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A 1.1-Mb Segmental Deletion on the X Chromosome Causes Meiotic Failure in Male Mice[1]

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ABSTRACT

The mammalian X chromosome contains a large number of multicopy genes that are expressed during spermatogenesis. The roles of these genes during germ cell development and the functional significance of gene multiplication remain mostly unexplored, as the presence of multicopy gene families poses a challenge for genetic studies. Here we report the deletion of a 1.1-Mb segment of the mouse X chromosome that is syntenic with the human Xq22.1 region and contains 20 genes that are expressed predominantly in testis and brain, including three members of the nuclear export factor gene family (*Nxf2*, *Nxf3*, and *Nxf7*) and five copies of preferentially expressed antigen in melanoma-like 3 (*Pramel3*). We have shown that germline-specific Cre/loxP-mediated deletion of this 1.1-Mb segment is efficient and causes defective chromosomal synapsis, meiotic arrest, and sterility in male mice. Our results demonstrate that this 1.1-Mb region contains one or more novel X-linked factors that are essential for male meiosis.

male fertility, male infertility, meiosis, meiotic arrest, segmental deletion, spermatogenesis, X chromosome

INTRODUCTION

Sex chromosomes have evolved from a pair of autosomes; their unique status, however, has shaped specific gene content during evolution. In mammalian males, the hemizygous state of the X chromosome exerts immediate selection for or against new X-linked alleles encoding sexually dimorphic traits [1]. The X chromosome also encodes a large number of genes that are highly expressed in the brain, and mutations in more than 100 of these genes have been associated with mental retardation syndromes in males [2, 3]. During male meiosis, the X and the Y chromosome are transcriptionally silenced in a process termed meiotic sex chromosome inactivation (MSCI), whereas autosomes remain transcriptionally active [4, 5]. In postmeiotic germ cells, the transcription of sex chromosome-linked genes remains substantially repressed [6, 7]. Consistent with these changes in transcriptional activity during spermatogenesis, the X chromosome is enriched for genes that are expressed prior to meiosis but depleted of genes that play roles during or after meiosis [8, 9]. Many X-linked micro-RNAs, however, escape from MSCI and are present during the pachytene stage of male meiosis [10]. Genetic studies in the mouse have shown that several X-linked genes regulate male meiosis and play critical roles during germ cell maturation [11].

Families of multiple or multicopy genes can arise from gene duplication, retrotransposition, or from segmental duplications. Approximately 5% of the human genome consists of large interspersed segmental duplications with high sequence identity, producing recombination hot spots that can result in genomic inversions, amplifications, and deletions [12]. Segmental duplications also pose a challenge for genome assembly due to their repetitive nature, leaving gaps in the ''finished'' genome [13]. The mouse X chromosome contains 22 ampliconic regions enriched for identical or nearly identical multicopy genes that are expressed in the testis [14]. Thirty-three such multicopy gene families are expressed preferentially in postmeiotic germ cells, suggesting that this increase in gene copy numbers evolved to counteract the transcriptional repression of the X chromosome in postmeiotic germ cells. Ampliconic regions make up 19.4 Mb of genomic sequence and approximately 12% of the X chromosome; however, little is known about their functional relevance in the male germline.

The International Knockout Mouse Consortium (IKMC) aims to mutate all protein-coding genes in the mouse using the Cre/loxP targeting approach, among others; however, multicopy genes are not amenable to genetic studies on a one-gene-at-a-time basis. A transgenic RNAi approach has been successfully used to knock down the X-linked multicopy *Slx*/*Slxl1* genes and the Y-linked multicopy *Sly* genes [15, 16]. Cre/loxP-mediated deletion of entire genomic regions could be an effective alternative strategy for studying multicopy genes. Meiotic recombination in mice with independent conditional null mutant alleles flanked by loxP sites (floxed) can generate alleles in which large genomic regions are flanked by loxP sites. Such floxed genomic segments can then be deleted using Cre recombinase, provided that the loxP sites are in the same orientation. The continually expanding number of conditional mutants available from the IKMC will eventually render this strategy feasible for many locations [17]. Here, we have generated a floxed allele for a 1.1-Mb segment of the murine X chromosome, an ampliconic region between *Nxf2* and *Nxf3* that contains 20 genes. We report that germline-specific deletion of this large genomic segment causes male sterility, revealing that it contains factors that are essential for meiotic progression in males.

MATERIALS AND METHODS

Isolation of Mouse Spermatogenic Cells

Cell populations that are highly enriched for specific spermatogenic cell types were isolated from the testes of male CD-1 mice (Charles River

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Laboratories) using the Sta Put method as described previously [18, 19]. Isolation of each of the spermatogenic cell types was done once [20]. The purity of each of the spermatogenic cell types was reported previously: >95% for pachytene spermatocytes and round spermatids, respectively, and >85% for all other cell types [20].

RNA Extraction and RT-PCR

Transcript levels were assessed using a semiquantitative RT-PCR method. Total RNA was extracted from adult mouse tissues, E13.5 fetuses, placenta, isolated spermatogenic cells, and testes from mice of various ages (1, 2, 4, and 8 wk or 4 mo) using TRIzol reagent (Invitrogen). Poly $(A)^+$ RNA was isolated using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech); 0.5 μg of total RNA from tissues or 70 ng of poly $(A)^+$ RNA from germ cells were used for reverse transcription primed with random hexamers or oligo (dT) 18. Bulk cDNA was diluted and used for PCR reactions as previously described [20]. To avoid saturation of PCR reactions, aliquots of products were removed after various cycles of PCR and examined by agarose gel electrophoresis. Each PCR reaction was performed at least twice.

Mice

Nxf2^{fl} and *Nxf3*^{fl} mice were on mixed genetic backgrounds (129xC57BL/6). *Nxf2*^{fl} and *Nxf3*^{fl} mice were intercrossed to generate *Nxf2*$^{fl/+}$ *Nxf3*$^{+/fl}$ females, which carried the *Nxf2*^{fl} and *Nxf3*^{fl} alleles on separate X chromosomes [21, 22]. Among 49 offspring of *Nxf2*$^{fl/+}$ *Nxf3*$^{+/fl}$ females mated to wild-type males, we identified one *Nxf2*$^{fl/Y}$ *Nxf3*$^{fl/Y}$ male that carried both *Nxf2*^{fl} and *Nxf3*^{fl} on the same X chromosome due to meiotic recombination between these two alleles in the *Nxf2*$^{fl/+}$ *Nxf3*$^{+/fl}$ female. This new *Nxf2 Nxf3* floxed allele is referred to as *Nxf*^{fl} in this study; the approved symbol for the allele after Cre recombinase-mediated excision of the Nxf fragment is Del (XNxf2-Nxf3)1Jw (MGI ID: 5297602; see Supplemental Fig. S2). *Nxf*$^{fl/+}$ females (backcrossed to C57BL/6J for two generations) were crossed with *Ddx4*-Cre mice (Jackson Laboratory; stock no. *Ddx4*-Cre, 006954) to produce *Nxf*$^{fl/Y}$ *Ddx4*-Cre males [23]. Genotyping was performed on genomic DNA isolated from tails. Mice were maintained and used in accordance with standards approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Generation of Antibodies

A carboxy-terminal segment of 200 aa (263–462) of the mouse PRAMEL3 protein was expressed as a GST fusion protein in *Escherichia coli* using the pGEX4T-1 vector. After affinity purification with glutathione sepharose, the GST-PRAMEL3 fusion protein was used to immunize rabbits (Cocalico Biologicals Inc.). Two antisera, UP2340 and UP2341, were obtained, from which antibodies were affinity purified by immunoblotting [24]. Western blot analysis was performed on 20 μg of testicular extract as previously described [22].

Sperm Count and Mating Tests

Cauda epididymides were dissected. Epidydimal sperm were fixed in 4% formaldehyde and counted using a hematocytometer. Sperm count was performed as previously described [25]. For mating tests, four wild-type and four *Nxf*$^{fl/Y}$ *Ddx4*-Cre males (2 mo of age) were individually housed with two wild-type virgin females (2–3 mo of age) for 2 mo, with daily inspection of cages. The wild-type males sired an average of 2.5 litters per male, with 8.6 pups per litter. The four *Nxf*$^{fl/Y}$ *Ddx4*-Cre males produced no offspring.

Histological Analysis

Testes were fixed in Bouin solution, embedded in paraffin, and sectioned, and slide-mounted sections were stained with hematoxylin and eosin. To quantify the seminiferous tubules, 100 cross sections of seminiferous tubules per mouse were examined and categorized into tubules with meiotic arrest (absence of spermatids) and tubules without meiotic arrest (presence of spermatids). Testes from five 8-wk-old and six 4-mo-old *Nxf*$^{fl/Y}$ *Ddx4*-Cre males were analyzed. The data were presented as average ± standard deviation.

Immunofluorescence, TUNEL, and Nuclear Spread Analyses

For immunofluorescence, testes were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated in 30% sucrose overnight, frozen in dry ice/95% ethanol, and sectioned at −20°C. TUNEL assays were performed on frozen sections using ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon) combined with immunostaining with anti-H1t antibodies. Surface spreads of spermatocyte nuclei were prepared as previously described [26, 27]. The following primary antibodies were used for immunofluorescence: anti-PRAMEL3 (this study), antihistone H1t (gift from Mary Ann Handel) [28], CREST antiserum (gift from Bill Brinkley) [29], anti-SYCP1 (Abcam, ab15090), and anti-SYCP2 [30]. Secondary antibodies were FITC- and Texas red-conjugated. Images were captured on an Axioskop 40 fluorescence microscope using a digital camera (Evolution QEi, MediaCybernetics).

RESULTS

A 1.1-Mb Nxf2–Nxf3 Segment on the X Chromosome Is Enriched for Testis and Brain-Expressed Genes

A 1.1-Mb segment of the murine X chromosome, located between the *Nxf2* and *Nxf3* genes, encodes 20 genes with testis- and brain-specific expression patterns (Fig. 1 and Supplemental Table S1; all Supplemental Data available online at www.biolreprod.org). This region, here termed Nxf segment, is syntenic with the human Xq22.1 region and maps to the cytogenetic bands XF1 (18 genes: *3632454L22Rik* to *Nxf3*) and XE3 (two genes: *Nxf2* and *Zmat1*; Fig. 1). Supplemental Table S1 summarizes the known or putative functions of the 20 genes, of which so far only *Nxf2*, *Nxf3*, and G protein-coupled receptor associated sorting protein 1 (*Gprasp1*) have been subject to targeted inactivation in the mouse. *Nxf2* and *Nxf3* are expressed in germ cells and Sertoli cells of the testis, respectively [21, 22]. Inactivation of *Nxf2* impairs male fertility [21], whereas *Nxf3*-deficient mice are grossly normal and fertile [22]. A double mutant of *Nxf2* and *Nxf3* lacks compound effects [22]. GPRASP1 has been implicated in regulating dopamine and cannabinoid receptor signaling, and *Gprasp1* null-mutant mice exhibit impaired striatum-dependent responses [31, 32]. The Nxf segment also contains a cluster of genes belonging to the *Prame* (preferentially expressed antigen in melanoma) family of genes that are expressed in testis and several types of cancers (cancer-testis genes): *Pramel*, *Pramel3* (five nearly identical copies), and *Prame*; two genes (*Tceal6* and *Bhlhb9*) encode transcription factors; *Gm6215* (three nearly identical copies) and *3632454L22Rik* are long intergenic noncoding RNA (lincRNA) genes; and *Mir1970* is a microRNA gene. In addition, the fragment contains three predicted pseudogenes (*Gm6207*, *Gm6228*, and *Gm15016*), for which RNA reference sequences are lacking.

We determined expression patterns of 18 genes encoded within the Nxf fragment in diverse tissues by semiquantitative RT-PCR analysis (Fig. 1) and found that eight of these were specifically or preferentially expressed in testis: *Nxf2*, *Pramel*, *Pramel3*, *Gm6215*, *Prame*, *Tcp11l3*, *Tmsb15a*, and *Nxf3*. *Nxf7* transcript levels were highest in placenta but detectable in other tissues such as testis and brain, whereas *Tceal6* expression was restricted to brain. Transcripts of the remaining eight genes (*Zmat1*, *3632454L22Rik*, *Armcx5*, *Gprasp1*, *Gprasp2*, *Bhlhb9*, *Arxes1/2*, and *Bex2*) were detected in a broad range of tissues, including testis and brain. The Nxf segment therefore includes both genes with highly tissue-specific expression patterns, restricted predominantly to testis and brain, and broadly expressed genes that may play roles in multiple tissues.

X-Linked Genes Exhibit Stage-Specific Expression Patterns During Spermatogenesis

For 16 genes in the Nxf fragment that were expressed in testis, we next characterized expression profiles throughout spermatogenesis by assessing transcript levels in seven specific subpopulations of spermatogenic cells isolated from testis (Fig. 2). These highly enriched cell populations included three types

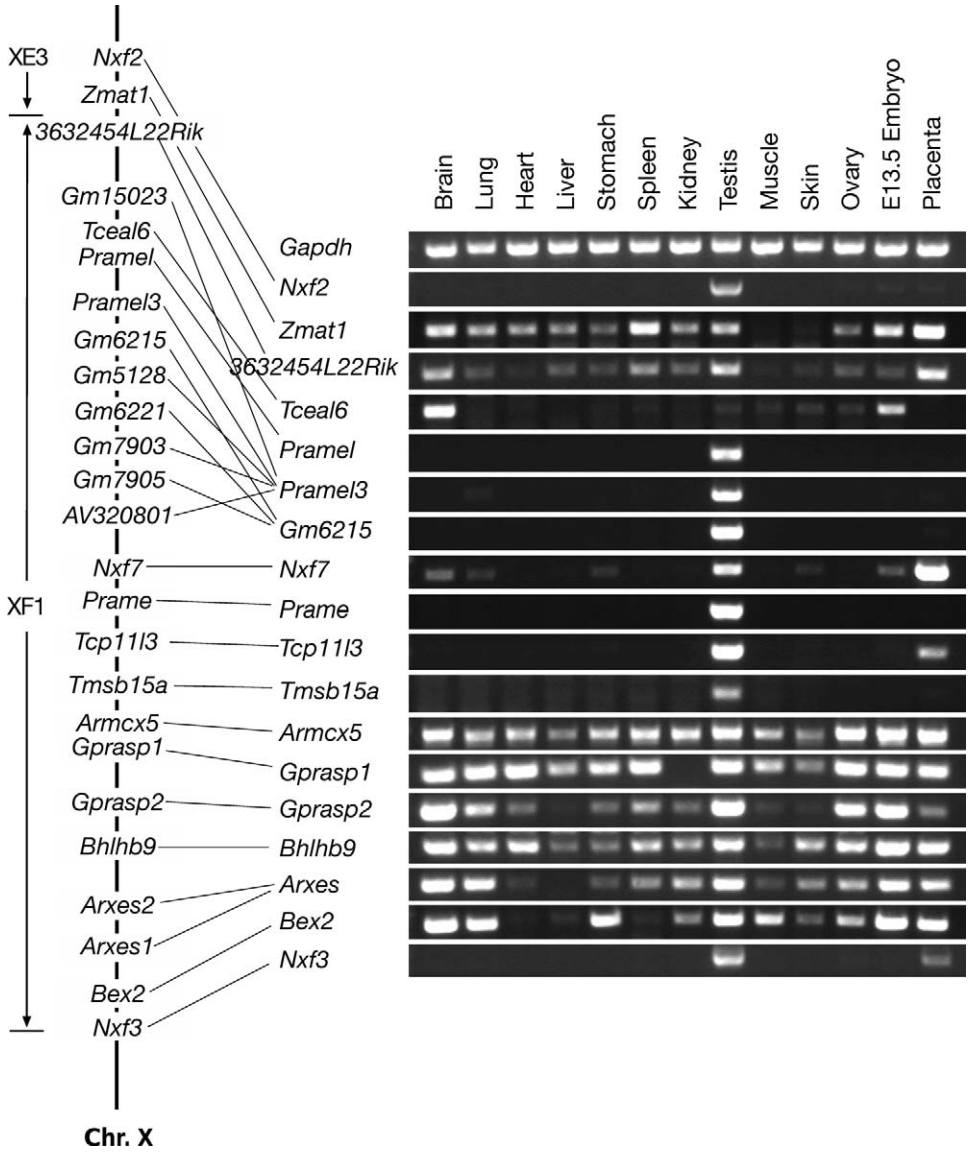

FIG. 1. Relative chromosomal locations and tissue-specific expression of genes contained within the 1.1-Mb Nxf fragment on the X chromosome. Expression was assayed by RT-PCR analysis of RNA isolated from adult mouse tissues and from E13.5 whole embryo and placenta. Chromosomal locations shown are based on the NCBI mouse reference genome. Almost identical gene copies of *Pramel3* (five copies) and *Gm6215* (three copies) are marked by multiple lines. Primer sequences are listed in Supplemental Table S2. *Gapdh* served as a positive control; reactions without reverse transcriptase were negative (data not shown).

of spermatogonia, that is, mitotically dividing diploid germ cells: primitive type A, which contain a subpopulation of self-renewing spermatogonial stem cells; type A, which proliferate and differentiate into intermediate spermatogonia, which then divide to become type B spermatogonia. Type B spermatogonia enter meiosis to become Prophase I spermatocytes (preleptotene, leptotene, zygotene, pachytene, and diplotene). Round spermatids are postmeiotic haploid germ cells that undergo a complex differentiation process to develop into mature spermatozoa. Eight of the 16 genes (*Zmat1*, *Armcx5*, *Gprasp1*, *Gprasp2*, *Bhlhb9*, *Arxes1*, *Arxes2*, and *Bex2*) were expressed in all spermatogenic subpopulations and in XX^{Y*} testis (Fig. 2), which are germ cell deficient [33], revealing activity of these genes in germ cells throughout spermatogenesis and in somatic cells of the testis. In addition to *Nxf2* and *Pramel3* [8], four genes (*Nxf7*, *Prame*, *Tcp11l3*, and *Tmsb15a*) were found to be specifically expressed in germ cells of the testis, lacking expression in germ cell-deficient XX^{Y*} testis. These genes also exhibited differential expression in spermatogenic subpopulations: transcripts were found in spermatocytes and round spermatids but not in spermatogonia. During male meiosis, sex chromosome-linked genes undergo transcriptional inactivation (MSCI) at the pachytene stage [4, 7]. We observed reduced expression of many of the 16 genes analyzed in pachytene spermatocytes, providing evidence that these X-linked genes were subject to MSCI.

Dynamic Expression and Subcellular Localization of PRAMEL3 During Spermatogenesis

Pramel3 is a cancer-testis gene of unknown physiological functions. Transcript analysis suggested that PRAMEL3 is restricted to germ cells of the testis and absent from somatic cells (Fig. 2). To confirm this finding on the protein level and to characterize PRAMEL3 protein localization during spermatogenesis, we raised polyclonal antibodies for PRAMEL3.

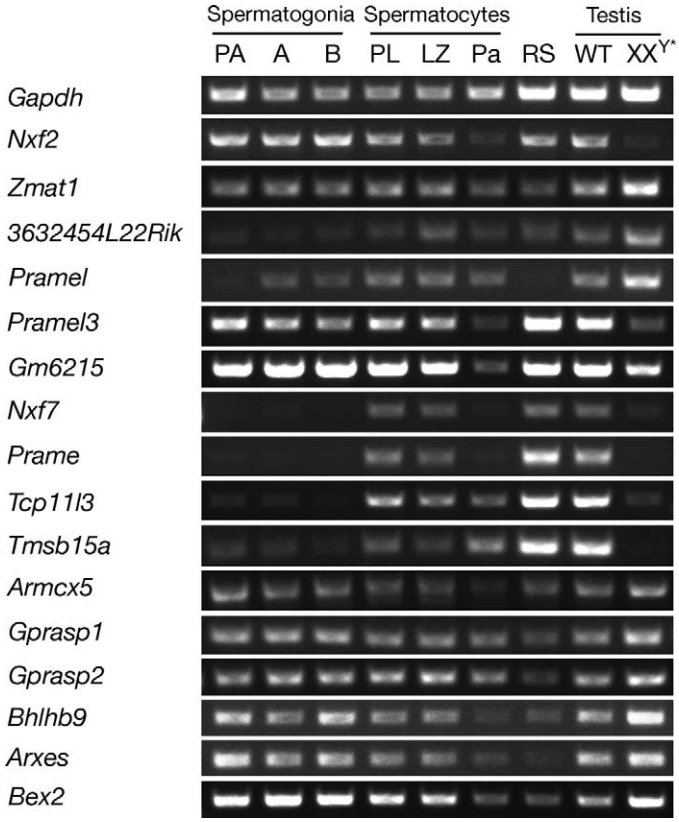

FIG. 2. Expression profiles of 16 genes of the 1.1-Mb Nxf segment during spermatogenesis. Relative transcript levels were determined by RT-PCR, with *Gapdh* as positive control for ubiquitous and *Nxf2* and *Pramel3* for testis-specific gene expression, respectively [20]. *Nxf3* is specifically expressed in Sertoli cells [22] and was therefore not included. Testes from XX^{Y*} mice lack germ cells and served to identify genes expressed in somatic cells. Abbreviations for spermatogenic cell types: PA, primitive type A spermatogonia; A, type A spermatogonia; B, type B spermatogonia; PL, preleptotene spermatocytes; LZ, leptotene plus zygotene spermatocytes; Pa, adult pachytene spermatocytes; RS: round spermatids. WT: wild type. Gene designations are as described in Figure 1. Primer sequences are listed in Supplemental Table S2.

Immunostaining of testis sections revealed a dynamic expression pattern of PRAMEL3 during spermatogenesis (see Supplemental Fig. S1): consistent with analyses of transcript distribution (Fig. 2), PRAMEL3 protein was detected in spermatogonia, early spermatocytes (preleptotene, leptotene, and zygotene), and round spermatids but absent from pachytene spermatocytes, metaphase spermatocytes, and elongated spermatids. In spermatogonia and round spermatids, PRAMEL3 was evenly distributed across cytoplasm and nuclei (see Supplemental Fig. S1A), whereas leptotene and zygotene spermatocytes exhibited higher protein levels in the cytoplasm (see Supplemental Fig. S1B, C). Absence of PRAMEL3 in pachytene spermatocytes suggests that *Pramel3*, like other X-linked genes, is subject to MSCI. Although most X-linked genes remain suppressed after meiosis and are not active in round spermatids, multicopy genes escape from postmeiotic silencing by an increase in copy number, thereby compensating for a low expression level per copy [14]. The *Pramel3* gene is present in five copies (Fig. 1). Consistent with the notion on the postmeiotic expression of multicopy genes, PRAMEL3 transcript and protein are abundantly expressed in round spermatids (Fig. 2 and see Supplemental Fig. S1).

Germline-Specific Segmental Deletion Causes Male Sterility and Meiotic Defects

To ascertain the consequences of the loss of a chromosomal segment enriched in testis-expressed genes, including multicopy genes (Fig. 1), we generated a conditional allele of the 1.1-Mb Nxf fragment, permitting its tissue-specific deletion. From intercrosses of mice with loxP-flanked alleles for *Nxf2* (*Nxf2*^{fl}) and *Nxf3* (*Nxf3*^{fl}), we obtained offspring with both *Nxf2*^{fl} and *Nxf3*^{fl} alleles on the same X chromosome as a consequence of meiotic recombination [21, 22]. In this new conditional allele (approved designation: Del [XNxf2-Nxf3] 1Jw [MGI:5297602]; here abbreviated *Nxf*^{fl}), *Nxf2* and *Nxf3* are flanked by loxP sites of the same orientation (see Supplemental Fig. S2). Ubiquitous deletion of the Nxf segment in males using *Actb*-Cre causes neonatal lethality. To obtain germ-cell specific deletion of this allele, we generated *Nxf*$^{fl/Y}$ *Ddx4*-Cre males. *Ddx4*-Cre (previously termed Vasa-Cre) expression is restricted to the germline; expression begins in embryonic germ cells at Embryonic Day 15 (E15) and continues throughout germ cell maturation, in males until the postmeiotic spermatid stage [23]. The *Ddx4*-Cre has been used to efficiently disrupt several genes specifically in male germ cells [34–36]. *Nxf*$^{fl/Y}$ *Ddx4*-Cre males were sterile, producing no offspring in mating tests. Testes from 8-wk-old *Nxf*$^{fl/Y}$ *Ddx4*-Cre males were significantly reduced in size, at 40% of the weight of wild-type testes (Fig. 3, A and B). The sperm count of *Nxf*$^{fl/Y}$ *Ddx4*-Cre males was less than 8% that of wild-type males (Fig. 3C). Therefore, deletion of the *Nxf2-Nxf3* segment on the X chromosome causes male sterility.

Histological analysis of testes from *Nxf*$^{fl/Y}$ *Ddx4*-Cre males revealed meiotic defects. Seminiferous tubules from wild-type adult testes contained a full spectrum of spermatogenic cells at different stages, including spermatocytes and spermatids (Fig. 3, D and F). In contrast, most seminiferous tubules (57 ± 10%) from 8-wk-old *Nxf*$^{fl/Y}$ *Ddx4*-Cre males contained spermatocytes but lacked postmeiotic germ cells, indicating meiotic arrest. The remaining tubules contained postmeiotic germ cells, such as round spermatids and/or elongated spermatids (Fig. 3E). We assume that the subset of tubules with apparent normal spermatogenesis failed to undergo complete Cre-mediated deletion of the *Nxf* segment due to its large size (1.1 Mb). In tubules with meiotic arrest, the most advanced germ cells were pachytene spermatocytes (Fig. 3G). Consistent with testis histology and sperm count data, epididymal tubules from *Nxf*$^{fl/Y}$ *Ddx4*-Cre males lacked sperm or contained visibly fewer sperm compared to those of wild type mice (Fig. 3, H and I).

As an approach to assess the efficacy of *Ddx4*-Cre-mediated deletion of the Nxf segment and to determine if the presence of spermatid-containing tubules in *Nxf*$^{fl/Y}$ *Ddx4*-Cre testes (Fig. 3E) was due to incomplete Cre-mediated deletion, we examined expression of PRAMEL3 protein (Fig. 4). Western blot analysis confirmed the presence of PRAMEL3 protein in wild-type but not germ cell-depleted XX^{Y*} testes (Fig. 4A). In testes from *Nxf*$^{fl/Y}$ *Ddx4*-Cre males, PRAMEL3 was not detectable by Western blot, suggesting that Cre-mediated deletion of the *Nxf* fragment was overall efficient (Fig. 4B). Immunofluorescent analysis confirmed that in *Nxf*$^{fl/Y}$ *Ddx4*-Cre mutant tubules with meiotic arrest, germ cells of all stages lacked detectable PRAMEL3 protein (Fig. 4D). In *Nxf*$^{fl/Y}$ *Ddx4*-Cre mutant tubules that contained spermatids, PRAMEL3 was expressed in the majority of round spermatids (Fig. 4E), while other round spermatids of the same tubule lacked PRAMEL3 protein (Fig. 4E, arrowheads). These observations suggest incomplete *Ddx4*-Cre-mediated deletion of the Nxf fragment in

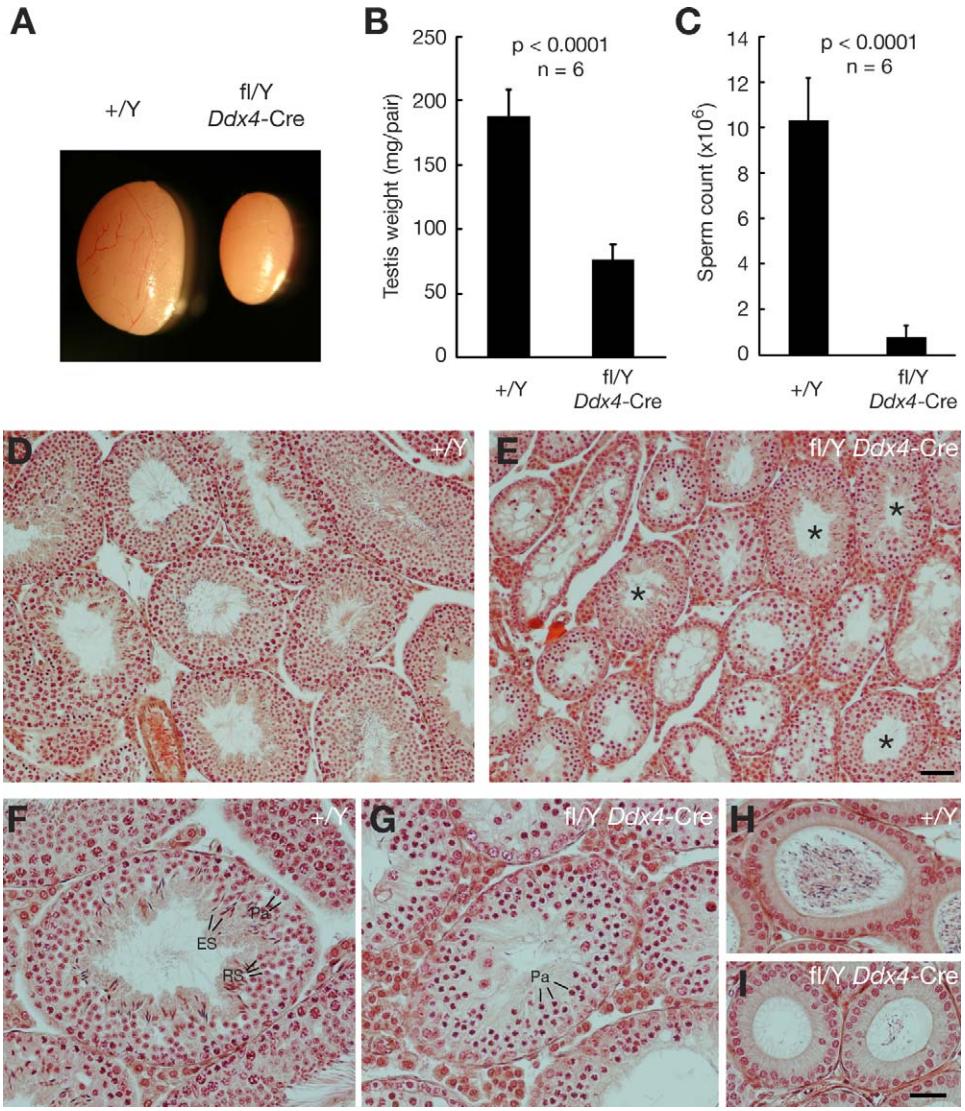

FIG. 3. Germline-specific segmental deletion causes meiotic failure and male sterility. **A**) Significant size reduction in 8-wk-old *Nxf*$^{fl/Y}$ *Ddx4*-Cre testis. **B** and **C**) Significantly reduced testis weight and sperm production in 8-wk-old *Nxf*$^{fl/Y}$ *Ddx4*-Cre mice. Data were analyzed by Student *t*-test. **D** and **E**) Hematoxylin and eosin stained sections of testes from 8-wk-old wild-type and *Nxf*$^{fl/Y}$ *Ddx4*-Cre mice (low magnification). Asterisks mark tubules of the mutant testes that contain postmeiotic germ cells (**E**). **F**) Wild-type tubule contains a full spectrum of germ cells. **G**) Mutant seminiferous tubule exhibiting meiotic arrest at the pachytene stage. **H**) Wild-type epididymal tubule filled with sperm. **I**) Epididymal tubules from *Nxf*$^{fl/Y}$ *Ddx4*-Cre mice, devoid of sperm. Pa, pachytene spermatocytes; RS, round spermatids; ES, elongating spermatids. Bars = 50 μm (**D** and **E**) and 25 μm (**F**–**I**).

spermatogonia, possibly due to its large size, permitting germ cell maturation in some seminiferous tubules (Fig. 3E). In some of these cells, *Ddx4*-Cre may mediate deletion of the Nxf fragment at postmeiotic stages.

The ongoing expression of *Ddx4*-Cre in spermatogonia of *Nxf*$^{fl/Y}$ *Ddx4*-Cre males may lead to an increase in Cre-mediated deletion of the Nxf fragment over time. Consistent with this, the testis weight of mutant males was further reduced at 4 mo (see Supplemental Fig. S3). We observed that, in testes from 4-mo-old *Nxf*$^{fl/Y}$ *Ddx4*-Cre males, the majority of tubules (93 ± 2%; n = 6 mice) exhibited complete meiotic arrest (see Supplemental Fig. S3), compared to 57 ± 10% (n = 5 mice) in 8-wk-old mutant testes (Fig. 3E).

We monitored the *Ddx4*-Cre-mediated deletion of the Nxf segment by analyzing the expression of genes within this segment in testes at different times after birth from 1 wk to 4 mo (see Supplemental Fig. S4). The expression of *Pramel3* is most informative, as it is germ cell-specific and is expressed in spermatogonia. The lower or absent expression of *Pramel3* in *Nxf*$^{fl/Y}$ *Ddx4*-Cre testes showed that the deletion of the Nxf segment was substantial (in spermatogonia) beginning at 1 wk after birth but was not complete until 4 mo. This conclusion was further supported by the expression profiles of other genes, such as *Gm6215*, *Prame*, *Tcp11l3*, and *Tmsb15a*. The genes that are highly expressed in testicular somatic cells, such as *Zmat1*, *Gprasp2*, and *Bhlhb9* (Fig. 2), appeared to be expressed at a higher level in *Nxf*$^{fl/Y}$ *Ddx4*-Cre testes than wild type at 4 wk and beyond (see Supplemental Fig. S4) due to the reduced percentage of germ cells in the mutant testes caused by meiotic arrest. In addition, we examined the expression of three genes on either side of the Nxf segment, respectively. The levels of their expression were either unchanged (*Bex4*) or higher (*Armcx6*) in *Nxf*$^{fl/Y}$ *Ddx4*-Cre testes compared to wild-type controls, mostly likely due to their ubiquitous expression or predominant expression in testicular somatic cells.

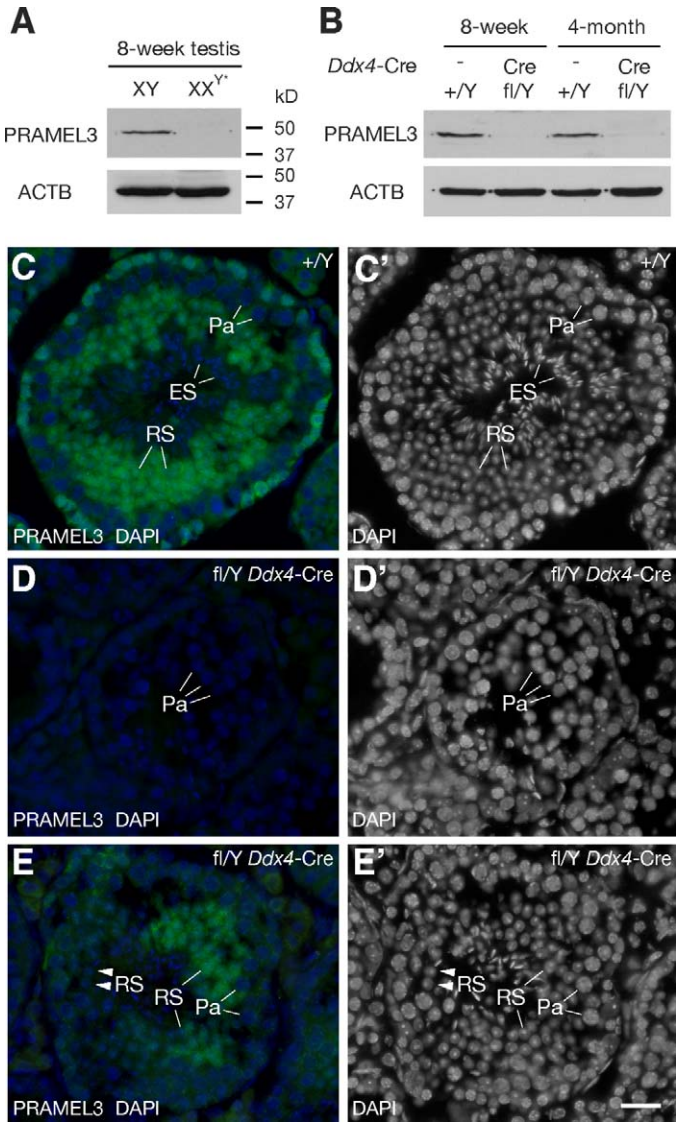

FIG. 4. Cre-mediated recombination of the Nxf fragment in germ cells assayed by the expression of PRAMEL3 protein. **A**) PRAMEL3 is restricted to germ cells and absent in in germ cell-deficient XX^{Y*} testes. **B**) Testes from $Nxf^{fl/Y}$ *Ddx4*-Cre lack detectable PRAMEL3 protein. ACTB serves as a loading control. **C**–**E**) Expression of PRAMEL3 in testicular germ cells from 8-wk-old wild-type (**C**) and $Nxf^{fl/Y}$ *Ddx4*-Cre (**D** and **E**) mice. Testis sections were immunostained with anti-PRAMEL3 antibody (green). Chromatin stained with DAPI (blue) is also shown in black and white to visualize nuclear morphology. PRAMEL3 is absent in mutant tubules with meiotic arrest (**D**). In the mutant tubule with spermatids (**E**), PRAMEL3 is present in some round spermatids but absent in other round spermatids (arrowheads), demonstrating partial Cre-mediated deletion of the Nxf genomic fragment. Bar = 25 μm.

Defective Chromosomal Synapsis in $Nxf^{fl/Y}$ Ddx4-*Cre Males*

Seminiferous tubules from $Nxf^{fl/Y}$ *Ddx4*-Cre testes contained substantially more apoptotic cells than tubules from wild-type testes (Fig. 5, A and B), suggesting effective elimination of abnormal spermatocytes through the pachytene checkpoint that operates at the midpachytene stage [5, 37]. Supporting this hypothesis, we found that prophase I spermatocytes from 20-day-old $Nxf^{fl/Y}$ *Ddx4*-Cre males contained proportionally fewer mid- to late (H1t-positive) pachytene cells [28] than early (histone H1t-negative) stages (Fig. 5C).

To determine the cause of meiotic arrest in $Nxf^{fl/Y}$ *Ddx4*-Cre germ cells, we examined the formation of the synaptonemal complex (SC), which mediates the alignment and synapsis of homologous chromosomes during meiosis. Immunostaining for SYCP2, a component of the axial/lateral elements of the SC (Fig. 6A) [30, 38, 39], showed that SC lateral elements were present in spermatocytes from $Nxf^{fl/Y}$ *Ddx4*-Cre males (Fig. 6, B–D). However, visualization of SYCP1, a component of SC transverse filaments that localizes to the synapsed regions of SC [40, 41], revealed defects in chromosomal synapsis in pachytene spermatocytes from $Nxf^{fl/Y}$ *Ddx4*-Cre testes (Fig. 6E). In most (90%) wild-type pachytene spermatocytes, the 19 pairs of autosomes were fully synapsed, and the XY chromosomes synapsed at the pseudoautosomal regions (Fig. 6, A and E). In contrast, 46% of mutant spermatocytes from 20-day-old testes exhibited synaptic defects: 8% displayed asynapsis of only the XY chromosomes (Fig. 6B), 13% of cells contained incompletely synapsed autosomes with split ends (Fig. 6C), and in 25% of cells both sex chromosomes and autosomes were affected (Fig. 6D).

Mouse chromosomes are telocentric, and their centromeres are the last regions to synapse [42, 43]. Combined immunostaining with anti-SYCP2 antibodies and the centromere-specific CREST antiserum [44] showed that among 33 pachytene spermatocytes from $Nxf^{fl/Y}$ *Ddx4*-Cre testes with unsynapsed autosomal ends, 22 cells exhibited asynapsis only at some centromeric ends, three cells displayed asynapsis only at some noncentromeric ends, and in eight cells asynapsis affected both centromeric and noncentromeric ends, showing that Nxf deletion could affect synapsis at either chromosomal end. In summary, these data suggest the presence of a single or multiple X-linked factors in the *Nxf2-Nxf3* segment that are important for chromosomal synapsis.

DISCUSSION

The 1.1-Mb *Nxf2-Nxf3* genomic region on the X chromosome is enriched for testis-specific single-copy and multicopy genes. Deletion of this segment in the male germline causes male sterility, revealing that this region contains new factors that are essential for male meiosis. This finding is intriguing considering that the mammalian X chromosome is transcriptionally inactivated during male meiosis due to MSCI [4, 7]. We previously reported that *Tex11*, an X-linked germ cell-specific gene, is essential for male meiosis [45]. TEX11 interacts with SYCP2 and regulates both chromosomal synapsis and meiotic recombination. TBP-associated factor 7 like (*Taf7*l), which is also X-linked, functions synergistically with *Tex11* in the regulation of male meiosis [11]. These X-linked genes are transcriptionally silenced at the pachytene stage of meiosis as a consequence of MSCI. However, continued availability of their respective protein products is essential for meiotic progression and may rely on accumulation of transcripts prior to MSCI.

The question remains as to which gene in the 1.1-Mb Nxf region is responsible for meiotic defects. It might be a single gene or more than one gene. The testis-specific genes (*Pramel*, *Pramel3*, *Prame*, *Tcp11l3*, and *Tmsb15a*) are strong candidates, as they are expressed in early spermatocytes. However, we cannot exclude the broadly expressed genes. Future genetic studies are needed to further narrow down the genomic region and identify the gene or genes critical for male meiosis. It is also possible that the synaptic abnormality observed in the Nxf^{fl} *Ddx4*-Cre mice is a downstream effect of other

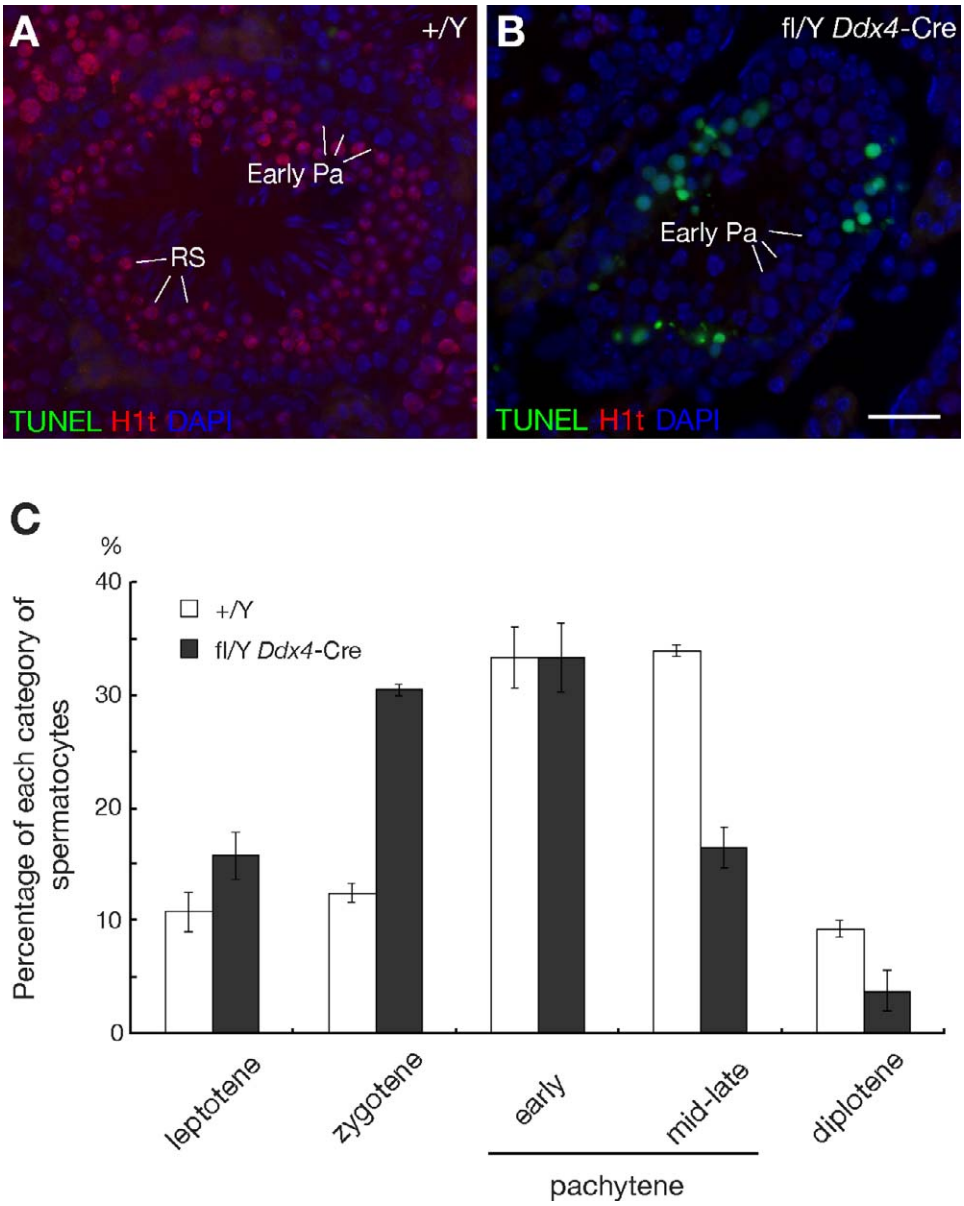

FIG. 5. Apoptosis of spermatocytes in $Nxf^{fl/Y}$ *Ddx4*-Cre testes. **A** and **B**) Spermatocytes in $Nxf^{fl/Y}$ *Ddx4*-Cre testis undergo apoptosis. TUNEL assay of frozen testis sections from 8-wk-old wild-type and $Nxf^{fl/Y}$ *Ddx4*-Cre males, combined with immunostaining for H1t. Chromatin is visualized with DAPI. RS, round spermatids (H1t-positive); Early Pa, early pachytene spermatocytes (H1t-negative). Bar = 25 μm. **C**) Reduced proportion of mid-late pachytene spermatocytes among prophase I spermatocytes from $Nxf^{fl/Y}$ *Ddx4*-Cre males. Surface spreads of spermatocytes from 20-day-old mice were immunostained with antibodies against SYCP2 and histone H1t; 200 spermatocytes from each mouse (three mice per genotype) were counted.

deficiencies in germ cells. Recently, aneuploidy (loss or gain of a chromosome) causes genomic instability and proteotoxic stress in yeast mitotic cells [46–48]. The deletion of the 1.1-Mb Nxf region on the X chromosome could have a more global effect on the transcriptome or proteome or even on the expression of the adjacent genes on either side of this segment. Notably, there is abnormal synapsis between X and Y in the In (X)1H male mice harboring a segmental inversion on the X chromosome [49]. Because the loxP sites are located within the introns of *Nxf2* and *Nxf3* genes (Supplemental Fig. S2), the 1.1-Mb Nxf segmental deletion does not delete the promoters of the neighboring genes. However, it remains possible that the deletion may interfere with the expression of genes outside the deleted region. Our attempt to address this possibility using testis samples was inconclusive due to the wide expression patterns of the neighboring genes (Supplemental Fig. S4).

Chromosomal segmental deletion is a frequent cause of human disease. Such chromosomal microdeletion regions span a few Mb genomic DNA sequences and contain multiple genes. Deletion of multiple genes often leads to haploinsufficiency syndromes. The 22q11 deletion syndrome, typically caused by a 3-Mb deletion, is the most common human microdeletion syndrome, occurring in 1:4000 live births [50, 51]. Medical cytogenetics and array comparative genomic hybridization have identified and defined the deletion intervals for a number of human deletion syndromes, including 1p36 [52, 53], 2q37 [54], 3p [55], 9p [56, 57], 13q [58], 18q23 [59], and Yq [60]. While studies of human patients are important for understanding the pathogenesis of these complex syndromes, there is a

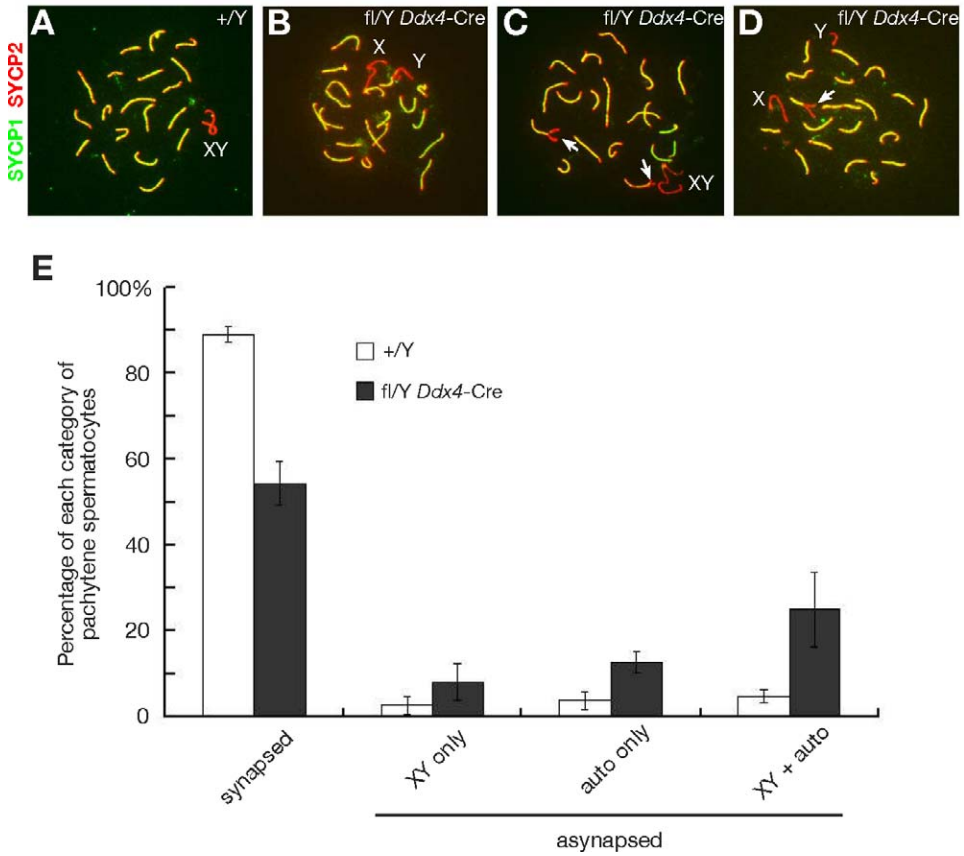

FIG. 6. Defective chromosomal synapsis in germ cells from $Nxf^{fl/Y}$ *Ddx4*-Cre males. **A**) Wild-type pachynema; 19 pairs of autosomes are fully synapsed. XY chromosomes are synapsed only in the pseudoautosomal regions. **B**) Mutant pachynema with asynapsed XY chromosomes, autosomes are not affected. **C**) Mutant pachynema with partially asynapsed autosomes (white arrows). **D**) Mutant pachynema with synaptic defects affecting both autosomes (white arrow) and sex chromosomes. **E**) Analysis of chromosomal synapsis in pachytene spermatocytes from 20-day-old wild-type and $Nxf^{fl/Y}$ *Ddx4*-Cre testes. Testes from three wild-type and three $Nxf^{fl/Y}$ *Ddx4*-Cre mice were analyzed by surface nuclear spread analysis using anti-SYCP1 and anti-SYCP2 antibodies; 100 pachynema were analyzed per mouse, and grouped into the categories depicted in **A**–**D**.

pressing need for generating animal models of human deletion syndromes. The mouse model of the 22q11 deletion syndrome has been valuable for characterizing this human syndrome [61–63].

In a recent report, a familial 1.1-Mb deletion in human chromosome Xq22.1 was found in heterozygous female patients with epilepsy, mental retardation, and other developmental defects [64]. Our 1.1-Mb deletion region (*Nxf2*-*Nxf3*) on the mouse X chromosome is syntenic to the 1.1-Mb deletion region in human Xq22.1. We have generated $Nxf^{+/-}$ heterozygous females using a ubiquitously expressed *Actb*-Cre [65]. The characterization of $Nxf^{+/-}$ heterozygous females will be published in a separate study. Therefore, our Nxf segmental deletion mouse mutant is an animal model for this new human syndrome: the Xq22.1 deletion syndrome.

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