TauCstF-64 Mediates Correct mRNA Polyadenylation and Splicing of Activator and Repressor Isoforms of the Cyclic AMP-Responsive Element Modulator (CREM) in Mouse Testis

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TauCstF-64 Mediates Correct mRNA Polyadenylation and Splicing of Activator and Repressor Isoforms of the Cyclic AMP-Responsive Element Modulator (CREM) in Mouse Testis

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ABSTRACT

Spermatogenesis is coordinated by the spatial and temporal expression of many transcriptional and posttranscriptional factors. The cyclic AMP-responsive element modulator (CREM) gene encodes both activator and repressor isoforms that act as transcription factors to regulate spermiogenesis. We found that the testis-expressed paralog of Cstf-64, tauCstF-64 (gene symbol Cstf2t), is involved in a polyadenylation site choice switch of Crems mRNA and leads to an overall decrease of the Crems mRNAs that are generated from internal promoters in Cstf2t−/− mice. More surprisingly, loss of tauCstF-64 also leads to alternative splicing of Crems exon 4, which contains an important activation domain. Thus, testis-specific CREMtau2 isoform protein levels are reduced in Cstf2t−/− mice. Consequently, expression of 15 CREM-regulated genes is decreased in testes of Cstf2t−/− mice at 25 days postpartum. These effects might further contribute to the infertility phenotype of these animals. This demonstrates that tauCstF-64 is an important stage-specific regulator of Crems mRNA processing that modulates the spatial and temporal expression of downstream stage-specific genes necessary for the proper development of sperm in mice.

alternative splicing, cleavage and polyadenylation, CREM, spermatogenesis, τCstF-64

INTRODUCTION

The most common form of male infertility is idiopathic impairment of spermatogenesis [1–3]. Many idiopathic cases of male infertility are likely caused by autosomal recessive genes, including those involved in transcriptional and posttranscriptional control of gene expression [4–7]. During spermatogenesis, the expression of specialized transcription factors ensures that the complex processes of male gamete formation will be accomplished properly. One of those transcription factors is the cyclic AMP-responsive element modulator (CREM; gene symbol: Crems), which plays a central role in the control of spermatogenesis [8–10]. While CREM is expressed in many tissues, in testis it interacts with tissue-specific factors such as the activator of CREM in testis (ACT; gene symbol Fhl5) that support its central function to promote spermatogenesis [11]. CREM and ACT expression are spatially and temporally correlated during spermatogenesis [12], making correct expression of these transcription factors critical for male fertility [13].

In the adult mouse testis, the major protein isoform of CREM is called CREMt [14]. During testicular development, CREMt expression increases strikingly between 13 and 14 days postpartum (dpp). During that time period, many alternatively spliced and polyadenylated mRNA forms become prominent [14–19]. Some Crems mRNA isoforms also initiate from alternative transcription start sites in male germ cells [20]. One CREM protein isoform, the transcriptional repressor S-CREM, results from use of an internal translational start site [21]. CREMt functions as an activator of gene transcription of stage-specific genes required for male gamete formation [9]. Two additional isoforms, CREMt1 and CREMt2, are produced by alternative splicing of exons 4 and 7 in different tissues [14, 15, 22, 23]; these isoforms are also transcriptional activators. Consistent with these roles, male mice deficient for the Crems gene lack spermatogenesis, have reduced seminiferous tubule diameter, and halt spermatogenesis at early stages of round spermatid formation [24].

Alternative polyadenylation is a major contributor to the isoform diversity of the Crems gene. A developmental switch in polyadenylation site choice during spermatogenesis increases the stability of the Crems mRNA by truncating control elements in its 3′ untranslated region (UTR) [19]. One key cleavage and polyadenylation protein, the RNA-binding subunit (Cstf-64; gene symbol Cstf2 [25, 26]) of the cleavage stimulation factor (Cstf) has a testis-expressed paralog, τCstF-64 (gene symbol Cstf2t [27, 28]), necessitated by meiotic silencing of its X-linked paralog Cstf2 [29]. In somatic cells, Cstf-64 acts to bind to the downstream sequence element (DSE) during early recognition steps of polyadenylation so that cooperation between the Cstf complex and the cleavage and polyadenylation specificity factor (CPSF) can direct cleavage and subsequent poly(A) addition to occur at the favored site [30, 31]. In male germ cells, τCstF-64 appears to replace the functionality of Cstf-64 during meiosis and subsequent spermiogenesis [25, 32–34]. Thus, male mice lacking τCstF-64 are infertile, demonstrating low sperm counts and severe abnormalities in sperm morphology and motility [35–37]. These abnormalities appear to be due to large-scale alterations in genome expression [35, 38], suggesting that τCstF-64...
regulates multiple levels of gene expression in these animals beyond mRNA biogenesis.

We report here on experiments to determine how loss of Cstf2t-/- leads to male infertility through its action on important spermatogenic regulators like Crem. Overall, loss of Cstf2t-/- resulted in reduced expression of a specific CREM activator protein isoform (CREMt2) in mouse testes. Analysis of high-throughput sequencing of regions adjacent to polyadenylation sites (A-seq) revealed polyadenylation site switching in Crem mRNA to a more distal site in Cstf2t-/- mouse testes. However, changes in polyadenylation were not sufficient to account fully for the altered CREM protein expression in Cstf2t-/- mice. High-throughput cDNA sequencing (RNA-seq) and confirmatory quantitative RT-PCR revealed that additional reductions could be accounted for by reduced Crem mRNA isoforms initiating from internal promoters. Strikingly, Crem gene transcripts that omitted exon 4 were consistently reduced in knockout animals, suggesting that Cstf2t-/- was involved, either directly or indirectly, in exon 4 exclusion in mice. Consistent with reduced CREMt2 expression, 15 of 58 genes that were reported to be directly regulated by CREM were down-regulated at least 2-fold in Cstf2t-/- mice. Thus, Cstf2t-/- is important for the stage-specific regulation of Crem isoforms that further regulate the correct expression of downstream stage-specific genes required for the proper development of sperm in mice.

MATERIALS AND METHODS

Animal Use and Isolation of Seminiferous Tubules

Wild-type and Cstf2t-/- mice in a C57BL/6 (Charles River) background were bred in-house. Except where noted, all mice were 25 dpp [38]. All animals and testis tissues used in the study were obtained according to protocols approved by the Institutional Animal Care and Use Committee at the Texas Tech University Health Sciences Center in accordance with the National Institutes of Health animal welfare guidelines.

Seminiferous tubules were isolated as follows: a small incision was made in the tunica albuginea of the testis, and the contents (mainly seminiferous tubules) were gently collected in ~5 ml [34] of ice-cold Dulbecco Phosphate-Buffered Saline (DPBS; Life Technologies) supplemented with phenylmethanesulfonyl fluoride (PMSF). The contents were vigorously shaken, 5 min on ice, and the supernatant (containing mainly Leydig cells) was aspirated. The process was repeated three times. After the final settlement, the tissue was spun briefly in a microcentrifuge at 500 g for 3 min, and the supernatant (containing mainly Leydig cells) was aspirated. The process was repeated three times. After the final settlement, the tissue was spun briefly in a microcentrifuge at ~500 x g. The obtained seminiferous tubules were used to isolate either total RNA or protein.

Protein Isolation and Immunoblots

Seminiferous tubules from one animal were lysed in either 250 µl extraction buffer (DPBS, 0.5% Triton X-100 [v/v], 2 mM PMSF, 0.02% Na2O), or RIPA buffer (50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 0.1% sodium deoxycholate, 0.5% deoxycholate, 0.5% NP-40), briefly sonicated, and incubated on ice for 10 min. Lysates were spun down for 10 min at 400 g at 4°C for 10 min. Equal amounts of protein from wild-type and Cstf2t-/- animals were loaded on precast NuPAGE Novex 4%–12% Bis-Tris Gels (Life Technologies), followed by a semidry transfer to polyvinylidene fluoride membranes. Membranes were incubated with primary antibody as indicated, followed by the appropriate secondary antibody conjugated to horseradish peroxidase. The following primary antibodies were used: rabbit anti-CREM (Santa Cruz Biotechnology; X-12, sc-440, used at a dilution of 1:500), rabbit anti-SRP40 (Millipore; 06-1365, dilution of 1:1000), and rabbit anti-DAZAP1 (Bethyl Laboratory; A303-905A-T, dilution of 1:2000). Mouse anti-Csf6-64 (3A7) and mouse anti-cstf-64 (6A9) were used as described [25, 32]. The E7 anti-fl-bulb monoclonal antibody (developed by Michael Klymkowsky and obtained from the Developmental Studies Hybridoma Bank) was a gift from Daniel Webster and used at a dilution of 1:1000. Anti-SRSF10 (formerly SRP38, dilution of 1:1000) was a gift from Dr. James L. Manley [39].

RNA Isolation and RNA-Seq

RNA-seq was carried out as described previously [34]. Total RNA from seminiferous tubules from one animal was extracted using 1 ml TRIzol reagent (Life Technologies). Quantity of the isolated total RNA was determined by spectrophotometric measurement using the NanoDrop device. The quality of the RNA was determined using standard 1% agarose gel electrophoresis. For the RT-PCR and quantitative RT-PCR (qRT-PCR), the RNA was treated with RQ1 (RNA Qualified) RNase-Free DNase (Promega) as recommended. RNA-seq libraries from three wild-type and three knockout animals were prepared using Illumina’s TruSeq RNA Sample Preparation Kits v2 (Illumina Inc.). Brieﬂy, 4 µg of total RNA was poly(A)-selected, followed by generation of double-stranded cDNA. Adapters were ligated, and resulting fragments were amplified by limited number of PCR cycles. RNA-seq libraries were sequenced on HiSeq 2000 platform with 90-nucleotide coverage of each end (PE90). Over 41 million individual sequences from each library were collected with a Q20% larger than 97%.

A-Seq Library Preparation

A-seq was carried out essentially as described previously [34, 40]. Brieﬂy, 40 µg of total RNA were used to isolate the poly(A) fraction using the Dynabeads mRNA Direct Kit (Life Technologies). Poly(A)-selected RNA was partially digested with three different concentrations of RNase I (Ambion), resuspended for poly(A), and the 5' ends were phosphorylated on the Dynabeads. Subsequently, RNAs were eluted and 3' ends blocked using corydzipine 5'-triphosphate (Sigma) and Escherichia coli poly(A) polymerase (New England Biolabs). Simultaneously, the RNAs were treated with RQ1 RNase-Free DNase (Promega). After phenol-chloroform extraction and ethanol precipitation, a 5' adapter (RNA: Illumina) was ligated. RNAs were reverse transcribed to complementary DNA using a 3'adapter labeled primer. The cDNAs were resolved on 5% denaturing polyacrylamide gel, and the cDNAs between ~120 and ~150 nucleotides (nt) were isolated. PCR was performed with primers adapters similar to the TruSeq Small RNA Sample Preparation Kit (Illumina). The resulting cDNA libraries representing the 3' ends of polyadenylated RNAs were sequenced on Illumina platform with 50-nucleotide coverage (SE50). The obtained reads were strand specific and coincided with the sense strand of the mRNAs.

HITS-CLIP Library Preparation

Cross-linking and immunoprecipitation combined with high-throughput sequencing (HITS-CLIP) was performed exactly as previously described [41] except using both the Csf6-64-specific 3A7 and cStF-64-specific 6A9 monoclonal antibodies [25].

Bioinformatics

RNA-seq reads obtained from each biological replicate were independently aligned on the mouse reference genome (Mouse Genome v37.2, MGSC v37.2, mm9) using the SequMan v10 software (DNASTAR Inc.). The assembly files generated by SequMan were used to identify the differentially expressed genes (2-fold change) using the QSeq package of ArrayStar package (DNASTAR).

A-seq data were initially processed with the FASTQC package [42] to identify the nature of the typical spacing of 5' and 3' adapter sequences. Custom in-house software was then used to remove sequences that contained only the adapter with no inserts. As the amount of sequence between adapters was variable, a common target length of 24 nt anchored on the putative poly(A) site was chosen as a compromise between retaining genomic uniqueness and retention of the largest possible number of sequences. Sequences as short as 16 nt were also kept but then collapsed to a matching 24-nt tag. There was also variability across samples noted in the presence of the 3' adapter, indicating the precise site of poly(A) addition. In some libraries, a majority (>75%) of the sequences failed to show the adapter. However, analysis of the sequence content across the whole library suggested that the adapter sequence was immediately or very near the downstream of the end of the sequenced insert. These tags were labeled “near” rather than “exact” and subsequently assigned to the nearest ‘‘exact’’ wild-type and three knockout animals. To reduce computational time, the number of each tag was noted, and a working file was generated with only one representative copy of each putative tag. Representative sequences were aligned to a database of reference repetitive element sequences such that those sequences that matched the repetitive elements could be removed from downstream analysis. The remaining representative tags were then aligned to the mouse genome GIRC v38 with BLAT [43]. Tags were
assigned to the nearest properly oriented exon according to the Ensembl mouse v70 annotations [44].

HITS-CLIP sequence data was processed initially by the Beijing Genome Institute by removing low-quality sequences, identifying 5' and 3' adapters, extracting the insert sequence between these, filtering based on a minimum length, and reducing the file to only single instances of each unique sequence, retaining the number of times each sequence as part of its annotation. The resulting file was aligned to a database of reference repetitive element sequences such that those sequences that matched the repetitive elements could be removed from downstream analysis. Sequences that passed this filtering were then aligned to the mouse genome GRC v38 with BLAT [43]. Empirical analysis revealed that sequences could be grouped based on common 3'-end positions, and that position was subsequently used to identify and quantify putative binding site usage. Genomic alignments were further carried out and compared with the PIPE-CLIP analysis software [45], using standard parameters.

Analysis of Alternative Splicing

We manually created 40-nt-long query sequences that represented the exon-exon junctions in the ENSMUST00000150235 transcript (Tx1; see Fig. 3). Using a local BLAST engine, RNA-seq data sets were converted to a custom searchable library. Exon-exon junction sequences were used to query the custom data sets, and the number of raw hits was recorded. Raw hits were normalized using RPKM normalization method [46, 47], using 40 nt as length for the gene, consistently between the samples. Average RPKM was calculated, and a two-tailed Student t-test between similar pairs was performed to show statistical significance.

Reverse Transcription, RT-PCR, and qRT-PCR

Complementary DNA for RT-PCR and qRT-PCR was prepared from 80 ng of total RNA using SuperScript VILO MasterMix (Life Technologies) to verify gene expression levels. To confirm the cleavage and polyadenylation site choice in wild-type and Cstf2t−/− mice, we used oligo(dT)-primed RNA. RT-PCR was performed using EmeraldAmp GT PCR Master Mix (Clontech) for 25–30 cycles with exon-specific primers as indicated (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org). PCR products were resolved on 2% agarose gel electrophoresis stained with ethidium bromide and visualized with UV-transilluminator. Percent-spliced-in (PSI) values were calculated by measuring the relative intensity of the PCR product including exons 4 and 5 plus the intensity of the PCR product excluding exon 4 multiplied by 100%. Background intensity value was subtracted from the intensity of the PCR products; qRT-PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies) on the QuantStudio 12K Flex Real-Time PCR System in triplicate. The relative expression was calculated using the competitive Cq method using 18S rRNA as a reference for the gene expression study and Glud gene to normalize relative usage of cleavage and polyadenylation site choice of Crem gene [48].

RESULTS

CREMτ Protein Isoforms Are Reduced in the Seminiferous Tubules of Cstf2t−/− Mice

The balance of full-length CREMτ with its cognate protein isoforms is critical for progression through spermatogenesis [49]. These isoforms are controlled in part by hormonal regulation of selection among Crem 3' polyadenylation sites [11, 19]. Therefore, we examined CREM protein expression in lysates from seminiferous tubules from wild-type and Cstf2t−/− mice at 25, 31, 35, 38, and 77 dpp (Fig. 1A). The CREMτ protein was expressed in testes of wild-type and Cstf2t−/− mice of all ages (Fig. 1A). However, the CREMτ2 protein isoform was decreased relative to the major CREMτ isoform in seminiferous tubules from Cstf2t−/− mice at all ages (Fig. 1B). The S-CREM translational isoform [21] did not vary consistently between wild-type and Cstf2t−/− mouse seminiferous tubules (Fig. 1). This suggests that τCstF-64 supports both expression of CREMτ and expression of specific CREM isoforms in mouse testis but that not all isoforms are affected in the same manner. Consistent with previous reports, Cstf-64 expression was increased in the Cstf2t−/− mice likely due to posttranscriptional regulation [34, 35, 50]; 25 dpp was chosen for further studies because this age showed critical effects of Cstf2t loss while showing the least effects of later stages of germ cell loss (Supplemental Figure S1).

Crem Cleavage/Polyadenylation Switches from Proximal to Distal Sites in Seminiferous Tubules from Cstf2t−/− Mice

Altered CREM protein expression in seminiferous tubules from Cstf2t−/− mice suggested changes in Crem mRNA processing [10, 49]. To address polyadenylation changes, we constructed a genome-wide library representing 3'-ends formed by cleavage and polyadenylation in the seminiferous tubules of 25-dpp wild-type and Cstf2t−/− mice using the A-seq method [40]. Analysis of A-seq indicated that at least 11 cleavage and polyadenylation sites were present for Crem in testes of wild-type 25-dpp mice (Fig. 2A). Two of these sites (sites 3 and 8; Fig. 2A) were most prominent. Relative use of sites 3 and 8 differed between wild-type and Cstf2t−/− mice: site 3 was used most prominently in wild type mice, while site 8 was used most prominently in Cstf2t−/− mice (Fig. 2A). Relative use of each of these sites was confirmed by qRT-PCR (Fig. 2B).

To test whether τCstF-64 might play a direct role in Crem polyadenylation, we identified potential RNA binding sites for τCstF-64 by high-throughput sequencing of RNA isolated by cross-linking and immunoprecipitation (HITS-CLIP) using an antibody specific for mouse τCstF-64 [25, 32, 41]. HITS-CLIP did not identify unique τCstF-64 binding sites in the 3' exon of
FIG. 2. *Crem* cleavage/polyadenylation sites change in wild-type and *Cstf2t*−/− seminiferous tubules. A) A-seq reads representing cleavage/polyadenylation sites mapped on the mouse genome (mm10) from two wild-type (WT1, WT2, top orange bars) or two *Cstf2t*−/− (*Cstf2t*−/− 1, *Cstf2t*−/− 2, middle blue bars) mice. Values in orange (wild type) and blue (*Cstf2t*−/−) on right represent the highest number of reads assigned to a specific 3′ cleavage site in the corresponding tracks. Specific polyadenylation sites are designated 1 through 11 (pA#1–11, indicated at bottom). Bottom panels show τCstF-64 (red, top) and CstF-64 (green, bottom) binding sites determined using HITS-CLIP. The last intron and 3′ exon of *Crem* are shown (blue). Distal and proximal primer pair.

C

pA#3 UAG-53nt- AAUACAAUAUAAUGCCGGCAAGAAUUAUGGCUUUUUCUU

pA#8 UAG-684nt- AAUGAAAGAGACCAAUAUUUUCAGAAUCUGGAAUGAGUU

FIG. 2. *Crem* cleavage/polyadenylation sites change in wild-type and *Cstf2t*−/− seminiferous tubules. A) A-seq reads representing cleavage/polyadenylation sites mapped on the mouse genome (mm10) from two wild-type (WT1, WT2, top orange bars) or two *Cstf2t*−/− (*Cstf2t*−/− 1, *Cstf2t*−/− 2, middle blue bars) mice. Values in orange (wild type) and blue (*Cstf2t*−/−) on right represent the highest number of reads assigned to a specific 3′ cleavage site in the corresponding tracks. Specific polyadenylation sites are designated 1 through 11 (pA#1–11, indicated at bottom). Bottom panels show τCstF-64 (red, top) and CstF-64 (green, bottom) binding sites determined using HITS-CLIP. The last intron and 3′ exon of *Crem* are shown (blue). Distal and proximal primer pair.
FIG. 3. Crem transcript variants expressed in wild-type and Cstf2t−/− mouse seminiferous tubules. A) Map of the Crem gene showing promoters, exons, and introns. Encoded protein isoforms of CREM are shown on left and transcript variants (Tx 1–10) on right. Tx1 corresponds to Ensembl transcript ENSMUST00000150235, Tx7 corresponds to ENSMUST00000134715, and Tx9 corresponds to ENSMUST00000122958. Promoters P1–P4 are shown with their specific location within the gene. Exons numbered 1 through 9 and 10d (distal) encode variants of CREMt with initiation at P1 corresponding to transcripts 1 to 6 (Tx1–6); mRNA variants splicing at exon 10p (proximal) encode the CREMsa isoform (Tx4). Exons 3a and 3b represent transcripts initiating in promoters P3 (Tx7, Tx8) and P4 (Tx9–10), respectively. Exons 4 and 7 (encoding the glutamine-rich activator domains Q1 and Q2), the P-box (containing a phosphorylation site), the c domain, and two DNA binding domains (DBDI and DBDII) are indicated at top.

B) RPKM-normalized RNA-seq data of exon-exon junction for Tx1 in wild-type (in orange) and Cstf2t−/− (in blue) mouse testes. The asterisk (*) denotes $P < 0.05$ by a two-tailed Student $t$-test.

C) RPKM-normalized RNA-seq data of exon-exon junctions ex3–ex4 (Tx1), ex3a–ex4 (Tx7), and ex3b–ex4 (Tx9) in wild-type (in orange, yellow, and brown) and Cstf2t−/− (in blue, light blue, and dark blue) mice, respectively. Bar graph on right represents ex4–ex5 taken from B for comparative purposes.

D) RPKM-normalized RNA-seq data of exon-exon junction for transcripts skipping exons 2–9 in wild-type (in orange) and Cstf2t−/− (in blue) mouse testes derived from Tx1. E) RPKM-normalized RNA-seq data of exon-exon junction for transcripts skipping exon 4 in transcripts initiating in P1 (exon 3), P3 (exon 3a), and P4 (exon 3b) in wild-type (orange) and Cstf2t−/− (blue) mouse testes.

I primer pairs used to confirm cleavage and polyadenylation switch in the last exon of Crem in B are indicated. B) Relative mRNA abundance of transcripts terminating at pA#3 and pA#8 in seminiferous tubules from two independent wild-type (orange) and two independent Cstf2t−/− (blue) mice as determined by qRT-PCR (primers in Supplemental Table S1). C) 3′ cleavage sites, putative polyadenylation signals (underlined), and U-rich downstream sequence elements (italic) for pA#3 and pA#8. A-seq reads corresponding to the sites of poly(A) addition are indicated above the sequences in orange (wild type) and blue (Cstf2t−/−). Reads represent the sum of the A-seq reads shown in A for the two wild-type and two knockout animals, respectively.

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Crem in wild-type mouse testes (Fig. 2A). However, it did identify shared sCstF-64/CstF-64 binding sites 150–175 nt downstream of polyadenylation site 8 (Fig. 2A). The relative strengths of the polyadenylation signals (PAS) at sites 3 and 8 revealed that site 3 is “stronger” (i.e., it conforms better to the PAS consensus [51]). Typically, polyadenylation results in addition of poly(A) at one or two closely spaced nucleotides at each site [52]. Further examination of A-seq indicated that loss of τCstF-64 caused the specific sites of poly(A) addition to spread over a broadly spaced range of nucleotides near site 3 (Fig. 2C), a phenomenon we refer to as “defocusing.” We saw less evidence of defocusing at site 8 (Fig. 2C). However, we did not see HITS-CLIP evidence supporting binding of either CstF-64 or τCstF-64 to any of the nearby (≤25 nt) GU-rich downstream sequences at site 3 (Fig. 2, A and C).

Many Variant Crem Transcripts Are Expressed in Mouse Testes

The Ensembl transcriptome database [44] describes 41 transcript variants for the Crem gene, making analysis of the RNA-seq data more complex. In Figure 3A, the transcripts designated Tx1 (which corresponds to ENSMUST00000150235) and Tx4 encode CREMt and CREMtx [17], respectively. Using Tx1 as a template, we designed 40-nt-long exon-exon junction sequences (20 nt on each side of the junction) for each pair of adjacent exons (e.g., ex1–ex2, ex2–ex3, etc.). We mapped the RNA-seq data from wild-type mouse to Crem using local BLAST and identified the number of reads that mapped to the exon junctions. In wild-type mice, the highest RPKM observed was 207.3 for the ex4–ex6 junction (Fig. 3B), and the lowest was 32.3 for the ex3–ex4 junction. The decreased number of reads mapped to the Crem 5’ end suggested that there are Crem transcripts initiating from promoters P3 (exon 3a) and P4 (exon 3b) in our mice. This pattern was seen in both wild-type and Cstf2t/C0/C0 mice (Fig. 3E). GROZDANOV ET AL.

Expression of Crem mRNA Downstream of Exon 4 Is Reduced in Cstf2t+/− Mice

To examine overall expression levels of Crem mRNA in the testes of wild-type and Cstf2t+/− mice, we compared the abundances of the individual exon-exon junctions. Overall, Crem mRNA expression was reduced 1.4-fold in Cstf2t+/− mice compared to wild type (ex1–ex2 to ex9–ex10; Fig. 3B). However, when regions of Crem downstream of exon 4 (ex4–ex5 to ex9–ex10) were analyzed separately, Crem was reduced 1.5-fold. These transcripts initiate at promoters P3 and to a smaller extent P1 (Fig. 3A). Because the transcriptional activation domains Q1 and Q2 of CREM are encoded by exons 4 and 7, this suggests that the activation functions of CREMt might be reduced in Cstf2t+/− mice (Fig. 3B; see also Fig. 1).

Alternatively Spliced mRNA Variants of Crem in Testes of Wild-Type and Cstf2t+/− Mice

Crem expression is regulated extensively by alternative splicing [14, 15, 22, 23], so we chose to determine whether loss of τCstF-64 affected Crem splicing. CREMt1 (Fig. 3A; Tx2) and CREMt2 (Tx3) are formed by alternative cassette splicing of exons 4 and 7 of Crem [15, 17, 22]. In addition, skipping of exons 2 (Tx5, Tx6) and 8 has been reported [12, 54]. Therefore, we assessed the total number of normalized reads from exons that skipped the immediate downstream exon in Crem transcripts in testes of wild-type mice and expressed that as a percentage of the total (Fig. 3, D and E). For example, we created a sequence query that skipped exon 2; for example, the first 20 nt of the query were within the 3’ end of exon 1, and the remaining 20 nt were within the 5’ end of exon 3. We observed that exons 3, 5, 6, and 9 were never excluded from Crem transcripts. However, approximately 20% of transcripts excluded exon 4 in wild-type mice (Fig. 3A; Tx3, Tx6, Tx8). Approximately 17% of the mRNAs encoding CREMt (Tx1) excluded exon 2 (Fig. 3D). Only 0.7% of Crem transcripts excluded exon 7 (Tx3), and 1.2% of Crem transcripts excluded exon 8. Crem transcripts initiating at P3 (Tx7, Tx8) had an approximately equal number of reads that included or excluded exon 4 (Fig. 3, C and E). However, only 3.2% of reads corresponding to Tx9 (initiating at P4) skipped exon 4 (Fig. 3, C and E). Thus, the most abundant transcript produced in the seminiferous tubules of 25-dpp wild-type C57BL/6 mice is Tx9 (55.8%), followed by Tx1 (34.6%).

Crem Isoforms That Exclude Exon 4 Are Downregulated in Cstf2t+/− Mice

To assess differential expression of Crem mRNA isoforms that include (Tx1, Tx2, Tx4, Tx5, Tx7, Tx9) or exclude (Tx3, Tx6, Tx8, Tx10) exon 4, we examined the numbers of reads mapping to the junctions between exons 3 and 4, 3a and 4, and 3b and 4 (Fig. 3C). RNA-seq data indicated that expression of Tx9 did not differ significantly between wild-type and Cstf2t+/− mouse testes. However, reads representing Tx7 were 2.2-fold down-regulated in testes of Cstf2t+/− mice (Fig. 3C). Transcripts skipping exon 4 were down-regulated in all three variants analyzed (Tx1, Tx7, Tx9) in Cstf2t+/− mice (Fig. 3E).
tauCstF-64 CONTROLS CREM GENE EXPRESSION

With primer set B, we saw smaller PSI values than with primer set A (Fig. 4, B and E). This was consistent with exon 4 exclusion from exon 3a (initiating at promoter P3). PSI values for primer set B in Cstf2t−/− mice were also consistently greater than in wild-type mice. This finding agrees with the decrease of reads in the knockout mice skipping exon 4 (Fig. 3E). Because exon exclusion is observed for all transcripts initiating at exon 3a, this further suggests that the feature or features regulating exon 4 inclusion/exclusion are downstream of exon 3a (Fig. 3).

Amplification of mRNA between exon 3b and 7 (primer set C) showed almost no alternative splicing between exons 3b, 4, and 5 in either wild-type or Cstf2t−/− mice (Fig. 4C). The relatively small size of the intron that separates exons 3b (whose transcription initiates at P4) and exon 4 may preclude it from participating in alternative splicing of exon 4.

Alternative Splicing of Crem Does Not Involve Altered Expression of Known Splicing Factors

Alternative splicing of Crem has been studied in cell lines representing male germ cells and other tissues, and these events are under the control of specific splicing factors [15–18]. Thus, two models could account for τCstF-64-dependent exon 4 skipping in mouse testes: 1) τCstF-64 could affect expression of specific splicing factors that in turn control Crem exon 4 inclusion or exclusion, or 2) τCstF-64 could bind directly to Crem pre-mRNA to enhance exon 4 exclusion (probably in concert with other splicing factors). We first tested the expression of several splicing factors that have been reported to control splicing of Crem exons 4 and 7 [15–18]. Using antibodies recognizing either SRp38/NSSR1 (Srsf5), SRp40 (Srsf5), or DAZ associated protein 1 (Dazap1), we performed immunoblots on seminiferous tubule lysates from 25-dpp wild-type and Cstf2t−/− mice. We observed no consistent changes in expression of any of these splicing factors between three wild-type and three Cstf2t−/− mice at 25 dpp (Supplemental Figure S2B). This suggests that none of these splicing factors are likely to affect Crem splicing due to changes in their protein expression levels.

Our next question was whether τCstF-64 was associated with Crem pre-mRNA in the vicinity of exon 4. HITS-CLIP data indicated that τCstF-64 bound to the Crem pre-mRNA in several locations within the intron between exons 3 and 4 (Supplemental Figure S2A). Since our previous analysis suggested that transcripts initiating at exon 3a had a greater degree of exon 4 exclusion (Fig. 4B), this eliminated the τCstF-64 HITS-CLIP sites upstream of exon 3a. Thus, the strongest candidate for a direct influence of τCstF-64 on Crem alternative splicing is the site approximately 1850 nt downstream of exon 3a (Supplemental Figure S2A). Of interest, this HITS-CLIP site coincides with a long interspersed nuclear element (LINE) repeat within this intron. Our previous studies have shown that LINE expression increases in Cstf2t−/− mouse testis [38]. This suggests other roles in addition to polyadenylation for τCstF-64 in male germ cells.

Expression of a Subset of CREM-Regulated Genes Is Altered in Cstf2t−/− Mice

Changes in CREM expression in Cstf2t−/− mice appeared to alter the ratio between activator and inhibitor CREM isoforms (Fig. 1). Therefore, we investigated the expression of genes that are regulated by CREM in the wild-type and Cstf2t−/− mice. From the RNA-seq results, we examined the expression of 58 genes whose promoters were shown to be occupied by CREM in haploid germ cells and down-regulated in Crem knockout
animals [55]. Fifteen of the 58 genes were at least 2-fold down-regulated in testes of 25-dpp Cstf2t/C0/C0 mice; expression of the remaining genes was relatively unchanged (Fig. 5A). Four down-regulated genes that were important for spermatogenesis and another four that did not change expression were selected for further analysis by qRT-PCR (Fig. 5C). Gene ontology (GO) term analysis [56] of the down-regulated CREM-regulated genes indicated that six genes (Prm1, Tssk1, Tssk2, Tnp1, Tnp2, Hspa1l) were enriched in GO terms for both spermatogenesis (GO term: 0007283, P-value, 5.2 × 10^{-7}, and a false discovery rate [FDR] of 6.1 × 10^{-4}) and male gamete generation (GO term: 0048232, P-value, 5.2 × 10^{-7}, and FDR, 6.1 × 10^{-4}). Four of the genes (Tnp2, Prm1, Tssk1, Tssk2) are located in two ~10-kb clusters on mouse chromosome 16. No clustering was observed for the genes with unchanged expression. However, we noticed that the down-regulated genes consisted mainly of genes that had a smaller number of transcript variants (taken from the National Center for Biotechnology Information database) due to alternative promoters, splicing, and polyadenylation. The cohort of genes with reduced expression had a median of one transcript per gene, while genes with unchanged expression had a median of four transcript variants per gene (Fig. 5B). This suggests that other mechanisms of transcript regulation, such as intrinsic regulation of genes with only single polyadenylation sites, might be active in testis and in physiological and regulatory clustering of these genes as well as ontological clustering.

In addition, we examined the expression of the CREM-associated gene ACT (Fhl5), kinesin family member 17 (Kif17), and general transcription factor IIA (Gtf2a1) by RNA-seq and qRT-PCR (Fig. 5D). We found that two of these genes, Kif17 and Fhl5, showed decreased mRNA expression in
the Cstf2t/C0 mice, while Gif2a1 did not. This indicates that Kif17 and Fhl5 can limit the availability of CREM to promoter region of specific genes involved in spermatogenesis, contributing further to the male infertility phenotype of Cstf2t/C0 mice.

**DISCUSSION**

**τCstF-64 Controls CreM Polyadenylation in Mouse Testis**

Deletion of Cstf2t in mice results in male infertility due to cumulative defects in spermatogenesis and leads to altered expression of thousands of genes [35–38]. We proposed two mechanisms by which τCstF-64 controlled male fertility: 1) a smaller number of genes were affected directly by loss of τCstF-64 (“primary targets”), and 2) a larger number of genes (“secondary targets”) were affected by changes in expression of those primary targets. One primary target of τCstF-64 appears to be the transcriptional regulator CreM. To date, neither CSTF2T nor CREM has been directly implicated in human male infertility [57, 58], although gene deletions in mice have supported both possibilities [35, 49]. Multiple CREM and CREMt protein isoforms are reduced in testes of Cstf2t/C0 mice, although some were not affected (Fig. 1). This, combined with CstF-64’s known role in mRNA processing, suggested to us that different CreM transcripts were affected differentially by τCstF-64. We identified 11 cleavage/polyadenylation sites in the 3’ exon of the CreM gene, of which two were most prominent (Fig. 2A). The proximal site 3 is used most prominently in wild-type mice, while the more distal site 8 is most prominent in Cstf2t/C0 mice (Fig. 2B). Shortening of the 3’ UTR of CreM occurs during male germ cell development in mice between 13 and 14 dpp [19], coincident with the appearance of τCstF-64 [32]. This suggests that the appearance of τCstF-64 in pachyplast, the stage of meiotic prophase prior to spermiogenesis, is a significant contributor to the developmental shift in CreM polyadenylation and thus controls CREM protein isoforms necessary for spermiogenesis. Further, the developmental switch of CreM toward a shorter 3’ UTR reportedly eliminates AUUUA destabilizing elements, increasing the stability of the CreM mRNA [19]. A shorter 3’ UTR would also eliminate a conserved miR206/613 binding site [59]. Thus, we propose that major forms of CreM mRNA are less stable in Cstf2t/C0 mice, accounting for overall decreased CREMt1/2 protein isoforms (Fig. 1).

HITS-CLIP did not identify unique τCstF-64 binding sites in the 3’ exon of CreM, although it identified RNA sites that were bound to both τCstF-64 and CstF-64 (Fig. 2A). Thus, we cannot distinguish possible contributions of τCstF-64 from those of CstF-64. Furthermore, these sites were ~150 nt downstream of the distal site and more than 800 nt downstream of the proximal site. This suggested that polyadenylation of CreM does not correlate directly with nearby binding of τCstF-64. Other studies have similarly shown that only a subset of polyadenylation sites are correlated with Cstf-64 [60] or τCstF-64 [61] binding in HeLa cells.

To explain this, the model that we favor is that τCstF-64 (and, by extension, CstF-64) is dispensable for cleavage and polyadenylation of many genes. The role for τCstF-64 is instead in directing polyadenylation site choice; in its absence, polyadenylation sites are chosen by other default termination mechanisms [62–64]. We favor this model for several reasons. Early in vitro studies showed that CstF enhanced polyadenylation of “strong” sites [65] but was less important for “weak” sites [66]. Knockdown [50, 61, 67] and knockout [34, 35, 38] studies of both Cstf-64 and τCstF-64 have shown that each, while critical for cellular growth and development, was not necessary for expression of most genes. In further support of this model, we note that spermatogenesis, while greatly impaired in testes of Cstf2t/C0 mice, still produces a few motile spermatozoa [36, 37]. This demonstrates that gene expression is compromised but not arrested in male germ cells in the absence of τCstF-64-mediated polyadenylation. Similarly, embryonic stem cells lacking Cstf-64 are able to differentiate into some developmental cell types but not others [68], suggesting that some but not all gene products require Cstf-64 for their expression.

The A-seq technique allows us to assign exact sites of poly(A) addition in the 3’ exon. Thus, in further support of the role of τCstF-64 in directing polyadenylation site choice, we note that loss of τCstF-64 results in spreading of the frequency of the A-seq signal across a broader range of sites—“defocusing” (see Fig. 2C). Defocusing resembles an effect of Cstf-64 reported earlier on the SV40 late polyadenylation signal, wherein the site of polyadenylation bifurcated as the Cstf binding site was moved distally [30]. However, we cannot explain how defocusing in the CreM 3’ exon occurs with the lack of evidence for τCstF-64 binding at the GU-rich DSE at this polyadenylation site (pA#3; Fig. 2C). Defocusing might be evidence supporting the default role of termination in mRNA 3’ end formation in Cstf2t/C0 mice. However, we have not ruled out other models, including that the absence of τCstF-64 results in structural changes in the CstF or CPSF complexes that alter functions at the cleavage site, even in the absence of a measurable binding site for τCstF-64. Similar structural changes occur in the histone mRNA 3’ end complex in the absence of Cstf-64 [34], lending support to this model.

**Alternative Splicing of CreM in Cstf2t/C0 Mice**

More unexpectedly, loss of τCstF-64 in Cstf2t/C0 mouse testes altered the splicing of CreM, specifically by reducing exclusion of exon 4. Exon 4 encodes a glutamine-rich, Q1 domain that interacts with ACT, a potent transcriptional activator [11–13]. The interaction between CREMt and ACT bypasses the requirement for a phosphorylation of the P-box domain of CREMt by PKA and facilitates the activator function of CREMt on transcription in male germ cells [9, 69]. We propose that τCstF-64 mediates the exclusion of the Q1 domain of CREMt (which includes exon 4), forming CREMt1/2 (which exclude exon 4). Thus, splicing-mediated reductions in CREMt1/2 contribute to the male infertility in Cstf2t/C0 mice.

The mechanism of τCstF-64’s involvement in exclusion of exon 4 is not yet clear. The reduction is greater in transcripts initiating at promoter P3 (exon 3a; Fig. 3A) than in transcripts initiating at P1 or P4. The exclusion of exon 4 might be due to τCstF-64 effects on other splicing factors. However, expression of two serine/arginine-rich splicing factors, SRp38/NSSR1 (Srsf5) and SRp40 (Srsf5), and deleted in azoospermia-associated protein 1 (Dazap1), reported to participate in regulation of the alternative splicing of CreM mRNA, is unchanged between wild-type and Cstf2t/C0 mice (Supplementary Figure S2B). This suggests that other splicing factors might be involved or that splicing of CreM mRNA may be dependent on direct interaction of τCstF-64 with nascent CreM mRNAs. The CreM transcripts initiating in P3/exon 3a (Tx7 and 8; Fig. 3) are the most affected by the alternative splicing of exon 4 (Fig. 4). We identified a unique HITS-CLIP site for τCstF-64 ~1850 nt downstream of exon 3a (Supplemental Figure S2A). Therefore, it is plausible that direct binding of τCstF-64 to the nascent mRNA of CreM (probably in cooperation with other spliceosomal factors) regulates exclu-
sion of exon 4. Future experiments will detail the role of CSTF-64 in alternative splicing of the CREM gene.

CSTF-64 Regulates CREM, Which Further Modulates Transcription of Genes Required for Male Germ Cell Development

During spermatogenesis, CREM and its isoforms regulate spatial and temporal expression of male germ-specific genes to ensure development of mature spermatooza [8, 10]. Mice lacking CREM show complete male infertility characterized by azoospermia, reduced seminiferous tubule diameter, and severely impaired spermatogenesis [49]. Similarly, CSTF-64-ablated mice demonstrate infertility with impaired spermatogenesis [35, 36]. The reduced expression of CREMtx2 in testes of Cstf2r−/− mice (Fig. 1) led us to test whether expression of genes regulated by CREM was also reduced. However, of 58 CREM-regulated genes [55], 15 were reduced in Cstf2r−/− mice (Fig. 5A), while no genes showed significantly increased expression. As an explanation, we note that the ratio between activator and repressor isoforms of CREM is altered in Cstf2r−/− mice, resulting in complex effects on downstream gene expression. The 15 down-regulated genes were specifically involved in male gamete formation and development, supporting a deeper role for CSTF-64 in control of spermatogenesis via involvement with CREM.

The reduction in expression of genes regulated by CREM led us also to examine whether Fhl5 (ACT), Kif17, or Gtf2a1, all of which act synergistically with CREM, were affected in Cstf2r−/− mice. While expression of Gtf2a1 was unchanged, expression of Fhl5 and Kif17 was reduced in Cstf2r−/− mice (Fig. 5D), suggesting that proper qualitative and quantitative transport of ACT into the nucleus by kinesin might be disrupted. However, spermatogenesis was not impaired in mice ablated of Fhl5, suggesting that other members of the Fhl5 gene family might compensate for the function of ACT in male germ cells [13, 70]. The function of kinesin 17 as a transport vehicle of members of the Fhl gene family is still important, and down-regulation of expression may influence the availability of CREM to transcription sites. These results suggest that the influence of Cstf2r on male fertility ranges beyond direct effects on CREM and includes ancillary effects on other genes.

CSTF-64 is important for spermatogenesis through several mechanisms. One mechanism is to support the critical balance of CREM mRNA isoforms as we explore here. However, there are likely many other critical steps in male germ cell development that are affected as well. For example, previously we determined that loss of CSTF-64 controls testis genome expression [38]. These results are supported by other studies where we show that histone and testis-specific histone-like genes are affected in testes of Cstf2r−/− mice (P.N. Grozdanov and C.C. MacDonald, manuscript in preparation). Because Cstf2r arose [27, 28] when mammals established sexual diocacy by X- and Y-chromosomal heterogamy [71], it is parsimonious to suggest that many mammalian features of spermatogenesis coevolved to take advantage of its properties to control of germ cell-specific genes and gene isoforms. This fine-tuned control of gene expression would lend special plasticity to mammalian spermatogenesis that is not available in other vertebrate classes. Future studies will examine those mechanisms.

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