice are notoriously strain dependent (1, 45). The mice evaluated in the current study had a mixed 129/B6 genetic background such that progressive introgression onto a pure background such as B6 could result in a strain with significant phenotypic differences.

Based on previous studies of BORIS and CTCF and their abilities to recognize common target sites, it is likely that BORIS will be found to have functions other than regulating spermatogenesis. Methylation-sensitive binding of CTCF to ICRs of *H19/Igf2* (24), *Rasgrf1* (58), and *Kcnq1* (13) regulates allele-specific gene expression of these imprinted loci in mouse and human somatic cells. However, ICRs alone are neither necessary nor sufficient for resetting of imprinting upon germ line transmission (32). Additional ICR-targeting sequences have been mapped for the *Rasgrf1* ICR (58) but remain unknown for *H19/Igf2* and other ICRs. Therefore, remethylation may indeed occur upon replacement of BORIS to CTCF as suggested (37) and be targeted to paternal ICRs from a distant BORIS binding site that remains to be mapped *in vivo* (32). Although offspring of *BORIS*$^{-/-}$ mice develop normally, indicating that at least some sperm can develop normally without *BORIS*, there might be defective germ cells with aberrant imprinting destined to die in *BORIS*$^{-/-}$ testis, a suggestion consistent with the high rate of apoptosis seen in testes of BORIS-deficient mice.

Most promising, perhaps, is the finding that expression of *Tsp50* is markedly altered in BORIS-deficient testes. TSP50 is known as a CTA (47, 59), is overexpressed in a high proportion of breast cancers (59), is a gene with promoter methylation associated with expression (22), and has a near-canonical CTCF target sequence in the promoter (data not shown). Further studies of this gene, its regulation, and its importance as a CTA may link it functionally to BORIS regulation of CTA and to BORIS as a CTA target for cancer treatment (36).

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