Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID

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The establishment and maintenance of spermatogenesis in mammals requires specialized networks of gene expression programs in the testis. The gonad-specific TAF4b component of TFIID (formerly TAF_{II}105) is a transcriptional regulator enriched in the mouse testis. Herein we show that TAF4b is required for maintenance of spermatogenesis in the mouse. While young *Taf4b*-null males are initially fertile, *Taf4b*-null males become infertile by 3 mo of age and eventually exhibit seminiferous tubules devoid of germ cells. At birth, testes of *Taf4b*-null males appear histologically normal; however, at post-natal day 3 gonocyte proliferation is impaired and expression of spermatogonial stem cell markers *c-Ret*, *Plzf*, and *Stra8* is reduced. Together, these data indicate that TAF4b is required for the precise expression of gene products essential for germ cell proliferation and suggest that TAF4b may be required for the regulation of spermatogonial stem cell specification and proliferation that is obligatory for normal spermatogenic maintenance in the adult.

[Keywords: Spermatogonial stem cell; *Taf4b*; germ cell; *Stra8*; *c-Ret*]

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Spermatogenesis is a complex process requiring the specialized function of multiple cell types including somatic and germ cells that collectively results in the continuous production of functional sperm in adult males. The unlimited production of male gametes is largely accomplished through the ability of spermatogonial stem cells to self-renew in the adult testis. These complex and multifaceted events are dependent on appropriate expression and action of specific genes at multiple stages of germ cell and testicular development (Matzuk and Lamb 2002; McLaren 2003). The precise temporal and spatial expression of specific transcription factors is also essential for proper execution of spermatogenesis (Sassone-Corsi 1997). Emerging evidence now suggests that in addition to gonad-specific transcription factors, specialized components of the basal RNA Polymerase II machinery are also critical for the execution of gonad-specific programs of gene expression (Hochheimer and Tjian 2003).

The TFIID complex is a core RNA polymerase complex that contains the TATA-binding protein (TBP) and

14 TBP-associated factors (TAFs) that function in core promoter recognition and activator-dependent RNA Polymerase II recruitment (Verrijzer and Tjian 1996). While most TFIID subunits are expressed and function broadly in most cell types, there are selective TFIID subunits that apparently have evolved to function in the specification of gonadal-specific programs of gene expression. In the mouse, TAF4b is a component of TFIID that is highly enriched in gonadal tissues and is required for ovarian follicle development (Freiman et al. 2001). TAF4b is similar in structure to its broadly expressed paralog TAF4 (TAF_{II}130). While TAF4 and TAF4b display overlapping expression patterns in certain cell types, TAF4b is essential for regulating the selective expression of ovarian-specific gene expression patterns required for female fertility (Freiman et al. 2001).

Several other members of the basal transcription machinery also exhibit cell-specific expression and regulation of genes in the testis. The *Drosophila* testes contain several unique isoforms of TAFs that are essential for fertility (Hiller et al. 2004). In mammals, TFIID components are expressed at high levels at specific stages of spermatogenesis (Schmidt and Schibler 1995) and the testis contains a unique paralog of TAF7, TAF7L (Poin-tud et al. 2003). TRF2/TLF, a TBP-related factor, acts as

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a surrogate transcription factor for TBP in pachytene spermatocytes and post-meiotic spermatids (Martianov et al. 2002). Loss of *Trf2/Tlf* causes infertility in mice (Zhang et al. 2001). ALF (TFIIA τ) is a germ cell-specific isoform of TFIIA, another component of the basal RNA Polymerase II machinery, that can substitute for TFIIA at specific promoters (Ozer et al. 2000). The requirement for specialized transcription machinery in spermatogenesis is a highly conserved phenomenon across species and likely involves several yet-to-be-characterized factors.

To identify the function of TAF4b in male fertility, we characterized reproduction and testis development in the *Taf4b*-null males. Herein we show that TAF4b is obligatory for the maintenance of spermatogenesis in adult mice. Mice null for *Taf4b* initially appear normal and fertile; however, following the first round of spermatogenesis, seminiferous tubules degenerate, eventually becoming devoid of germ cells. Phenotypic abnormalities are first apparent at post-natal day 2 when germ cell stem cell differentiation and proliferation are disrupted and several genes involved in spermatogonial stem cell function including *Stra8* and *c-Ret* are misregulated. These data suggest that early expression of TAF4b in spermatogonial stem cells in the post-natal testis may be required for the proper maintenance of spermatogenesis in the adult.

Results

Taf4b-null males become infertile by 11 wk of age

Taf4b-null males are initially fertile, but display multiple reproductive defects by 11 wk of age. To investigate the underlying cause for loss of fertility, 4-wk-old *Taf4b*-null or heterozygous male mice were placed with fertile females. The experiment began on the day that the first litter was born, and the number of subsequent litters born was recorded for 6 mo. During the first month, *Taf4b* heterozygous and null males sired a comparable number of litters (Fig. 1A). When the number of pups born to each female was counted at the time of weaning, *Taf4b* heterozygotes and null mice sired a similar number of pups (Fig. 1B). During months 2–6, *Taf4b* heterozygotes sired an average of four litters. In contrast, no litters were born to *Taf4b*-null males after the first month of mating (Fig. 1A).

To determine more precisely when *Taf4b*-null males become infertile, immature wild-type females were treated with a superovulatory regime of hormones and placed in cages with a single 8-wk-old *Taf4b* heterozygous or null male. On the following morning, vaginal plugs were observed, and on the next day females were sacrificed, oviducts were removed, and the number of fertilized versus nonfertilized oocytes was counted. In superovulated females placed with either 8-wk-old *Taf4b* heterozygotes or knockouts, 71% and 59% of oocytes were fertilized, respectively (Fig. 1C). When the experiment was repeated with the same males but new females at 11 wk of age, 78% of the oocytes were fertilized.

Figure 1. (A) The number of litters born during the first month or months 2–6 from breeding pairs with *Taf4b* heterozygous or null males were counted. (B) The average number of pups born in the first litter to *Taf4b* heterozygous or null males was counted. (C) The percentage of oocytes fertilized in superovulated wild-type females placed with *Taf4b* heterozygous or null males at 8 and 11 wk of age was determined. Means were calculated (as shown by a solid bar), and statistical significance was determined using the Student's *t*-test. (D) *Taf4b* heterozygous and null males were weighed. (E) Testes weights from the same mice were weighed. Means were compared by two-way ANOVA. An asterisk (*) indicates $p < 0.01$. (F) Serum from *Taf4b* heterozygous and null males was isolated and analyzed by radioimmunoassay for FSH. Statistical analysis was performed using two-way ANOVA. Statistical significances of $p < 0.01$ (*) and $p < 0.001$ (**) are indicated.

ized by *Taf4b* heterozygotes. In contrast, only 13% of the oocytes collected from superovulated females placed with 11-wk-old *Taf4b*-null males were fertilized (Fig. 1C). Therefore, *Taf4b* heterozygotes and knockouts are able to fertilize oocytes in vivo at comparable levels when they are 8 wk of age but 11-wk-old *Taf4b*-null males are essentially infertile.

To assess testes development, body weight and testes weights were measured. At all time points there was no significant difference in the body mass of *Taf4b*-null and heterozygous mice (Fig. 1D). However, there was a significant difference in testis weights between *Taf4b* heterozygotes and knockouts as early as 3 wk of age ($p < 0.01$) (Fig. 1E). By 6 wk of age, *Taf4b*-heterozygous testes reached near maximal mass, weighing 81.1 mg (± 1.4) on average, that was maintained through 12 wk. In contrast, *Taf4b*-null testes were smaller at 6 wk of age (58.1 mg ± 2.2 mg) and weighed only 40% that of heterozygotes at 12 wk.

To determine if endocrine abnormalities might underlie or contribute to the *Taf4b* male phenotype, serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) were measured. In mice younger than 7 wk of age, no differences were ob-

served in serum levels of FSH, LH, or T (Fig. 1F; data not shown). However, by 8–9 wk of age, FSH levels become elevated in *Taf4b*-null males relative to heterozygous littermates. Serum levels of LH and T remained comparable in the heterozygous and null animals at all ages assayed (data not shown). Fertile 8-wk-old *Taf4b*-null males exhibited minor defects in epididymal sperm; however, by 12 wk sperm motility and acrosome formation were severely impaired (Fig. 1G).

TAF4B is expressed in gonocytes in the post-natal testes and in spermatogonia and spermatids in the adult testes

To determine the cell type or stage-specific expression of TAF4B, immunohistochemistry using a polyclonal TAF4B antiserum was performed. The results demonstrate that TAF4b protein is expressed highly in gonocytes in neonatal testes (Fig. 2A, day 3). In the adult testes (Fig. 2B) specific punctate nuclear staining was observed in premeiotic spermatogonia and post-meiotic spermatids. TAF4b protein was not detected in meiotic spermatocytes or somatic cells. TAF4b protein was not detected in *Taf4b*-null testes (Fig. 2C) or in wild-type testes incubated with preimmune serum (Fig. 2D). Immunohistochemical analysis in wild-type adult testes ranging in age from 12 wk to 1 yr showed that TAF4b protein was detected in the nuclei of spermatogonia and spermatids but not other testis cell types at all ages assayed (data not shown). A Western blot using the same antibody produced a 105-kDA band in adult heterozygous testes but not *Taf4b*-null testes (Fig. 2E). Thus, TAF4b protein is expressed in germ cells of neonatal as well as adult mice, suggesting that TAF4b might function at several stages of testes development and spermatogenesis.

Taf4b-null testes degenerate as males age

To determine if changes in germ cell number or maturation occur and underlie the defects in *Taf4b*-null testes, sections from heterozygous and null animals of various ages were stained with hematoxylin and eosin or immunostained for germ cell nuclear antigen 1 (GCNA1) used as a marker for premeiotic and meiotic germ cells (Enders and May 1994). No overt histological differences were observed in neonatal (day 0) *Taf4b*-null and heterozygous testes (Fig. 3A–D). However, fewer germ cells were present in *Taf4b*-null testes at post-natal day 8 (Fig. 3E–H). In testes from day 21, *Taf4b*-null mice, the lumens of seminiferous tubules appear to be less densely packed compared to heterozygous males (Fig. 3I,K). GCNA1 staining of these same testes revealed that a few seminiferous tubules of *Taf4b*-null mice were devoid of germ cells and the tubules that did contain sperm were less densely packed with cells relative to heterozygous littermates (Fig. 3J,L). By 8 wk of age, the seminiferous tubules of *Taf4b* heterozygous and null mice contain

Figure 2. Testes from wild-type 3-d-old (A) and 8-wk-old (B) or *Taf4b*-null 8-wk-old (C) animals were immunostained with antiserum against TAF4b. Preimmune serum was used to stain wild-type 8-wk-old testes (D). The following cell types are indicated by arrows: gonocytes (black), spermatogonia (red), Sertoli cells (yellow), spermatocytes (white), and spermatids (green). (E) Western blots with whole-cell extracts from adult *Taf4b*-null or heterozygous testes were probed with TAF4b antiserum.

maturing sperm (Fig. 3M,O). However, some *Taf4b*-null seminiferous tubules were conspicuously devoid of sperm. At 12 wk of age normal spermatogenesis was observed in all tubules of heterozygous mice (Fig. 3Q,R), whereas *Taf4b*-null tubules contained reduced numbers of germ cells (Fig. 3S,T). By 8 mo seminiferous tubule degeneration including loss of germ cells was apparent even in *Taf4b* heterozygotes (Fig. 3U,V); however, heterozygous males remain fertile beyond 12 mo (data not shown). Conversely, by 8 mo *Taf4b*-null males are not only infertile but also present seminiferous tubules that with few exceptions are completely devoid of sperm (Fig. 3W,X). Leydig cell hyperplasia was also apparent but was not associated with abnormal levels of testosterone or LH and is therefore most likely secondary to germ cell loss (Fig. 3X).

Figure 3. Testes from heterozygous (columns 1 and 2) and *Taf4b*-null (columns 3 and 4) mice of various ages (day 8 [A–D], day 21 [E–H], 8 wk [I–L], 12 wk [M–P], and 8 mo [Q–T]) were stained with hematoxylin and eosin (columns 1 and 3) or immunostained with an antibody against GCNA1 (columns 2 and 4). Disrupted tubules in day 21 males are indicated by an asterisk (*). Black arrows indicate normal seminiferous tubule morphology. Tubules depleted of germ cells are designated with open arrows. Red arrows indicate tubules in which germ cells are sloughing off. Leydig cell hyperplasia is marked with a pound sign (#).

Germ cell proliferation is impaired in Taf4b-null males at post-natal day 2

To investigate the underlying cause of the severe loss of germ cells in the adult *Taf4b*-null males, gonads of neonatal mice were analyzed in greater detail. Immunostaining for GCNA1 revealed that on the day of birth (day 0) and on post-natal day 1, *Taf4b*-null males have the same number of gonocytes per seminiferous tubule as wild-type and heterozygous testes (Fig. 4A). However, by post-natal day 2, wild-type and heterozygous mice have more gonocytes per seminiferous tubule than the *Taf4b*-null mice. This difference persists through day 3. By day 8 when there were only 3.8 spermatogonia in seminiferous tubules of *Taf4b*-null mice, wild-type and heterozygous seminiferous tubules contained 14.4 and 14.9 spermatogonia per tubule, respectively (Fig. 4B).

In the mouse testes shortly after birth gonocytes migrate from the center of the lumen to the basement membrane of the seminiferous tubules, proliferate, and differentiate into spermatogonia. Functional spermatogonial stem cells are first evident at post-natal day 3 (McLean et al. 2003). Wild-type, heterozygous, and

Taf4b-null males were immunostained for phospho-histone H3 Ser 10, a marker of mitosis (Fig. 4D). At this important stage of germ cell proliferation, 45.4% and 47.6% of wild-type and heterozygous seminiferous tubules contain phospho-histone H3 positive cells, whereas only 26.6% of knockout seminiferous tubules contain positive cells ($p < 0.05$) (Fig. 4C). TUNEL staining to identify apoptotic cells failed to show differences in wild-type versus *Taf4b*-null testes. Therefore, apoptosis is not the underlying cause of low germ cell number in *Taf4b*-null testes on day 3 or in the adult (Fig. 4E; data not shown).

Loss of germ cells in Taf4b-null animals is cell autonomous

To determine if loss of germ cells in *Taf4b*-null males resulted from defective somatic cell support of germ cells or was inherent to germ cells, germ cell transplantation experiments were conducted. Germ cell stem cells isolated from Rosa26 mice were injected into the seminiferous tubules via the rete testes of 6-mo-old *Taf4b*-null testes. Germ cell-deficient *Kit^{w-v}/Kit^w* mouse testes were also transplanted to serve as positive controls. Three months after the surgery, the animals were sacrificed and the testes were stained for β -galactosidase. Successful germ cell colonization was observed in multiple animals in both *Kit^{w-v}/Kit^w* (Fig. 5A) and *Taf4b*-null (Fig. 5B) strains with donor germ cell derived spermatogenesis (Fig. 5E), suggesting that like the *Kit^{w-v}/Kit^w* mice, the defects in *Taf4b*-null mice are cell-autonomous and inherent to the germ cells. In fact, the overall colonization success was superior in the *Taf4b*-null mice with >50% of the tubules staining blue. These data show that *Taf4b*-null Sertoli cells are functional and capable of forming proper cell contact with wild-type germ cell stem cells and maintaining spermatogenesis. No spermatogenesis was observed in nontransplanted testes (Fig. 5C,F).

Genes involved in germ cell stem cell function, retinoic acid signaling, meiosis, and spermatogenesis are misexpressed in post-natal Taf4b-null testes

Because germ cell stem cells are first present at day 3, germ cell stem cell markers were assayed by RT-PCR and microarray. As early as the day of birth, *Stra8* (stimulated by retinoic acid 8), a germ cell stem cell marker (Giuli et al. 2002) is expressed at lower levels in *Taf4b*-null males (Fig. 6A). *Stra8* expression remained lower in *Taf4b*-null testes at day 8 (Fig. 6B) and in the adult (data not shown). *Plzf*, a transcriptional repressor required for germ cell stem cell self-renewal (Buas et al. 2004), is expressed at normal levels in newborn *Taf4b*-null testes but becomes deficient by day 3 and remains low in day 8 *Taf4b*-null testes. GDNF (glial cell line-derived neurotrophic factor) is a factor secreted by Sertoli cells that mediates the decision between germ cell stem cell renewal and spermatogonial differentiation (Meng et al.

Figure 4. Wild-type, heterozygous, and *Taf4b*-null testes were immunostained for GCNA1 at post-natal days 0, 1, 2, 3 (A), and 8 (B), and the number of positive cells per seminiferous tubule was counted (** indicates $p < 0.05$). The same testes were stained for phospho-histone H3 (Ser 10) (D) or TUNEL-positive cells (E). Positive cells are indicated by black arrows. (C) The percentage of tubules with phospho-histone H3 (Ser 10) positive cells was counted.

2000). *Gdnf* is expressed at normal levels in *Taf4b*-null males, however, the GDNF receptor *Gfr1α* and its effector tyrosine kinase receptor *c-Ret* are deficient in day 8, *Taf4b*-null testes (Fig. 6A,B) with lower *c-Ret* expression observed as early as the day of birth. These results suggest that early post-natal germ cell stem cell specification and proliferation are impaired in *Taf4b*-null males, likely resulting in fewer germ cell stem cells.

Several genes known to be important to meiosis and spermatogenesis are also expressed at reduced levels in the *Taf4b*-null testis at post-natal day 8, a time when the number of spermatogonia is significantly reduced (Fig. 4B). These include genes involved in retinoic acid signaling that are known to impact male fertility (Livera et al. 2002). Retinoic acid receptor γ , which has been shown to control expression of *Stra8* (Chiba et al. 1997), is expressed at lower levels in *Taf4b*-null testis (Fig. 6C). Other retinoic acid signaling molecules, including cellular retinoic acid-binding protein (*Crabp*) and retinol-binding protein (*Rbp*), are reduced in *Taf4b*-null testis as well (Fig. 6C).

Genes involved in meiosis that commences at day 9 in the mouse testes are lower in the *Taf4b*-null testes at post-natal day 8 including *Dmc1*, *Scp3*, and *Stag3* (Fig. 6D; Prieto et al. 2001; Hunt and Hassold 2002). *Bard1*, which has recently been identified as a pro-apoptotic factor in the testes (Feki et al. 2004), is low in day 8 *Taf4b*-null testes. *Bmp8b*, which is required for gonocyte proliferation (Zhao et al. 1996), is expressed at lower levels in *Taf4b*-null testes by RT-PCR at day 8 but not at

day 0 or day 3 (Fig. 6E; data not shown). JAGGED2, the ligand for NOTCH1, is also expressed at lower levels in *Taf4b*-null males. Mice null for *Jagged2* die just after birth, precluding any study of fertility (Jiang et al. 1998). However, JAGGED2 has been functionally implicated in spermatogenesis by in vitro studies where immunodepletion of JAGGED2 or NOTCH1 causes the disappearance of post-meiotic germ cells in culture (Fig. 6E; Hayashi et al. 2001). Thus, even before puberty, several molecular abnormalities are apparent in *Taf4b*-null testes.

Discussion

Spermatogenesis is a complex process that involves specialized transcriptional activity (Sassone-Corsi 2002). Here we show that TAF4b, a cell-type-specific component of the TFIID complex involved in RNA polymerase II transcription, is essential for continuous spermatogenesis and a requisite for fertility in mice. Although males null for *Taf4b* are able to sire one litter, they become infertile. The studies described herein were conducted in a pure C57BL/6 background, and in this background penetrance is 100%. The initial characterization of *Taf4b*-null mice was conducted in a mixed genetic background in which some of the males were fertile (Freiman et al. 2001). The *Taf4b*-null phenotype with the pure C57BL/6 background is characterized specifically by decreases in germ cell (gonocyte) number as early as day 2, lack of spermatogonial proliferation leading to progressive decreases in testes size, eventual depletion of sper-

Figure 5. Germ cell transplantation surgeries were performed using germ cell-deficient *Kit^{w-v}/Kit^w* (A) or *Taf4b*-null (B) males. (C) Uninjected *Taf4b*-null testes are also shown. (A–C) Recipient males were sacrificed 3 mo after transplantation, and their testes were stained β-galactosidase to detect donor cells. Successful colonization (blue-stained tubules) were seen in 6/8 *Kit^{w-v}/Kit^w* and 6/7 *Taf4b*-null testes transplanted. (D–F) Spermatogenesis was also observed in the colonized tubules but not in the nontransplanted testes.

matogonia, and defects in spermiogenesis clearly evident by 8 wk.

Successful maintenance of spermatogenesis requires the establishment of a pool of spermatogonial stem cells that self-renew and differentiate into mature sperm. In the newborn testes, gonocytes are located at the center of the seminiferous tubule. The initial colonization of the gonad by germ cells appears to be normal since at birth *Taf4b*-null males have comparable numbers of gonocytes per seminiferous tubule relative to wild type and heterozygous littermates. Thus, factors that regulate germ cell migration and early stages of proliferation appear intact (McLaren 2003). Shortly after birth gonocytes migrate to the basement membrane, differentiate into spermatogonial stem cells, and proliferate (de Rooij and Russell 2000), a process that leads to the appearance of functional spermatogonial stem cells at post-natal day 3–4 (McLean et al. 2003). These spermatogonial stem cells proliferate rapidly prior to puberty resulting in a 39-fold increase in the number of germ cell stem cells between birth and adulthood (Shinohara et al. 2001). The observed deficiency in germ cells in *Taf4b*-null males beginning at day 2–3 corresponds with this critical stage of stem cell development and expansion, suggesting that the inability of adult *Taf4b*-null males to maintain spermatogenesis might be due to defects in spermatogonial stem cell function. This hypothesis is further substantiated by the ability of transplanted wild-type germ cell stem cells to colonize in seminiferous tubules of adult germ cell-depleted *Taf4b*-null mice.

The phenotype observed in *Taf4b*-null males differs from other previously described mouse models of male infertility. Other knockout mice that exhibit germ cell deficiency just after birth including *Pin1*-null and *Bmp8b*-null mice exhibit defects in primordial germ cell proliferation prior to birth (Zhao et al. 1996; Atchison and Means 2003; Atchison et al. 2003). Since the *Taf4b*-null mice can complete spermatogenesis and spermiogenesis leading to the production of functional sperm and healthy offspring, the genes that mediate these events also seem to be largely unaffected at least in

young males. Increased apoptosis also does not appear to underlie the *Taf4b*-null phenotype, which is in contrast to many other mouse models of infertility (Matzuk and Lamb 2002). The endocrine system also appears to be largely intact since serum levels of the pituitary gonadotropins and testosterone are normal in young *Taf4b*-null mice. Although FSH levels increase in older mice, this could be a consequence of the altered function of the tubules with germ cell loss rather than a cause of the abnormal phenotype. The defects in spermiogenesis observed at 12 wk of age, but not at 8 wk of age, are most likely secondary to the degeneration of the testes that is characterized by loss of germ cells and elevated FSH levels.

The GDNF signaling pathway, which is regulated by FSH, has been shown to be critical for germ cell stem cell fate decision (Meng et al. 2000; Tadokoro et al. 2002). Mice with decreased *Gdnf* levels are fertile; however, spermatogonial stem cells differentiate at inappropriately high levels resulting in seminiferous tubules depleted of sperm in older animals. Conversely, mice overexpressing *Gdnf* are infertile because germ cell stem cells proliferate but do not differentiate (Meng et al. 2000). GDNF, which is secreted by Sertoli cells, signals through the receptor *GFR1α*, which activates the recep-

Figure 6. (A) Semiquantitative RT-PCR was performed using RNA isolated from day 0 and day 3 wild-type and *Taf4b* heterozygous and null testes using specific primer pairs against *Stra8*, *Plzf*, *c-Ret*, and *Gdnf*. For each sample at least five animals were pooled. Microarray analysis was also performed using day 8 testes. RT-PCR reactions were done two to three times. Representative autoradiographs are shown. (B–E) Semiquantitative RT-PCR was performed using RNA isolated from day 8 wild-type and *Taf4b* heterozygous and null testes. Statistical analyses were based on the results of RT-PCR with six individual samples for each group. All values are normalized to the internal control L19. Fold change was calculated comparing knockout to wild-type (left column) or heterozygous (right column) values. Representative autoradiographs are included. Statistical significance was calculated by two-way ANOVA. For *Gdnf* there is not a statistically significant difference. For *Rarα* and *Gfr1α*, $p < 0.05$; for all other genes assayed, $p < 0.01$. Genes that were not included on the array are designated as “n.d.”

tor tyrosine kinase *c-Ret* (Sariola and Saarma 2003; Kubota et al. 2004). Transgenic mice expressing a dominant-negative *c-Ret* mutation have normal testes on the day of birth. However, by post-natal day 10, mutant *c-Ret* mice have fewer germ cells, a phenotype similar to that observed in *Taf4b*-null males (Jain et al. 2004). The observed deficiency of *c-Ret* in *Taf4b*-null males coupled with the evidence that GDNF signaling is important to germ cell stem cell function directly implicates a role for TAF4b in these germ cell stem cells. Since *c-Ret* is deficient in *Taf4b*-null testes as early as the day of birth, when *Taf4b*-null males have comparable numbers of gonocytes relative to wild-type males of the same age, *c-Ret* could be a direct target of TAF4b.

PLZF (promyelocytic leukemia-associated protein), a zinc finger transcription factor involved in germ cell stem cell self-renewal (Buaas et al. 2004), is expressed at normal levels in *Taf4b*-null testes at day 0, but at lower levels in *Taf4b*-null testes at day 3. This deficiency of *Plzf* at day 3 is probably the result of the presence of fewer germ cell stem cells at this time in *Taf4b*-null testes. Thus, *Plzf* is most likely not a direct target of *Taf4b*; however, decreased expression of this germ cell stem cell marker in *Taf4b*-null males at day 3 provides further evidence that loss of TAF4B may cause germ cell stem cell deficiency due to impaired proliferation.

The role of retinoic acid signaling in male fertility is well established (Livera et al. 2002). *Stra8*, a RAR γ -responsive gene, (Chiba et al. 1997) is the most dramatically down-regulated gene in *Taf4b*-null testes by semi-quantitative RT-PCR. *Stra8* is deficient as early as the day of birth, when *Taf4b*-null testes have comparable numbers of germ cells compared to wild type and heterozygous littermates. Although the function of STRA8 is not yet known, STRA8 has been shown to be expressed in premeiotic spermatogonia and embryonic oocytes and has been used as a marker of spermatogonial stem cells (Oulad-Abdelghani et al. 1996; Menke et al. 2003; Lassalle et al. 2004). These results suggest that the infertility in *Taf4b*-null mice could also be caused in part by depletion of *Stra8*. Whether or not TAF4b is targeted to the promoter of RAR γ or *Stra8* remains to be determined.

Control of spermatogenesis requires specialized transcriptional machinery in multiple species including *Drosophila*, *Xenopus*, mouse, and human (Martianov et al. 2001; Zhang et al. 2001; Wang and Page 2002; Han et al. 2003; Hiller et al. 2004). These alternative general transcription factors may be direct targets of testis-specific factors or function in the initiation of expression-specific genes in the testis that are expressed from alternative promoters (Sassone-Corsi 2002). The observation that a specific TBP-associated factor, TAF4b, appears to be required for the expression of several genes thought to regulate spermatogonial stem cell proliferation provides evidence that the complex events controlling germ cell proliferation in the neonatal mouse testes between post-natal day 0 and day 3 unexpectedly require a specialized component of the preinitiation complex (Hochheimer and Tjian 2003). One would predict that TAF4b, like the

other TAFs, is an essential coactivator for specific transcription factors that impact chromatin structure and gene transcription (Veenstra and Wolffe 2001). Future studies at the molecular level will investigate what chromatin-directed events occur in spermatogonial stem cells and what specific functions TAF4b plays in these molecular events.

In summary, mice null for *Taf4b* exhibit a unique testicular phenotype that includes normal fertility at early ages followed by a complete loss of fertility by 12 wk of age, characterized spermiogenesis defects, loss of germ cells, and testicular degeneration. Although other components of the RNA Polymerase II basal transcription apparatus impact meiosis (Zhang et al. 2001; Hiller et al. 2004), TAF4b is required for mitosis specifically in gonocytes and spermatogonia. TAF4b appears to be essential for germ cell stem cell differentiation and proliferation, which are required for the maintenance of fertility in adult males. Thus, TAF4b, a component of the TFIID complex of RNA polymerase II basal transcription machinery, is essential for the maintenance of spermatogenesis, most specifically the proliferation of spermatogonial stem cells.

Materials and methods

Animals

Mice null for *Taf4b* were generated by an insertion of a neomycin-resistance cassette in the reverse orientation into the sixth exon of the *Taf4b* gene as previously described (Freiman et al. 2001). Heterozygous mice were backcrossed to the inbred C57BL/6 strain (Harlan Sprague Dawley, Inc.). Mice were maintained on a 14L:10D cycle with free access to food and water in the vivarium at Baylor College of Medicine. For natural breeding experiments, males were housed with one or two females. Neonatal males were collected from timed pregnancies. Tissues were fixed in Bouin's fixative or frozen for RNA isolation. Mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals with institutional oversight by Baylor College of Medicine.

Fertilization experiments

Males participating in mating experiments were housed in separate cages for the duration. Immature female C57BL/6 (d25) were obtained from Harlan Sprague Dawley, Inc. or from heterozygous crossings and injected with 5 IU of pregnant mare serum gonadotropin (PMSG, Gestyl) purchased from the Professional Compounding Center of America to stimulate follicle growth, followed 48 h later with 5 IU of human chorionic gonadotropin (hCG; Pregnyl) from Organon Special Chemicals to stimulate ovulation and luteinization. Following treatment with hCG, females were placed in cages with a single male. After 24 h vaginal plugs were observed, and after 52 h females were sacrificed and oviducts were collected. Oocytes were collected from the oviduct, counted, and examined by light microscopy for fertilization stage.

Germ cell transplantation

Cells for transplantation were isolated from the testes of the transgenic mouse line ROSA26 (originally from The Jackson

Laboratory), which are maintained on a C57BL/6 $\times$ 129/Sv genetic background and express the *Escherichia coli* LacZ gene. Many cell types, including germ cells of all stages of differentiation, stain positively for β -galactosidase (β -gal), a characteristic that allows accurate tracking of the transplanted cells. The male mice were surgically made cryptorchid at age 6–8 wk to minimize the number of haploid germ cells in the donor cell population. On average, six to eight donor mice were killed and their testes were harvested 6–8 wk post-operatively. A single-cell suspension was prepared using a two-step enzymatic digestion procedure as described previously (Ogawa et al. 1997). The cells were resuspended in medium containing DMEM, 5% HEPES, and 10% fetal bovine serum.

Transplantation surgeries were performed using 4–6-mo-old *Kit^{w-v}/Kit^w* (The Jackson Laboratory) or *Taf4b*-null testes. Mice were killed 3 mo after transplantation. The testes were immediately fixed in fresh CHO's fixative (3% paraformaldehyde, 0.2% glutaraldehyde, and 2% sucrose in PBS at pH 7.5) at 4°C for 2 h. Whole-mount colorimetric β -gal staining was performed using the modified protocols from Specialty Media. Because it is difficult to stain the tissue evenly with this method, we bivalved the testes for optimal staining. After rinsing with PBS and washing with buffer solutions, the testes were incubated in COMPLETE β -Gal Tissue Stain Solution (Specialty Media) for 3 h at 37°C. Positive colonization with donor cells is apparent from the blue color generated with this assay. The testes were then refixed with CHO's fixative for 12 h (or overnight). Histology sections were obtained as described below and counterstained with nuclear fast red.

Histology and immunohistochemistry

Testes were collected and fixed in Bouin's fixative overnight, dehydrated in 70% ethanol, and embedded in paraffin. Sectioned tissues were dehydrated and stained with hematoxylin and eosin then re-dehydrated and mounted. The cellular and subcellular localization of germ cell nuclear antigen and phospho-histone H3 were analyzed by immunostaining Bouin's-fixed and paraffin-embedded testes. Sections (7 μ M) were rehydrated and boiled in 0.1% hydrogen peroxide followed by PBS washes. Sections were then incubated with 20% nonimmune goat serum to block nonspecific sites, followed by incubation with the 10D9G11 (GCNA1) monoclonal antibody at 33°C for 90 min (kindly provided by G.C. Enders, University of Kansas, Kansas City, Kansas) or with a polyclonal antibody against Phospho-histone H3 (1:60) overnight at 4°C (Cell Signaling Technology). After washing in PBS, horseradish peroxidase-conjugated anti-Rat IgM μ diluted to a concentration of 1:450 was applied for 60 min at room temperature (Pierce Biotechnology, Inc.). Sections were incubated with DAB substrate (3,3'-diaminobenzidine) (Vector Laboratories) for 1 min, dehydrated, counterstained with hematoxylin and mounted. The number of positive cells per seminiferous tubule was counted for at least six individual animals and averaged. At least 60 seminiferous tubules were counted per testis. One-way ANOVA was used for statistical analysis.

Western blot

Total protein extracts were prepared from testes of adult *Taf4b* heterozygous and null mice. Testes were dounce homogenized in extraction buffer (100 mM HEPES at pH 7.6, 400 mM NaCl, 1.5 mM MgCl₂, 0.1% NP-40, 10% glycerol, 0.5 $\times$ COMPLETE [Roche]), 1 mM PMSF, 20 μ M leupeptin, 20 KIU of aprotinin, 10 mM NaF, 5 mM Na₃VO₄, and 2 mM Na₄P₂O₇), incubated on ice for 20 min, and centrifuged. A total of 30 μ g of protein was separated on a 7% SDS-polyacrylamide gel. Western blot analy-

sis was performed with anti-TAF4b rabbit polyclonal antisera diluted 1:2500, goat anti-rabbit IgG-horseradish peroxidase (Pierce) diluted 1:13,000 and visualized with SuperSignal Chemiluminescent Substrate (Pierce).

TUNEL assay

TUNEL assays were performed on Bouin's fixed paraffin embedded sections according to the manufacturer's instructions using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Serologicals Corporation).

Sperm assays

Epididymides were dissected and sheared in Modified BWW Medium (Irvine Scientific) and incubated for 30 min. For sperm motility counts, live sperm were immediately spread onto a slide, viewed, and scored visually as motile or immotile. For whole sperm counts, sperm was immobilized by treatment with water and put into a hemocytometer for counting. For acrosome reactions, sperm were spread onto slides and allowed to dry overnight. On the following morning, sperm were stained with Rhodamine-labeled Pisum Sativum Agglutinin for 10 min (Vector Laboratories), washed, and mounted with 50% glycerol. Positive-staining acrosomes were visualized and scored using a fluorescent microscope.

Hormone assays

Blood was drawn by cardiac puncture. Serum was separated by centrifugation (2 min, 16,000 rpm) in microtainer brand tubes (Becton Dickinson), frozen and stored at -80°C. Radioimmunoassays for FSH, LH, and T were performed by the Ligand Assay and Analysis Core (University of Virginia) as previously described (Gay et al. 1970; Fallest et al. 1995) or by using the commercially available testosterone RIA kit from Diagnostic Systems Laboratories, Inc. or Diagnostic Products Corporation.

RNA isolation

Whole testis RNA was obtained by tissue homogenization in TRIzol reagent (Line Technologies, Inc.), followed by RNA precipitation in isopropanol. Recovered RNA was then resuspended in DEPC-treated water and verified as DNA free by PCR. RNA was quantitated and stored at -80°C until use.

Microarray

Total RNA was purified from day 8 testes of *Taf4b* wild-type, heterozygous, and null littermates. For cDNA synthesis, 12 μ g of total RNA was used in a reverse transcription reaction at 42°C using a T7-(dT)₂₄ primer and superscript II reverse transcriptase (Invitrogen). In vitro transcribed cRNA was prepared using half of the cDNA sample, and 15 μ g of fragmented cRNA was hybridized to Affymetrix U74Av2 Murine Genome high-density oligonucleotide arrays as per the manufacturer's protocols (Affymetrix). Data analysis for individual arrays and single comparisons was performed using Microarray Suite 5.0, and cross-comparison analysis of replicate experiments was performed using Data Mining Tool 2.0 (Affymetrix). Microarray analyses were repeated with a second group of animals to verify accuracy.

RT-PCR

Total RNA (300 ng) was reverse-transcribed using poly(dT) (Amersham Pharmacia Biotech) and avian myeloblastosis virus-re-

verse transcriptase (Promega Corp.) at 42°C for 75 min and 95°C for 5 min. PCR primers were generated using a Web-based prediction algorithm (<http://www.genome.wi.mit.edu/genome-software/other/primer3.html>), and cDNA was amplified using Taq Polymerase (Promega Corp.). ³²P-CTP was added to the reaction mixture for incorporation and reactions were completed in 25–30 cycles of PCR at 95°C for 30 sec, 59°C for 45 sec, and 72°C for 60 sec. The amplified cDNA products were resolved on a 5% polyacrylamide gel, which was dried and exposed to film. The radioactive PCR product bands were quantified by using a Storm 860 PhosphorImager (Molecular Dynamics, Inc.). To determine the linear range of amplification for specific mRNAs, 300 ng of RNA was reverse-transcribed and amplified in a range of cycle numbers. The primer pairs and number of cycles used are listed in Supplementary Table 1. Sequences for the amplified PCR products were verified by subcloning and sequencing at the Baylor College of Medicine Sequencing Core.

Statistics

All statistics were analyzed with Prism GraphPad software. For all data sets, standard *t*-tests or one-way and two-way ANOVA were used, and standard error of means were calculated as appropriate. Statistical significance was defined as *p* < 0.05.

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