Subfertility in young male mice mutant for chromatin remodeller CECR2

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Abstract

Defects in spermatogenesis are an important cause of male infertility. Multiple aspects of spermatogenesis are controlled by chromatin remodelers, including regulating transcription. We previously described mutations in chromatin remodelling gene Cecr2 that resulted in the lethal neural tube defect exencephaly in most mutant mice and subfertility in mice that were non-penetrant for exencephaly. Here, we show that the severity of male subfertility is dependent on age. Cecr2GT/Del males contain two mutant alleles, one of which is hypomorphic and therefore produces a small amount of protein. These males sire the fewest pups just after sexual maturity (88% fewer than Cecr2+/+ at P42–60) but improve with age (49% fewer than Cecr2+/+ at P81–100), although never completely recovering to Cecr2+/+ (wild type) levels. When young, they also have defects in testis histology, in vivo fertilization frequency, spernum number and motility, and testis weight that show similar improvement with age. Immunostaining of staged seminiferous tubules showed CECR2 in type A, intermediate and B spermatogonia, and less in preleptotene and leptotene spermatocytes. Histological defects were first apparent in Cecr2GT/Del testes at P24, and RNA-seq analysis revealed 387 differentially expressed genes. This included 66 genes on the X chromosome (almost double the number on any other chromosome), all more highly expressed in Cecr2GT/Del testes. This inappropriate expression of X chromosome genes could be caused by a failure of effective meiotic sex chromosome inactivation. We identify several abnormally expressed genes that may contribute to defects in spermatogenesis at P24. Our results support a role for Cecr2 in juvenile spermatogenesis.

Reproduction (2022) 163 69–83

Introduction

The use of model organisms such as mice has proven invaluable in identifying the molecular factors involved in spermatogenesis, such as genes with roles in spermatogonial proliferation and self-renewal, meiosis, spermiogenesis, and fertilization (Jamsai & O’Bryan 2011). Despite this, the genetic causes of human infertility remain poorly understood; in infertile men, the cause of abnormal spermatogenesis remains undetermined in approximately 72% of cases even after genetic testing (Tüttelmann et al. 2018).

Spermatogenesis is a complex process that relies on precise timing to ensure the constant production of spermatocytes. While spermatogenesis in mice takes 35 days, new cells enter the cycle every 8.6 days in a cyclical manner (Oakberg 1956). This results in cell associations that are consistently found together, with cells from previous cycles/waves forming layers progressively closer to the lumen until they are released (Russell et al. 1990). These typical cell associations are known as the 12 stages of the cycle of the seminiferous epithelium. A pulse of retinoic acid occurs in a cyclical manner at stage VIII, committing a new group of spermatogonia to differentiation every 8.6 days (Griswold 2016). If retinoic acid signalling is disrupted, such as when retinoic acid receptor α (RARα) is mutated, the disruption of this precise cyclical timing can lead to delayed, asynchronized, and abnormal spermatogenesis including decreased numbers of expected cell types (Lufkin et al. 1993). This effect of RARα is likely indirect through the Sertoli cells (Zhang et al. 2011, Tong et al. 2013).

There are also many genes identified as critical for spermatogenesis that are specifically essential for meiosis (Matzuk & Lamb 2002). Successful completion of meiosis requires intrinsically linked processes, including the induction of DNA double-strand breaks, formation of crossovers, and synopsis of homologous chromosomes (Handel & Schimenti 2010). At pachytene stage, any chromosome regions that remain unsynapsed undergo transcriptional silencing. Since the sex chromosomes in males have no homolog to fully pair with, they are obligatorily silenced, a process referred to as meiotic sex chromosome inactivation (MSCI) (Turner 2007). The failure of synopsis, DSB repair, or MSCI can trigger the pachytene checkpoint and lead to apoptosis.
or abnormal spermatogenesis in cells which do not trigger apoptosis (Burgoyne et al. 2009).

Chromatid remodelers are known to play a role in spermatogenesis through control of DNA replication, repair, and transcriptional regulation. For example, in spermiogenesis, CHD5 is critical during the exchange of histones for protamines through both facilitating the removal of histones and by controlling the levels of transition proteins and protamines (Li et al. 2014). BRG1, a catalytic subunit of the BAF complex, is important for homologous recombination (Wang et al. 2012). BAZ1A, the defining subunit of the ACF and CHRC ISW1 complexes, regulates post-miotic gene expression (Dowdle et al. 2013). CECR2 is a critical member of the CERF complex, an ISWI chromatin-remodelling complex that has roles in male fertility and neurulation (Banting et al. 2005, Thompson et al. 2012), but the specific mechanisms are unknown.

We previously showed that female mice mutant for Cecr2 are subfertile due to implantation failure and embryo loss (Norton et al. 2021). Here, we study males which are compound heterozygotes for two Cecr2 alleles: a null allele (Cecr2<sup>Del</sup>) and a hypomorph allele (Cecr2<sup>GT</sup>), which produces a small amount of normal protein (the Cecr2<sup>Del/Del</sup> phenotype is almost always lethal). We show that this large reduction in Cecr2 protein levels leads to severe subfertility at sexual maturity that improves dramatically with age, an improvement that is not seen in females. CECR2 is detectable in type A, intermediate, and type B spermatogonia as well as in preleptotene (Pl) and leptotene (L) spermatocytes. Loss of CECR2 results in seminiferous tubules that have a highly variable loss of spermatogenic cells, with some tubules that lack patches or layers of cells while others appear normal. Using RNA-seq, we identified genes that are differentially expressed between Cecr2<sup>+/+</sup> and mutant testes that may contribute to these phenotypes. Interestingly, all differentially expressed X chromosome genes were upregulated, suggesting that MSCI may be affected.

Materials and methods

Mice and Cecr2 mutations

Approval was obtained from the University of Alberta Animal Care and Use Committee for all experiments involving mice (AUP000000094). Mice were housed at 22 ± 2°C with a 14 h light:10 h darkness cycle and fed PicoLab Diet #5053 except for breeders and plugged females, who were fed higher fat PicoLab Diet #5058. This study utilizes two previously generated mutations in Cecr2, the hypomorphic Cecr2<sup>GT</sup> allele (Cecr2<sup>GT/Hemo</sup>) and the presumptive null Cecr2<sup>Del</sup> deletion allele (Cecr2<sup>Del/Hemo</sup>), both of which result in the perinatal lethal neural tube defect exencephaly (Fig. 1A and B). A small percentage of compound heterozygotes are non-penetrant for exencephaly and are viable with normal lifespans. Genotyping was done as previously described (Banting et al. 2005, Fairbridge et al. 2010). The mutations are congenic on the BALB/cCrAI strain, which originated from Charles River Laboratories but then was bred independently at the University of Alberta since ~1988. This strain now differs in some respects from the original BALB/cCrI line.

Fertility testing

Cecr2<sup>+/+</sup> females were housed with males and monitored daily for post-copulatory plugs. Plugged females were separated until dissection at embryonic day 14.5–18.5 (E14.5–18.5). The number of live fetuses was recorded. Females that were not pregnant upon dissection were recorded as zero.

Superovulation and antibody staining of oocytes

Cecr2<sup>+/+</sup> females were superovulated as previously described (Thompson et al. 2012). The following morning, females were housed with males and monitored for a post-copulatory plug every 30 min. The presence of a post-copulatory plug in this experiment was used to evaluate mating behaviour of males. Successfully mated females were euthanized 5 h post-mating, and their oocytes were isolated. The zona pellucida was removed using Tyrode's Solution (Sigma). Oocytes were fixed using 4% w/v formaldehyde in PBS for 15 min and permeabilized in 0.5% Triton-X in PBS for 10 min. After blocking in 10% goat serum (Sigma), the samples were incubated with 1:40 AlexaFluor488 phallolidin (Invitrogen)

Figure 1 Mutant alleles of Cecr2 produce phenotypes of differing severity, as evidenced by the penetrance of exencephaly. Three alleles of Cecr2 are used in this study (A). The WT Cecr2 allele (Cecr2<sup>+</sup>) has 19 exons, with a DDT domain located in exons 1–2 and a bromodomain located in exons 12–14. The Cecr2<sup>GT</sup> (Cecr2<sup>GT/GT/Hemo</sup>) genetrap allele has an insertion of β-Gal between exons 7 and 8; therefore, most transcripts lack exons 8–19 including the bromodomain. Some full-length transcript is still produced, presumably by splicing around the genetrap and making a detectable amount of protein (Norton et al. 2021), thus making this allele a hypomorph. The Cecr2<sup>Del</sup> (Cecr2<sup>Del/Del/Hemo</sup>) deletion allele is a deletion of exon 1 and ~1 kb upstream, and is a presumptive null allele. The difference in the effect of these two mutations is reflected in the percentage of homozygous mutants that develop the perinatal lethal neural tube defect exencephaly (B). The production of compound heterozygous Cecr2<sup>Del/GT</sup> males (from the 16% non-exencephalic animals) allows the study of reproduction with the least amount of Cecr2 present (Modified from Leduc et al. 2017, Norton et al. 2021). The epitope used to produce a polyclonal antibody against CECR2 is shown above the Cecr2<sup>+</sup> allele (Norton et al. 2021).
and 1:200 anti-acetylated tubulin antibody (T6793, Sigma) followed by 1:200 Cy3 goat anti-mouse secondary antibody (Jackson ImmunoResearch). Oocytes were mounted in polyvinyl alcohol (PVA; Sigma) containing DAPI and imaged with a Nikon Eclipse 80i confocal microscope.

**Computer-assisted sperm analysis**

Immediately after euthanasia, both vasa deferentia and cauda epididymides were dissected into prewarmed M2 medium (Sigma). Sperm were squeezed out of each vas deferens using fine forceps, and each epididymis was shredded using insulin needles. After incubation at 37°C, the sperm solution was diluted and sperm number and motility were assessed using the Hamilton Thorne MouseTraxx Sperm Analysis System Version 14.

**Testis weights**

After determining whole body weight, testes were dissected out and the excess tissue was removed before weighing. The average of the testis weights was used to create a testis:bodyweight ratio.

**Histology**

Testes were fixed in Bouin’s fixative (Sigma) for 20–24 h except for the testes used in Fig. 2 which were fixed in neutral buffered formalin (BDH, Epping, NSW, Australia) instead to preserve DAPI staining. For all except E18.5 testes, the tunica albuginea was removed after 30–60 min to allow for better permeation of the fixative. Testes were processed through ethanol/toluene gradients for embedding in paraffin and sectioned at 5–7 μm. After haematoxylin and eosin (H&E) staining, samples were analysed blind to the genotype to detect histological differences. Alternatively, sections were stained using periodic acid-Schiff (PAS) and counterstained with H. The stage of the cycle of the seminiferous epithelium was determined using Russell et al. 1990 as a guide (Russell et al.). The number of pyknotic nuclei was counted in H&PAS-stained sections. Images were captured using SeBaView software (Laxco) and a SeBaCam5C digital camera with a Zeiss Axioscope.A1 microscope.

**Immunostaining**

Sections were deparaffinized and then heated in antigen retrieval buffer (10 mM Tris Base, 1 mM EDTA, 0.05% Tween-20) to boiling three times over 30 min. Sections were blocked in 10% normal goat serum (Sigma) and 0.6% Triton-X (Sigma) in PBS for 1 h before incubation with 1:10,000 anti-CECR2 (Norton et al. 2021) in antibody dilution buffer (0.1% BSA, 0.3% Triton-X in PBS) overnight at 4°C. After washing in PBS, sections were incubated with 1:200 AlexaFluor-488 goat anti-rabbit secondary antibody (Life Technologies) for 2 h at room temperature and then in 0.1% DAPI for 5 min. After washing, Fluoromount-G (Southern Biotech) was used for mounting, and imaging was done as described for oocyte samples.

**Figure 2**

CECR2 is localized to gonocytes in E18.5 testes and cells at the outer edge of each seminiferous tubule in adults. Immunofluorescence using CECR2 antibody shows staining in the gonocytes at E18.5 in Cecr2+/+ testes (A, B, and C). Staining in adult testes is observed only in a percentage of cells localized near the outer edge of the tubules, consistent with spermatogonia and early spermatocytes (G, H, and I). See Supplementary Figure 2 and Supplementary Table 2 for a detailed analysis by stage of the cycle of the seminiferous epithelium. Only background staining is observed in E18.5 (D, E, and F) and adult (J-L) Cecr2GT/Del seminiferous cords and tubules, including non-specific staining in the lumen of all adult seminiferous tubules. Scale bar = 50 μm. Insets are 2.5x more magnified. Dashed boxes show where the insets originate.

**Testosterone ELISA**

Serum samples were collected using tail bleeds and stored at −80°C. Serum testosterone levels were measured in duplicate for each mouse using a testosterone ELISA kit (ENZO, Farmingdale, NY, USA) following the manufacturer’s instructions, with a CV of <7%.

**RNA-seq**

Testes were isolated from five Cecr2+/+ and five Cecr2GT/+ Del males at post-natal day 24 (P24), flash frozen in liquid nitrogen, and stored at −80°C. RNA was extracted using the RNeasy Lipid Tissue MiniKit (Qiagen). RNA integrity was ≥9.3, measured using the Agilent 2100 Bioanalyzer. cDNA libraries were prepared for sequencing by Delta Genomics (Edmonton, Canada) using the TruSeq RNA Sample Prep Kit (illumina,
San Diego, CA, USA). Sequencing was done by the McGill University and Génome Québec Innovation Centre on the Illumina HiSeq 4000 (paired-end sequencing; 100 bp reads). Read quality was assessed (using FastQC v0.11.5 software: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) prior to and after performing quality-based read trimming and adapter removal using Trimmomatic (Bolger et al. 2014) with default settings. Reads that passed quality control were mapped to the mm9 (NCBI m37, GCA_000001635.18) mouse reference genome assembly using STAR v2.5.2b (Dobin et al. 2013). Gene expression metrics were generated using featureCounts v1.5.0-p3 (Liao et al. 2014) and Ensembl release 67 annotations (Zerbino et al. 2018). Genes expressed at very low levels in more than five samples were removed, where the expression threshold was determined based on the counts per million mapped reads value corresponding to five reads in the sample with the lowest library size. The resulting expression dataset of 19,395 genes was normalized using the TMM method in edgeR v3.18.1 (Robinson & Oshlack 2010) and tested for differentially expressed genes between Cecr2<sup>GT/Del</sup> and Cecr2<sup>+/+</sup> using a generalized linear model with one factor, treatment with two levels, mutant and Cecr2<sup>+/+</sup>. Multiple testing correction was conducted using a false discovery rate (FDR) calculation (Benjamini & Hochberg 1995). Differentially expressed genes with a FDR < 0.05 and a fold change of >1.5 determined based on a power analysis using R package RNASeqPower (Hart et al. 2013) were considered significant.

### qRT-PCR
RNA was extracted as above and cDNA was synthesized using the qScript cDNA SuperMix (Quanta Biosciences). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was done on a minimum of three biological and three technical replicates using the QuantStudio 6 Flex Real-Time PCR System given at the end of this article). The qRT-PCR Mastermix (*Dynamite*) used is a proprietary mix (Molecular Biology Service Unit, Department of Biological Sciences, University of Alberta), containing Tris (pH 8.3), KCl, MgCl₂, Glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye, SYBR Green (Molecular Probes) as the detection dye, and an antibody-inhibited Taq polymerase. Samples were normalized to Gapdh as an endogenous control, and the ΔΔCT method was used for relative quantification. Primers are listed in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

### Statistics
Results are expressed as mean ± S.E.M., and significance was evaluated using Student’s t-test except where noted otherwise. Fisher’s exact test was used when comparing the frequency of two distinct outcomes between genotypes.

### Results
**CECR2 is localized to gonocytes perinatally and spermatogonia and early spermatocytes in adults**

To determine where CECR2 is found within the testis, we used antibody staining (Norton et al. 2021) on testis sections from E18.5 fetuses and adults. We have previously shown by X-gal staining that CECR2 is detected in gonocytes/prospermatogonia as early as E16.5 (Thompson et al. 2012). In E18.5 fetuses, CECR2 immunostaining is localized to the gonocytes in the middle of each seminiferous cord (Fig. 2A, B and C). In adult males, CECR2 staining is seen in cells along the outer edge of each tubule (Fig. 2G, H and I). This was consistent at all juvenile and adult ages examined (P7, P21, P28, and P42, see Supplementary Fig. 1) to >P200 (Fig. 2G, H and I). Tests of Cecr2<sup>GT/Del</sup> males showed no specific staining (Fig. 2D, E, F, J, K, L). Cecr2<sup>GT/Del</sup> mice combine a Cecr2<sup>GT</sup> hypomorphic allele and a Cecr2<sup>Del</sup> presumptive null allele (Fig. 1A). Only 16% of Cecr2<sup>GT/Del</sup> mutants (6/38) survive and reproduce, while 84% die at birth due to exencephaly. The lack of testis staining in these surviving adult mice indicates that the level of CECR2 from one hypomorphic allele is below fluorescence detection.

To determine in which cells CECR2 was localized to in adult testes, we performed a detailed analysis of CECR2 localization in each stage of the cycle of the seminiferous epithelium using CECR2 antibody staining followed by H&EPAS staining on the same sections (Supplementary Fig. 2). CECR2 is found in type A, intermediate, and B spermatogonia. Weaker staining can also be seen in PL and L spermatocytes in stages VII–X, but is absent in pachytene spermatocytes. A comparison between the number of CECR2-positive cells per tubule and published numbers of these cell types expected at each stage (Nakata et al. 2015) also shows CECR2 localization to type A, intermediate, and B spermatogonia, as well as PL and L spermatocytes (Supplementary Table 2). No substantial staining was observed in more advanced germ cells, Sertoli cells, or interstitial cells.

### Loss of CECR2 results in subfertility that is most severe in young adults and improves with age

To test the effect of severe CECR2 deficiency on fertility, Cecr2<sup>GT/Del</sup> and Cecr2<sup>+/+</sup> males were fertility tested at several ages. No difference was observed in mating behaviour between Cecr2<sup>+/+</sup> males (25/49, 51%) and Cecr2<sup>GT/Del</sup> males (36/67, 54%, Fisher’s exact test P > 0.05) as measured by the presence of a post-copulatory plug when mated to superovulated females for 3 h. Strikingly, Cecr2<sup>GT/Del</sup> males sired very few pups when mated at the start of sexual maturity, P42–60, which includes sperm produced during the first wave of spermatogenesis (average of 0.6 pups per confirmed mating; 5.1 for Cecr2<sup>+/+</sup>). However, fertility improved significantly with age (Fig. 3A). By P81–100, Cecr2<sup>GT/Del</sup> males sired an average of 3.1 pups (P = 0.007 compared to Cecr2<sup>GT/Del</sup> P42–60). This is still significantly lower than Cecr2<sup>+/+</sup> males, with an average of 6.0 pups at P81–101 (P = 0.003). No further improvement was observed...
in older (P101–216) Cecr2GT/Del males. The average litter size of Cecr2+/+ males did not significantly differ between ages.

We previously reported that Cecr2GT/GT males (homozygous for the hypomorphic allele) sire ~31% smaller litters than Cecr2GT/+ (Thompson et al. 2012), however, that study combined males from P42 to 216. We reassessed this data based on age and added 21 Cecr2GT/+ and 10 Cecr2GT/GT males. Cecr2GT/GT males also sired smaller litters at P42–60 (average of 1.9 pups) and later improved significantly but remained subfertile even at P121–251 (average of 3.8 pups compared to 6.0 for Cecr2GT, \( P = 0.005 \)).

Although the Cecr2Del allele produces no detectable protein, the Cecr2GT allele produces some protein detectable by Western blot (Norton et al. 2021). Consequently, improvement with age could be due to upregulation of the Cecr2GT allele in older males. We therefore used qRT-PCR to measure Cecr2 levels in the testes at P24 and P103–104. Although we did observe a small increase with age in both genotypes, the levels in Cecr2GT/Del males did not increase in proportion to Cecr2GT/+ levels (54% increase in Cecr2GT/+ and 26% increase in Cecr2GT/Del (Fig. 3B).

Multiple fertility measures are reduced in young males and improve with age; Cecr2GT/Del males had smaller testes than Cecr2GT/+ males of the same age (Fig. 3F). Although no difference was observed between average Cecr2GT/+ and Cecr2GT/Del male bodyweight, the testis:bodyweight ratio was used to account for variation between mice. At P42, the average testis:bodyweight ratio of Cecr2GT/Del males was 56% of Cecr2GT/+ (\( P < 0.001 \)). At P140–221, the Cecr2GT/Del ratio had increased to 82% of Cecr2GT/+ (\( P < 0.001 \)). The Cecr2GT/Del tests:bodyweight ratio was significantly larger at P140–221 than P42 (\( P = 0.002 \)).

Fertilization frequency was assessed by mating Cecr2GT/Del males with Cecr2GT/+ females and fluorescently detecting paternal DNA in the oocyte as evidence of successful fertilization (Supplementary Fig. 3). Only 2.8% of oocytes were fertilized in vivo by P42–60 Cecr2GT/Del males, whereas 52.9% were fertilized by Cecr2GT/+ males of the same age (\( P < 0.0001 \), Fig. 3C). The fertilization frequency of Cecr2GT/Del males improved significantly by P61–80 (26.3% fertilized, \( P < 0.0001 \)) and was comparable to Cecr2GT/+ in P101–154 males (61.3% for Cecr2GT/Del and 67.1% for Cecr2GT/+; \( P > 0.05 \)).

Since CECR2 is a chromatin-remodelling protein and may affect gene expression, defects in the early stages of spermatogenesis may affect later sperm production or function. We therefore used computer-assisted sperm analysis to assess sperm number and motility of Cecr2GT/Del and Cecr2GT/+ males (Fig. 3D and E). Cecr2GT/Del males showed both lower sperm concentration and poor sperm motility compared to Cecr2GT/+ males. These defects were most prominent in P42–60 males and improved with age, with comparable sperm concentration to Cecr2GT/+ in P101–160 males. Cecr2GT/Del sperm motility remained lower than Cecr2GT/+ at P101–160 but significantly improved from P42 to P60 Cecr2GT/Del levels (\( P = 0.003 \)).
Overall, all measures suggest pronounced spermatogenesis defects at the onset of maturity and improvement with age.

**Histological abnormalities of the testis are most severe in young adults and improve with age**

We began by determining when after birth seminiferous tubules could first be seen as abnormal. *Cecr2<sup>+/+</sup>* and *Cecr2<sup>GT/Del</sup>* testes at P7, P14, and P19 were indistinguishable (Fig. 4A, B and Supplementary Fig. 4). By P24, a small number of seminiferous tubules in *Cecr2<sup>GT/Del</sup>* testes showed abnormal spermatogenesis (19/120 compared to 4/120 *Cecr2<sup>+/+</sup>* tubules, Fisher's exact test \( P = 0.002 \)), with fewer advanced germ cells than expected and often unequal germ cell loss in different areas of the same tubule (Fig. 4C and D). While the presence of these abnormal tubules allowed the identification of these testes as abnormal, the majority of tubules in *Cecr2<sup>GT/Del</sup>* testes (101/120 or 84%) appeared normal at P24 (Fig. 4C and D). A quantification of the numbers of spermatogonia, spermatocytes, and spermatids in *Cecr2<sup>+/+</sup>* and *Cecr2<sup>GT/Del</sup>* testes at P24 showed that these abnormal tubules resulted in fewer spermatocytes (average of 86.3 vs 96.1 in *Cecr2<sup>+/+</sup>* tubules, \( P = 2.4E-3 \)) and spermatids (average of 27.2 vs 32.9 in *Cecr2<sup>+/+</sup>* tubules, \( P = 0.045 \)) in *Cecr2<sup>GT/Del</sup>* testes but no difference in the number of spermatogonia (average of 9.3 vs 9.3 in *Cecr2<sup>+/+</sup>* tubules, \( P > 0.05 \)) (Fig. 5). The number of pyknotic nuclei at P24 was also counted as a preliminary measure of cell death, revealing a small increase in cell death in *Cecr2<sup>GT/Del</sup>* testes.

**Figure 4** Histological abnormalities in *Cecr2<sup>GT/Del</sup>* testes appear between P20 and P24, are most severe at maturity (P42), then improve with age. H&E-stained *Cecr2<sup>+/+</sup>* (A, C, E, and G) and *Cecr2<sup>GT/Del</sup>* (B, D, F, and H) testes. No difference was observed between genotypes at P19 (A and B), but *Cecr2<sup>GT/Del</sup>* testes could be clearly distinguished at P24 (C and D) by the presence of some tubules lacking spermatocytes in portions of the tubule (16% compared to 4% of *Cecr2<sup>+/+</sup>* tubules). (D’) is typical of the most severe cell loss we observed, while other abnormal tubules were missing fewer cells. More severe defects were observed at P42 (E and F), when many tubules exhibited disorganized or missing cell patches or layers. These defects were less pronounced by P170–210 (G and H). Magnified portions of each image are also shown (A’–H’). Scale bar = 50 µm.
Subfertility in young Cecr2 males

The coordination of the seminiferous tubules (compared to 0/150 tubules from each of the genotypes at P42 (A) and P118–210 (B). Tubules were staged and classified as appearing normal or having one of the five categories of defects (example images shown in C, D, E, F, and G): having disorganized cell layers (C), missing most cells in one area of the tubule (D), missing many or all of the most mature cell type expected at that stage (E), missing many or all of a less mature cell type such as spermatocytes or round spermatids but not missing the most mature cell type in that stage (F), or missing most cells (G). Tubules that were too abnormal to be assigned a stage were also assessed and are shown as stage N/A. n = 49–50 tubules from each of three Cecr2+/+ and Cecr2GT/Del males at each age.

Once again was not observed in a specific cell type or tubule morphology.

In older Cecr2GT/Del males, most seminiferous tubules appeared normal (Fig. 4G and H). At P118–210, only 9/150 Cecr2GT/Del tubules (compared to 5/150 Cecr2+/+ tubules, Fisher’s exact test P > 0.05, Fig. 6B and C) had disorganized layers of cells, and 19/150 (compared to 6/150 Cecr2+/+ tubules, Fisher’s exact test p = 0.01, Fig. 6B and D) had missing patches of cells. There were 4/150 Cecr2GT/Del tubules (compared to 0/150 Cecr2+/+ tubules, Fisher’s exact test P > 0.05, Fig. 6B, E and F) missing all or most of a particular cell type, and 6/150 tubules (compared to 1/150 Cecr2+/+ tubules, Fisher’s exact test P > 0.05, Fig. 6B and G) that were missing the majority of cells.

**Figure 6** Histological analysis of Cecr2GT/Del staged spermatogenic tubules at P42 and P100+ shows frequent abnormal tubules which improve with age. Seminiferous tubules from Cecr2+/+ and Cecr2GT/Del males were examined at P42 (A) and P118–210 (B). Tubules were staged and classified as appearing normal or having one of the five categories of defects (example images shown in C, D, E, F, and G): having disorganized cell layers (C), missing most cells in one area of the tubule (D), missing many or all of the most mature cell type expected at that stage (E), missing many or all of a less mature cell type such as spermatocytes or round spermatids but not missing the most mature cell type in that stage (F), or missing most cells (G). Tubules that were too abnormal to be assigned a stage were also assessed and are shown as stage N/A. n = 49–50 tubules from each of three Cecr2+/+ and Cecr2GT/Del males at each age.

**Figure 5** Analysis of cell types in the P24 testis shows a small but significant difference between Cecr2GT/Del and Cecr2+/+. The number of spermatogonia, spermatocytes, and spermatids per tubule were counted for Cecr2+/+ and Cecr2GT/Del testes at P24. There is a small but significant decrease in the number of spermatocytes and spermatids in Cecr2GT/Del testes, but not spermatogonia. n = 250 tubules from three Cecr2+/+ and three Cecr2GT/Del males. Levels of significance: **0.001 ≤ P < 0.01, *0.01 ≤ P < 0.05, and no asterisk indicates a lack of significance (P > 0.05).
As Cecr2<sup>GT/Del</sup> males produce a small amount of CECR2 due to the hypomorphic Cecr2<sup>GT</sup> allele (Norton et al. 2021), we also examined the histology of Cecr2<sup>Del/Del</sup> tests (complete CECR2 deficiency). As almost all of these mice die perinatally due to exencephaly, we could only assess E18.5 testes. Although the seminiferous cords appeared normal, the testis architecture of Cecr2<sup>Del/Del</sup> males was disrupted, with larger areas of the testis containing no cords (Fig. 7A and B). While the number of gonocytes per seminiferous cord was not different between Cecr2<sup>+/+</sup> and Cecr2<sup>Del/Del</sup> testes (Fig. 7C, P > 0.05), Cecr2<sup>Del/Del</sup> testes had significantly fewer cords than Cecr2<sup>+/+</sup> (Fig. 7D, P = 3.3E-3) and therefore also fewer germ cells (average of 6.0 gonocytes per 100 µm<sup>2</sup> compared to 7.4 in Cecr2<sup>+/+</sup> testes, P = 3.1E-3).

**Testosterone levels are not affected by loss of CECR2**

Testosterone is required for spermatogenesis (O’Donnell et al. 2006), and mouse mutants with lower serum testosterone levels have decreased testis size and abnormalities in spermatogenesis ranging from decreased sperm count to arrest prior to the first meiotic division (Wang et al. 2009). We therefore measured serum testosterone levels in males when they have severe abnormalities in spermatogenesis (P42–60) (Supplementary Fig. 5). There was no significant difference in testosterone levels between Cecr2<sup>GT/Del</sup> and Cecr2<sup>+/+</sup> males, suggesting that testosterone is not contributing to the abnormal phenotype.

**Differentially expressed genes in Cecr2<sup>GT/Del</sup> testes**

As Cecr2 is a chromatin remodeler, it could cause the misregulation of gene expression critical for spermatogenesis. We therefore did a bulk RNA-seq comparing the transcriptomes of Cecr2<sup>+/+</sup> and Cecr2<sup>GT/Del</sup> testes at P24. We chose this age because at P24, abnormal spermatogenesis was present in a low level of tubules (16% vs 3% observed in Cecr2<sup>+/+</sup> P24 testes). Overall, the numbers of spermatocytes and spermatids were only decreased by ~12% in Cecr2<sup>GT/Del</sup> testes compared to Cecr2<sup>+/+</sup> testes at P24 (Fig. 5), thus minimizing any secondary effects due to a loss of these cell types. RNA-seq analysis revealed 387 genes that were differentially expressed, 238 (61%) of which were expressed at a lower level in Cecr2<sup>GT/Del</sup> testes and 149 (39%) of which were higher. Strikingly, 18% of differentially expressed genes were on the sex chromosomes (66 on X, 2 on Y), representing a notable enrichment (Fig. 8A). Furthermore, differentially expressed sex chromosome genes exclusively had higher expression in Cecr2<sup>GT/Del</sup> samples, representing 46% of all genes with a higher abundance of mRNA. Visualization of the steady-state mRNA levels of all expressed genes in these samples (outer heatmap, Fig. 8B) rather than only significantly differentially expressed genes (inner heatmap, Fig. 8B) also revealed a general trend of higher gene expression in sex chromosomes across multiple Cecr2<sup>GT/Del</sup> samples. This suggests that MSCI may not be occurring efficiently in Cecr2<sup>GT/Del</sup> testes, leading to abnormally high transcription from the improperly silenced sex chromosomes. Several of the genes (Him1, Msh4, Spata22, Meiob, Tex11, and Hormad2) are known to be involved in MSCI, recombination, or synopsis (see Table 1), therefore, their altered expression could lead to inefficient MSCI and the abnormally high X chromosome transcript levels in Cecr2<sup>GT/Del</sup> testes.

Since the small number of abnormal tubules in the mutant resulted in a small decrease in the number of spermatocytes (~10%) and spermatids (~17%), it is unlikely that the differential levels of mRNAs revealed in the RNA-seq analysis are due to the loss of pachytene or post-pachytene cells in abnormal tubules. To support this, we used published single-cell transcriptome data (Green et al. 2018, Hermann et al. 2018, Grive et al. 2019) to determine which cell types the 387 differentially expressed genes from our RNA-seq analysis are expressed in. After removing genes that were found in different cell types in different datasets...
Figure 8 A disproportionately large number of sex chromosome genes are more highly expressed in Cecr2<sup>GT/Del</sup> testes than Cecr2<sup>+/+</sup> testes at P24 as determined by RNA-seq. The distribution of differentially expressed genes across the chromosomes (A) reveals an enrichment in chromosome X. In addition, while the majority of significantly differentially expressed autosomal genes are expressed at lower levels in Cecr2<sup>GT/Del</sup> testes, genes located on the sex chromosomes are exclusively higher. Circular heatmaps (B) provide a visualization of the expression levels of all expressed genes (outer heatmap) and statistically differentially expressed genes (inner heatmap) in Cecr2<sup>GT/Del</sup> vs Cecr2<sup>+/+</sup> mice testes at P24. The order of samples is the same in both heatmaps and matches the order in the sample clustering dendrogram (plotted in the centre) observed when samples were hierarchically clustered using Z-score transformed expression values from differentially expressed genes. In the outer heatmap, a strong general trend of higher gene expression in Cecr2<sup>GT/Del</sup> vs Cecr2<sup>+/+</sup> samples is evident for the sex chromosomes (visualized as a tendency for red in the first five rows and blue in the last five rows for the sex chromosome sectors of the outer heatmap), beyond what the differential expression analysis was able to detect within the statistical power constraints of the analysis. The inner heatmap gives a visual representation of the distribution of differentially expressed genes over all chromosomes and confirms the disproportionate number of higher-expressed genes on chromosome X (evident by the narrower columns indicating a large number of genes and by red in the first five rows and blue in the last five rows in and genes that were also expressed in somatic cells, we were able to cross-reference 58 genes. We divided these into expression in spermatogonia to early pachytene spermatocytes (35 genes, hereafter referred to as ‘early’ spermatogenic cells) and post-meiotic cells up to midround spermatids which are present at P24 (23 genes, hereafter referred to as ‘late’ spermatogenic cells’). If the differential detection of mRNA abundance in the RNA-seq analysis were due to a relative overabundance of ‘early’ cells (due to an absence of ‘late’ cells), we would expect the transcripts of the 35 genes expressed in the ‘early’ cells to be at increased levels. Instead, we found 25 autosomal genes at lower levels in ‘early’ cells and only 1 autosomal gene and all 9 X-chromosome genes at a higher level. This supports our hypothesis that the abnormal cells are not numerous enough to be responsible for the differences we see with RNA-seq, as well as supports an unusual skew of genes on the X-chromosome. In ‘late’ spermatogenic cell types, 14 genes showed decreased mRNA levels while 9 autosomal genes showed increased mRNA levels. This again does not fit with a strong skew from a loss of later cell types, which would predict a decrease in mRNA levels overall.

Based on known functions and/or expression data from previous literature, we tested the abnormal mRNA abundance of 16 genes using qRT-PCR, 6 of which are located on the X chromosome (Table 1). Of these, all except Slx2 were validated in P24 testes (Fig. 9 and Table 1). We also measured their relative mRNA levels in P103–104 testes to see if mRNA abundance levels normalized. While 12/16 genes remained differentially expressed in the testes of older males, 11/16 were significantly closer to Cecr2<sup>+/+</sup> levels than at P24 (Fig. 9), including all 8 expressed at lower levels in Cecr2<sup>GT/Del</sup> testes.

**Discussion**

**CECR2 is expressed in spermatogonia and early spermatocytes**

Analysis of CECR2 immunostaining throughout the stages of spermatogenesis indicated that CECR2 is expressed in type A, intermediate, and B spermatogonia. Weaker staining is seen in Pl and L spermatocytes, which likely

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Table 1 Differentially expressed genes between Cecr2<sup>-/-</sup> and Cecr2<sup>GT/Del</sup> mouse tests at P24 and P103–104.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr.</th>
<th>RNA-seq FDR</th>
<th>RNA-seq FC</th>
<th>P24 qPCR FC</th>
<th>P103–104 qPCR FC</th>
<th>Relevant functions and features from literature</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgr</td>
<td>4</td>
<td>2.0E-07</td>
<td>0.34</td>
<td>0.27*</td>
<td>0.73*</td>
<td>Highest expression in 2–3-week-old tests, still moderately expressed in adult</td>
<td>Goupil et al. 2011</td>
</tr>
<tr>
<td>Hlim1</td>
<td>5</td>
<td>1.4E-08</td>
<td>0.59</td>
<td>0.45*</td>
<td>0.75*</td>
<td>Required for normal CO numbers and complete synopsis</td>
<td>Guiralderelli et al. 2013</td>
</tr>
<tr>
<td>Mshb4</td>
<td>3</td>
<td>1.2E-15</td>
<td>0.51</td>
<td>0.48*</td>
<td>0.74*</td>
<td>Required for normal CO numbers and complete synopsis, mutations associated with spermatogenic failure in humans</td>
<td>Kneitz et al. 2000, Snowden et al. 2004, Terribas et al. 2010</td>
</tr>
<tr>
<td>Hromad2</td>
<td>11</td>
<td>1.6E-09</td>
<td>0.60</td>
<td>0.46*</td>
<td>0.83*</td>
<td>Required for ATR recruitment to unsynapsed axes and MSCI</td>
<td>Fukuda et al. 2012, Kogo et al. 2012, Wojtasz et al. 2012</td>
</tr>
<tr>
<td>Spata22</td>
<td>11</td>
<td>1.3E-06</td>
<td>0.61</td>
<td>0.49*</td>
<td>0.85*</td>
<td>Required for normal CO numbers and complete synopsis, forms a complex with MEIOB</td>
<td>La Salle et al. 2012, Hays et al. 2017, Xu et al. 2017</td>
</tr>
<tr>
<td>Meiob</td>
<td>17</td>
<td>4.0E-11</td>
<td>0.61</td>
<td>0.50*</td>
<td>0.86</td>
<td>Required for normal CO numbers and complete synopsis, mutations associated with spermatogenic failure in humans</td>
<td>Luo et al. 2013, Souquet et al. 2013, Gershoni et al. 2019</td>
</tr>
<tr>
<td>Ptdh3</td>
<td>11</td>
<td>1.3E-17</td>
<td>0.53</td>
<td>0.57*</td>
<td>0.74*</td>
<td>Tests-specific Ptdh3, predicted role in Hh signalling for sperm motility, not essential for spermatogenesis in humans</td>
<td>Fan et al. 2007, Ghahramani Seno et al. 2011</td>
</tr>
<tr>
<td>Cbs</td>
<td>17</td>
<td>3.2E-03</td>
<td>0.57</td>
<td>0.53*</td>
<td>1.30</td>
<td>Generates H2S, which is important for sperm motility. Associated with asthenospermia (poor sperm motility) in humans</td>
<td>Nuño-Ayala et al. 2012, Li et al. 2015, Wang et al. 2018</td>
</tr>
<tr>
<td>Clp1</td>
<td>9</td>
<td>9.5E-03</td>
<td>1.50</td>
<td>1.34*</td>
<td>1.31*</td>
<td>Expressed in Leydig cells, mutation leads to spermatogenic arrest</td>
<td>Li et al. 2007</td>
</tr>
<tr>
<td>Bmp7</td>
<td>2</td>
<td>5.7E-05</td>
<td>1.61</td>
<td>1.39*</td>
<td>0.80</td>
<td>Expressed in spermatogonia and primary spermatocytes in immature tests, but spermatids in adults. Role in proliferation of germ cells in embryo, then in maintenance of spermatogenesis in adults</td>
<td>Zhao et al. 2001, Ross et al. 2007, Ciller et al. 2016</td>
</tr>
<tr>
<td>Tex11</td>
<td>X</td>
<td>2.2E-08</td>
<td>1.57</td>
<td>1.37*</td>
<td>1.34*</td>
<td>Expressed in spermatogonia, regulates recombination frequency, and possibly proliferation of spermatogonia. Mutations cause azoosperma in humans</td>
<td>Adelman &amp; Petrini 2008, Yu et al. 2012, Yang et al. 2015, Yatsenko et al. 2015, Sha et al. 2018</td>
</tr>
<tr>
<td>Scml2</td>
<td>X</td>
<td>1.3E-10</td>
<td>1.58</td>
<td>1.41*</td>
<td>1.30*</td>
<td>Expressed in spermatogonia and early meiotic cells, plays a role in maintaining active histone modifications on sex chromosome genes needed in spermatids</td>
<td>Hasegawa et al. 2015, Luo et al. 2013, Adams et al. 2018</td>
</tr>
<tr>
<td>Sfk2</td>
<td>X</td>
<td>5.2E-04</td>
<td>1.54</td>
<td>1.26</td>
<td>0.96</td>
<td>Expressed in primary spermatocytes and localizes to sex body</td>
<td>Shi et al. 2013, Zhuang et al. 2013, Geyer &amp; Eddy 2008, Busada et al. 2016</td>
</tr>
<tr>
<td>Rhox13</td>
<td>X</td>
<td>3.0E-08</td>
<td>1.78</td>
<td>1.72*</td>
<td>1.51*</td>
<td>Required for a histologically normal first wave of spermatogenesis, but not for normal adult spermatogenesis. No difference in litter sizes at any age.</td>
<td>Goupil et al. 2011</td>
</tr>
<tr>
<td>Ccnb3</td>
<td>X</td>
<td>5.1E-21</td>
<td>2.52</td>
<td>2.06*</td>
<td>1.43*</td>
<td>Early meiotic cyclin normally downregulated at transition to pachytene. Continued expression causes aberrant spermatogenesis.</td>
<td>Nguyen et al. 2002, Reif-Rogers et al. 2006</td>
</tr>
</tbody>
</table>

*Indicates qPCR significance between Cecr2<sup>-/-</sup> and Cecr2<sup>GT/Del</sup> tests; Bolded qRT-PCR values indicate significance between P24 and P103–104. FC, fold change, where FC>1 indicates higher expression in Cecr2<sup>GT/Del</sup> tests; FDR, false discovery rate-adjusted significance.
represents a progressive loss of the protein, that is not present in pachytene spermatocytes. The presence of chromatin remodeller CECR2 protein in spermatogonia and early spermatocytes suggests there may be an effect on expression of other genes affecting spermatogonial differentiation and meiosis, which could be responsible for the variable phenotype of the mutant tubules. Histology of P42-staged tubules shows no developmental block at a particular stage. Rather, there can be missing patches within the tubule, or missing layers of spermatocytes, round spermatids, or elongating spermatids. One possibility is that the variation in this phenotype may be the result of a general disruption to the timing and/or synchronization of spermatogonial differentiation or entry into meiosis. If some or most spermatogonia do not commit to differentiation at the beginning of a new round of spermatogenesis, this could lead to a missing cohort of cells in some or most of that tubule. The small increase in the number of pyknotic nuclei in Cecr2\textsuperscript{GT/Del} testes that were not localized to a specific cell type and a lack of an obvious developmental block makes it difficult to pinpoint the mechanism by which a lack of CECR2 results in the disruption of spermatogenesis.

**Cecr2\textsuperscript{GT/Del} males have a defect in juvenile and early-adult spermatogenesis that becomes milder with age**

We have shown that compound heterozygote Cecr2\textsuperscript{GT/Def} males have severe subfertility just after sexual maturity and improve as they age. Both the average size of litter sired and the percentage of oocytes fertilized in vivo are close to zero in P42–60 males, and sperm concentration, sperm motility, and testis weight are also significantly decreased. All of these parameters show improvement with age. While some measures return to approximate Cecr2\textsuperscript{+/+} levels in older Cecr2\textsuperscript{GT/Del} males, they continue to sire significantly smaller litters with no further improvement after P100. A reexamination of the Cecr2\textsuperscript{GT/GT} hypomorph mutants showed a similar pattern of subfertility. Cecr2\textsuperscript{GT/GT} testis histology, sperm number, and sperm motility were reported as normal (Thompson et al. 2012), but this could be explained by their age (P128–243) combined with higher levels of CECR2 from two copies of the hypomorphic allele creating a milder phenotype.

Severe subfertility in young males just after sexual maturity and subsequent recovery is indicative of a defect during the synchronized first wave of spermatogenesis that occurs in juvenile males (Geyer 2017). This initial wave produces the first sperm available to adult males at sexual maturity and establishes the basis for continuous spermatogenesis in adults. Male mice with a germ cell-specific Sox3 knockout have a strikingly similar phenotype to Cecr2\textsuperscript{GT/Del} males. Sox3 mutant newborn testes initially appear normal but they have a severe germ cell depletion by P20 which subsequently partially recovers in adults to ~80% of normal (Laronda & Jameson 2011). As fertility was not tested in these animals, it is unknown whether the recovery in testis histology translates into increased litter size. Interestingly, Sox3 mutants have a block at the point of spermatogonial differentiation, while Cecr2\textsuperscript{GT/Del} testes still appear normal at P19 and have a histologically abnormal phenotype later in the first wave. A conditional knockout of retinol dehydrogenase 10 (Rdh10) in the germ and Sertoli cells also shows juvenile testes defects with subsequent adult recovery (Tong et al. 2013). Cell loss is evident by P7, and by 3 weeks over 85% of seminiferous tubules have no meiotic cells due to the inability to produce retinoic acid. Mutant males are infertile or subfertile prior to 7 weeks. However, by 9 weeks, these males show recovered histology, are fertile, and produce litters the same size as controls, unlike older Cecr2 mutants which still have smaller litters than controls. This indicates that while Rdh10 is critical for the first wave of spermatogenesis, it is not necessary for steady-state adult spermatogenesis, when presumably retinoic acid is produced through a different gene.

It is unclear why the Cecr2 mutant phenotypes disproportionately affect juvenile and early-adult spermatogenesis and partially recover as the males age. Analysis of Cecr2 expression in P24 and P103–104 indicates that this is not due to a relative change in expression as they age. The fertility defect in Cecr2\textsuperscript{GT/Del} males and subsequent partial recovery of this defect with age are likely largely due to the differences in the sperm count and motility that we observed. These measures reflect the histological defects seen in Cecr2\textsuperscript{GT/Del} tubules, which also change in prevalence and severity with age. While some tubules produce significantly fewer sperm, others appear relatively normal, yet the sperm produced has abnormal motility. This suggests that CECR2 may be playing multiple roles in meiosis and spermiogenesis.
As CECR2 is a chromatin remodeller that may affect the expression of multiple genes and some CECR2 protein is residually present in Cecr2\textsuperscript{GT/Del} animals, it would not be surprising if the phenotype was due to a complex mixture of mechanisms that may contribute to the partial, but not complete, nature of the recovery.

One intriguing possibility that may help explain the recovery of Cecr2 and Sox3 mutants with age is that there are unique pathways or regulatory differences in spermatogenesis that differ between the first wave and steady-state adult spermatogenesis (Laronda & Jameson 2011, Geyer 2017). CECR2 may play a role specifically during juvenile spermatogenesis that is no longer required in adults. Consequently, the disruption of juvenile spermatogenesis, particularly the establishment of the spermatogenic wave and the stage-specific cell associations of the seminiferous epithelium, may lead to the milder but continued abnormal spermatogenesis we observe into later adulthood. CECR2, a chromatin remodeller, may also have multiple functions and be required for normal steady-state adult spermatogenesis, since Cecr2 expression and antibody staining remains in normal aging males and older males still show some abnormal tubules and smaller litters that the controls. Interestingly, the disrupted testis architecture observed in Cecr2\textsuperscript{Del/Del}E18.5 testes completely lacking CECR2 suggests an earlier role for CECR2 during testis development, before we first observe abnormal spermatogenesis in P24 Cecr2\textsuperscript{GT/Del} testes with measurable CECR2 present. While almost all Cecr2\textsuperscript{Del/Del} fetuses have exencephaly that could theoretically affect development of other organs, such fetuses appear grossly normal outside of the brain. Additionally, one rare Cecr2\textsuperscript{Del/Del} fetus used in this study was not exencephalic, yet displayed the same testis phenotype. Finally, improvement over age may reflect increasing compensation over time by other genes or pathways (as suggested by Laronda & Jameson 2011).

As CECR2 is a chromatin remodeller, when and how it affects spermatogenesis may also involve misregulation of one or more genes. Although the phenotype of Sox3 mutants closely resembles that of Cecr2\textsuperscript{GT/Del} males, Sox3 is not differentially expressed in P24 Cecr2\textsuperscript{GT/Del} testes. However, Rhox13, loss of which results in a juvenile defect in spermatogenesis in mice, is differentially expressed and may contribute to the Cecr2\textsuperscript{GT/Del} phenotype (see below).

**Transcriptional changes in Cecr2\textsuperscript{GT/Del} testes**

To find differentially expressed genes that could shed light on the phenotypes we see, we used RNA-seq to compare Cecr2\textsuperscript{GT/Del} and Cecr2\textsuperscript{+/+} testes at the age we first observed defects in a small number of P24 tubules, to minimize secondary effects. Furthermore, we showed that the changes in mRNA abundances did not fit the predictions for skewed expression due to the loss of cells of later-stage spermatogenesis. RNA-seq analysis revealed 387 genes that had differences in mRNA abundance in Cecr2\textsuperscript{GT/Del} testes, of which we further tested 16. The abnormal expression of all but Sh2 was validated. All 16 genes have functions in spermatogenesis (Table 1). Additionally, while the mRNA abundance of 5/16 genes was no longer significantly different from Cecr2\textsuperscript{+/+} levels at P103–104, the mRNA abundance of the other 11/16 genes had not fully returned to Cecr2\textsuperscript{+/+} levels by P103–104 (Fgr, Hlim1, Msh2, Hormad2, Spata22, Ptchd3, and Glp1), consistent with older males still showing subfertility.

Three of these 16 genes likely play a more important role in the testes prior to sexual maturity than in adult spermatogenesis, possibly explaining why the defect is more severe in young males. Fgr, reduced to ~27% of Cecr2\textsuperscript{+/+} levels in P24 Cecr2\textsuperscript{GT/Del} testes, normally has highest expression levels in P14 and P21 testes and lower levels in adults (Goupil et al. 2011), suggesting an important role in juvenile spermatogenesis. Similarly, Bmp7 is expressed in spermatogonia and spermatocytes before sexual maturity, but transitions to mid-late stage spermatids in adults (Zhao et al. 2001). Since CECR2 is found only in spermatogonia, it is possible that CECR2 only affects this early stage expression. This is supported by the qRT-PCR analysis, which shows Bmp7 is significantly more highly expressed in Cecr2\textsuperscript{GT/Del} testes at P24 but is not significantly different from Cecr2\textsuperscript{+/+} at P103–104. Rhox13 is required for a normal first wave of spermatogenesis, and without it, histological abnormalities occur in the seminiferous tubules (Busada et al. 2016). Despite the continued expression of Rhox13 into adulthood in Cecr2\textsuperscript{+/+} testes, older Rhox13 KO mice have normal testis histology. Therefore, the overexpression of Rhox13 in Cecr2\textsuperscript{GT/Del} testes at both P24 and P103–104 could disproportionately affect the first wave of spermatogenesis and contribute to the age-specific phenotype.

Our RNA-seq analysis showed a clear upregulation of many genes on the sex chromosomes, leading us to hypothesize that MSCI may not be occurring efficiently. In addition, six of the validated genes are associated with the intricately linked processes of homologous recombination, synopsis, or MSCI. Defects in any of these processes could lead to abnormal high expression of sex chromosome genes that we observed. Him1, Msh4, Spata22, and Meiob are all required for normal crossover numbers and complete synopsis (Kneitz et al. 2000, Snowden et al. 2004, La Salle et al. 2012, Guiraldeil et al. 2013, Luo et al. 2013). Transcripts of all of these genes were found at lower levels in P24 Cecr2\textsuperscript{GT/Del} testes but significantly increased towards Cecr2\textsuperscript{+/+} levels in P103–104 testes. Tex11 regulates recombination frequency and therefore synopsis and mutations in TEX11 have been linked to infertility in humans (Adelman & Petroini 2008, Yatsenko et al. 2015). Hormad2 plays a more direct role in MSCI, as it is required for the recruitment of ATR (Ataxia Telangiectasia and Rad3 related) to unsynapsed chromosomes (Kogo et al. 2012). A decrease in Hormad2 could impair sex body formation and MSCI, elevating transcription from the sex chromosomes. To
further address whether a defect in MSCI is contributing to the phenotype in Cecr2\(^{GT/Del}\) testes, transcriptional inactivation of the sex chromosomes should be measured in late pachytene spermatocytes.

The remaining six RNA-seq genes we tested further all have proposed or confirmed functions in other parts of spermatogenesis. Scml2, more highly expressed in Cecr2\(^{GT/Del}\) testes, plays a role in regulating gene expression during spermatogenesis, including maintaining active histone modifications on sex chromosome genes required post-meiotically (Hasegawa et al. 2015, Adams et al. 2018). Ptchd3 and Cbs are both predicted to be important for sperm motility (Fan et al. 2007, Wang et al. 2018), while mutations in Glp1 lead to spermatogenic arrest (Li et al. 2007). Tafl1 is required for the normal expression of post-meiotic genes (Zhou et al. 2013). Ccnb3 is an early meiotic cyclin that is found at significantly higher levels in Cecr2\(^{GT/Del}\) testes. Ccnb3 is normally downregulated at the zygote to pachytene transition, and its continued expression leads to abnormal spermatogenesis (Refik-Rogers et al. 2006). The misregulation of any or all of these genes could contribute to the histological defects, lower sperm count, and lower sperm mobility that we observed.

**Female and male Cecr2 mutant subfertility appears to have different mechanistic origins**

While male Cecr2\(^{GT/Del}\) mice have clear defects in spermatogenesis, we did not detect any abnormalities in Cecr2 mutant oogenesis in a previous study (Norton et al. 2021). Cecr2 mutant females do not show the lower follicle numbers or decreasing fertility with age (Norton et al. 2021) that often accompanies meiotic defects (Burgoyne et al. 2009). In addition, mutant females do not show improvement of fertility with age as was seen in Cecr2 mutant males.

**Overall conclusions**

Our results support a role for CECR2 in juvenile and early-adult spermatogenesis, possibly through an effect on MSCI. As a chromatin-remodelling protein, CECR2 in spermatogonia and early spermatocytes could be directly affecting gene regulation, the effects of which are not seen until later in meiosis when CECR2 is not present. CECR2 has also been suggested to have a role in DSB repair (Lee et al. 2012), which could affect chromosomal synopsis and MSCI. However, no CECR2 is observed in pachytene spermatocytes, so a direct role for CECR2 binding to chromatin in homologous recombination is unlikely. In addition, our recent work in neurosphere primary cultures from mice does not support a role for CECR2 in DSB repair in these cells (Elliott et al. 2020). Overall, loss of CECR2 chromatin remodelling affects juvenile and early-adult spermatogenesis and may also play a role in ongoing adult spermatogenesis.

**Supplementary materials**

This is linked to the online version of the paper at https://doi.org/10.1530/REP-19-0507.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This work was funded by a grant from the Natural Sciences and Engineering Research Council (NSERC). K A N held scholarships from NSERC and Alberta Innovates Technology Futures. K A N and F N held QEII scholarships from the Government of Alberta. V V N held a USRA scholarship from NSERC.

**Author contribution statement**

K A N was a lead graduate student for both the research and writing of this work. R H, C W, K D, and V V N were undergraduate students who contributed to specific experiments. F N provided the unpublished CECR2 antibody. A K and P S analysed the RNA-seq data. H E M supervised all students and contributed to specific experiments and writing of the paper. All authors provided intellectual input and critically edited the paper.

**Acknowledgements**

The authors would like to thank Hiroki Inoue, Troy Locke, Arlene Oatway, Dr Serene Wohlgemuth, and Dr David Westaway for their invaluable help and discussions. The authors thank Dr Toni Bayans, Carol Boechler, Sarah Collard, Simmone Kerswell, Annette Morin, Jessica Tansey, and Brittany Whitemore for excellent assistance with the mice and helpful discussions. RNA-seq sequencing was done by GenomeQuebec in collaboration with Michelle Miller and Delta Genomics.

**References**


Elliot J, Norton KA, Niri FH & McDermid HE 2020 Reported DNA repair protein CECR2, which is associated with neural tube defects in mice, is not required for double-strand break repair in primary neurospheres. DNA Repair 94 102876. (https://doi.org/10.1016/j.dnarep.2020.102876)


Luo M, Yang F, Leu NA, Landache J, Handel MA, Benavente K, La Salle S & Wang PJ 2013 MEIOB exhibits single-stranded DNA-binding and

Reproduction (2022) 163 69–83

K A Norton and others
exonuclease activities and is essential for meiotic recombination. Nature Communications 4:2288. (https://doi.org/10.1038/ncomms3788)


Matzuk MM & Lamb DJ 2002 Genetic dissection of mammalian fertility pathways. Nature Cell Biology 4 (Supplement) s41–s49. (https://doi.org/10.1038/ncb-nm-fertility541)


Received 23 October 2019
First decision 11 December 2019
Revised manuscript received 6 December 2021
Accepted 14 December 2021

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