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The role of spermatogonially expressed germ cell-specific genes in mammalian meiosis

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Abstract

Meiosis, a hallmark of sexual reproduction, reduces the chromatin complement by half to cope with genome doubling at fertilization and permits exchange of genetic material between parental genomes. Recent functional studies of novel proteins have greatly enhanced our understanding of the regulation of meiosis. The unique status of sex chromosomes in the male germ line may have shaped their content of germ line-intrinsic genes during evolution. Previously, a unique set of 36 spermatogonially expressed, mouse germ cell-specific genes was identified in one genomic screen. Thirteen of these genes have been disrupted in mice and two-thirds of these mouse mutants exhibit meiotic defects. Therefore, we hypothesize that the majority of uncharacterized germ cell-specific genes identified in the same screen, including 11 X-linked genes, might also play important roles in meiosis. In particular, we cite previously unpublished studies demonstrating that the NXF2 protein, an X-encoded factor, is present in early spermatocytes.

Unique status of the X chromosome in the male germ line

In germ cells the X chromosome and the autosomes behave differently. In particular, during meiosis of mammalian spermatogenesis, the sex chromosomes undergo meiotic sex chromosome inactivation (MSCI), while autosomes remain transcriptionally active (Solari 1974, Handel *et al.* 1994). MSCI appears to be part of a common process that silences unsynapsed chromatin during the pachytene stage of meiosis I, a phenomenon termed MSUC (meiotic silencing of unsynapsed chromatin) (Baarends *et al.* 2005, Turner *et al.* 2005). (For an in-depth discussion, refer to the review on meiotic silencing and the epigenetics of sex in the current issue by Kelly & Aramayo (2007)). MSCI does not require the *Xist* gene that is essential for X-inactivation in females (McCarrey *et al.* 2002) but involves a BRCA1-dependent mechanism. The BRCA1 tumor protein recruits ATR to asynapsed sex chromosomes, which in turn phosphorylates the H2AX histone variant (Turner *et al.* 2005). BRCA1-dependent silencing also applies to asynapsed autosomal chromatins in pachytene germ cells in both sexes. Asynapsed chromatin undergoes extensive histone modifications and nucleosome replacement beginning at the pachytene stage (Khalil *et al.* 2004, Greaves *et al.* 2006, van der Heijden *et al.* 2007). While some sex-linked genes are reactivated post-meiotically, transcriptional silencing of most X-linked genes persists in spermatids (Wang *et al.* 2005, Namekawa *et al.* 2006, Turner *et al.* 2006).

In contrast with their differential transcriptional activities during the pachytene and post-meiotic stages, both sex chromosomes and autosomes in

mouse are transcriptionally active in mitotically dividing germ cells (spermatogonia) and early meiotic spermatocytes such as leptotene and zygotene.

The unique and dynamic transcriptional states of the X chromosome during the various phases (mitotic, meiotic, and post-meiotic) of spermatogenesis may have influenced its gene content during evolution, particularly those involved in germ cell development (Vicoso & Charlesworth 2006). A genomic study using *Spo11*-deficient mouse testes revealed two contrasting gene distribution patterns on the X chromosome. Topoisomerase SPO11 is required for homologous recombination and its loss causes meiotic arrest prior to the pachytene stage in mouse (Baudat *et al*. 2000, Romanienko & Camerini-Otero 2000). Expression profiling of *Spo11*-deficient testes showed that genes expressed before the *Spo11* block are enriched on the X chromosome, whereas those expressed after the block are under-represented (Khil *et al*. 2004). It has been hypothesized that male beneficial genes (for example, genes expressed in early spermatogenesis) tend to accumulate on the X chromosome during evolution because of its hemizygosity in males (Rice 1984). However, the depletion of meiotic and post-meiotic genes on the X chromosome can be explained by MSCI (Turner *et al*. 2006).

Enrichment of early spermatogenesis genes on the mammalian X chromosome

The importance of the X chromosome in mammalian spermatogenesis was first suggested by its abundant representation in mouse spermatogonia. When 36 germ cell-specific genes were identified from mouse spermatogonia in a systematic genomic screen (Table 1), nearly one-third of these genes were X-linked, demonstrating that early spermatogenesis genes are enriched on the X chromosome in mouse (Wang *et al*. 2001). [Such enrichment is also substantiated by genomic studies of the mouse *Spo11* mutant and human germ cell-restricted genes (Khil *et al*. 2004, Koslowski *et al*. 2006)]. To date, 13 out of these 36 genes (*Sycp1*, *Sycp2*, *Sycp3*, *Sall4*, *Figla*, *Stra8*, *Tex14*, *Ddx4*, *Rnf17*, *Piwil2*, *Dazl*, *Tdrd1*, and *Taf7l*) have been characterized by targeted inactivation in mice (Table 1). These genetic studies have uncovered functions of most genes during spermatogenesis that could not be predicted based mainly on their expression patterns.

This review describes the characteristics of this unique set of 36 genes (Table 1), whose properties might indicate some of the functions of the uncharacterized genes identified in the same screen (Wang *et al*. 2001). Even though they are expressed in spermatogonia, these 36 genes are also expressed in meiocytes, suggesting a possible role in meiosis (Wang *et al*. 2005). Genetic studies of 13 genes in mice by targeted inactivation have demonstrated that the majority of them are involved in the regulation of meiosis (Table 1). In particular, at least two molecular/genetic networks of meiosis (the synaptonemal complex and post-transcriptional regulatory network) have emerged from these mouse studies and are reviewed as follows.

The synaptonemal complex (SC)

The synaptonemal complex, a tripartite multi-protein structure unique to meiotic cells, consists of two axial/lateral elements (AE/LE), one central element (CE), and numerous transverse filaments (TF) (Page & Hawley 2004). The assembly and disassembly of SC ensure chromosome synapsis, homologous recombination, and faithful chromosome segregation during meiosis. (For an in-depth discussion, refer to the review on the synaptonemal complex structure in the current issue by Cooke 2007). Interestingly, four genes identified in the genomic screen (*Sycp1*, *Sycp2*, *Sycp3*, and *Tex12*) encode components of the SC (Table 1): SYCP1, SYCP2 and SYCP3 are known SC proteins, whereas TEX12 is a novel SC constituent (Heyting *et al*. 1989, Hamer *et al*. 2006).

Recent studies of SC proteins have provided novel mechanistic insights into the regulation of meiosis, in particular the assembly of SC. SYCP1 is a TF protein. In mice lacking *Sycp1*, axial elements appear to be assembled normally; homologous chromosomes pair with each other but fail to undergo synapsis; meiotic recombination is initiated, but crossovers are not formed (de Vries *et al*. 2005). Recent biochemical and cell biological studies have shown that TEX12 and two SYCP1-interacting proteins (SYCE1 and SYCE2) localize exclusively to the CE and their localization to CE is SYCP1-dependent (Costa *et al*. 2005, Hamer *et al*. 2006).

Table 1. Thirty-six spermatogonially expressed, germ cell-specific genes in mouse[a].

Gene symbol[b]	Chromosome	Comments	References
Sycp1	3	SYCP1 is a TF component; loss of function leads to chromosomal asynapsis in both sexes.	de Vries *et al.* 2005
Sycp2	2	SYCP2 interacts with SYCP3; both are integral components of AE/LE; disruption of either	Yang *et al.* 2006
Sycp3	10	gene causes meiotic arrest at the zygotene stage in males; mutant females are subfertile.	Yuan *et al.* 2000
Tex12	9	TEX12 co-localizes with SYCE2 in the central element.	Hamer *et al.* 2006
Sall4 (Tex20)	2	*Sall4* is required for proliferation of embryonic stem cells and early embryogenesis.	Sakaki-Yumoto *et al.* 2006
Pramel1	4	Unknown.	
Lin28 (Tex17)	4	*Lin28* is regulated by micro-RNA.	Moss & Tang 2003
Figla	6	*Figla* encodes a transcription factor required for formation of ovarian follicles.	Soyal *et al.* 2000
Stra8	6	*Stra8* is required for meiotic commitment.	Baltus *et al.* 2006
Stk31	6	Unknown.	
Tuba3/7	6	*Tuba3*, *Tuba7*, and *Tex12* are transcriptionally silenced in somatic tissues by E2F6.	Pohlers *et al.* 2005
Nlrp4c (Rnh2)	7	Unknown.	
Tex15	8	Unknown.	
Tex18	10	Unknown.	
Tex19	11	Unknown.	
Tex14	11	TEX14 is an essential component of intercellular bridges in testis.	Greenbaum *et al.* 2006
Ddx4 (Mvh)	13	MVH is an RNA helicase and localizes to chromatoid body; disruption leads to zygotene arrest in males.	Tanaka *et al.* 2000
Rnf17	14	*Rnf17* encodes a component of a novel germ cell nuage and is required for spermiogenesis.	Pan *et al.* 2005
Piwil2 (Mili)	14	Disruption causes meiotic arrest at the zygotene/early pachytene stage in males.	Kuramochi-Miyagawa *et al.* 2004
Mov10l1	15	*Csm* is a cardiac-specific alternative isoform of *Mov10l1*.	Ueyama *et al.* 2003
Dazl	17	Disruption of *Dazl* leads to depletion of germ cells in both sexes.	Ruggiu *et al.* 1997
Tdrd1 (Mtr-1)	19	TDRD1 is a component of intermitochondrial cement (nuage); disruption leads to meiotic and post-meiotic defects in males.	Chuma *et al.* 2006
Taf7l (Taf2q)	X	TAF7L is a germ cell-specific TBP-associated factor; disruption leads to reduced sperm production in mice.	Cheng *et al.* 2007
Nxf2	X	NXF2 exhibits distinct localization patterns in spermatogonia and early spermatocytes.	This study
Nine X-linked genes: *Fthl17*, *Usp26*, *Tktl1*, *Tex11*, *Tex16*, *Pramel3*, *Tex13*, *Ott*, *Mage*.			
Three Y-linked genes: *Ube1y, Usp9y, Rbmy.*			

[a]All 36 genes were identified in the same cDNA subtraction screen (Wang *et al.* 2001).
[b]Symbols in parentheses are aliases.

Apparently, TEX12 forms a complex with SYCE2, which in turn interacts with SYCE1, and SYCE1 anchors this CE protein complex to TF via interaction with SYCP1. These new studies have uncovered an intriguing molecular network within the central element of SC.

SYCP2 and SYCP3 are integral components of the AE/LE. Genetic studies of mutant mice demonstrate that both proteins are required for formation of axial elements and thus chromosomal synapsis (Yuan *et al*. 2000, Yang *et al*. 2006). Not surprisingly, SYCP2 interacts with SYCP3, suggesting that they exist as heterodimers in the axial elements. An evolutionarily conserved coiled coil domain in SYCP2 is required for its interaction with SYCP3. Studies of a unique *Sycp2* mouse mutant lacking the coiled coil domain reveal that the assembly of SYCP2 and SYCP3 into axial elements might be tightly regulated (Yang *et al*. 2006). In *Sycp3*-deficient meiocytes, SYCP2 fails to localize to the axial chromosomal cores (Pelttari *et al*. 2001, Yuan *et al*. 2002). In *Sycp2* mutant mice the truncated SYCP2 protein lacking the coiled coil domain still localizes to the axial chromosomal cores, whereas SYCP3 fails to do so but forms large nuclear aggregates that are not associated with chromatin (Yang *et al*. 2006). Collectively, these studies suggest that SYCP2 is a primary determinant of AE/LE and SYCP3 might play a yet-unknown role in the stabilization of SYCP2 to the axial chromosomal cores.

Post-transcriptional regulation in meiosis

Several germ cell-specific genes (Table 1) encode putative RNA-binding proteins, such as *Dazl*, *Ddx4* (*Mvh*), *Piwil2*, *Tdrd1*, *Nxf2*, and *Rnf17*, underlining the complexity of post-transcriptional control in proliferation and differentiation of germ cells. Targeted inactivation studies have shown that at least four of these proteins (DAZL, MVH, PIWIL2 and TDRD1) play a role in meiosis (Table 1). These proteins have highly conserved sequence homologues in metazoans.

The role of DAZL in translational regulation has been intensively studied. Mice lacking DAZL are depleted of germ cells in both sexes (Ruggiu *et al*. 1997). However, the precise timing of germ cell defects in *Dazl* mutant mice appears to be influenced by genetic backgrounds; in hybrid backgrounds spermatogenesis fails to progress beyond the leptotene stage of meiosis I, whereas in C57BL/6 inbred background *Dazl* is required for embryonic development of male germ cells (Saunders *et al*. 2003, Lin & Page 2005). Biochemical studies in *Xenopus* oocytes show that DAZL family proteins are associated with poly(A)-binding proteins (PABP), which regulate the initiation of translation (Collier *et al*. 2005). Strikingly, a number of gene transcripts in Table 1 appear to be DAZL-binding targets (*Sycp3*, *Tex19*, *Mvh*, *Stk31*, *Tex14*, *Tuba3*, and *Fthl17*). Furthermore, it has also been shown that DAZL stimulates translation of *Mvh* by binding to its 3′ untranslated region (UTR) (Reynolds *et al*. 2005).

The Piwi gene family is involved in stem cell renewal, RNA silencing, and germ cell development in diverse organisms. *Piwil2* (also known as *Mili*) is required for male meiosis in mice (Kuramochi-Miyagawa *et al*. 2004). Recently it has been found that PIWIL2 binds to a novel class of small RNA (26–31 nucleotides) that accumulate during meiosis (Aravin *et al*. 2006). In addition, PIWIL2 forms a complex with MVH in germ cells, suggesting the functional association of these two proteins in post-transcriptional regulation of meiosis (Kuramochi-Miyagawa *et al*. 2004).

Intriguingly, several proteins with RNA-binding activity or putative RNA binding domains localize to nuage. Nuage in mouse germ cells refers to a number of electron-dense non-membrane bound structures of unknown functions. MVH localizes to the chromatoid bodies, which are a multi-lobular nuage prominent in the cytoplasm of spermatocytes and spermatids (Toyooka *et al*. 2000). TDRD1, a tudor repeat-containing protein, is a component of inter-mitochondrial cement in spermatocytes and chromatoid bodies in spermatids (Chuma *et al*. 2003). RNF17 also contains multiple tudor repeats and localizes to a novel germ cell nuage (referred to as RNF17 granules) in spermatocytes and spermatids, which is distinct from chromatoid bodies (Pan *et al*. 2005). The molecular functions of various germ cell nuages in germ cell development (chromatoid body, inter-mitochondrial cement, and RNF17 granules) remain elusive. Notably, Dicer interacts with MVH and is concentrated in chromatoid bodies, suggesting that chromatoid body might be involved in the microRNA pathway (Kotaja *et al*. 2006).

An abundance of meiosis factors

Out of the 36 germ cell-specific genes identified from mouse spermatogonia, 13 genes have been disrupted in mice to date (Table 1). Strikingly, two-thirds of these mutant mice (*Sycp1*, *Sycp2*, *Sycp3*, *Mvh*, *Piwil2*, *Dazl*, *Tdrd1*, *Tex14,* and *Stra8*) exhibit defects in meiosis. This number likely represents an underestimate, since disruption of *Sall4* causes premeiotic defects and thus precludes studies of a possible role in meiosis (Sakaki-Yumoto *et al*. 2006). With the benefit of hindsight, we extrapolate that the majority of the remaining 23 genes may also play a role in early meiosis (Table 1), which will be tested by targeted inactivation of these genes in mice in the future. In support of this extrapolation, all of these 23 genes are expressed in meiocytes (Wang *et al*. 2005). In further support, a recent study shows that TEX12 appears to be a meiosis-specific factor (Hamer *et al*. 2006).

However, it is also possible that some of these 36 genes are involved in the development of mitotic germ cells including spermatogonia, as they are indeed expressed in spermatogonia (Wang *et al*. 2001). In fact, *Mvh* also plays a role in the proliferation of primordial germ cells (Tanaka *et al*. 2000). Disruption of *Dazl* causes arrest of mitotic germ cells in an inbred genetic background (Lin & Page 2005). *Tex14* is required for formation of intercellular bridges of germ cells including spermatogonia (Greenbaum *et al*. 2006). Therefore, these three genes (*Mvh*, *Dazl*, and *Tex14*) function in both the mitotic and meiotic phases of germ cell development, which might also be the case for other genes in Table 1.

Regulation of meiosis by X-linked genes in mammals?

If the aforementioned extrapolation holds, the majority of the 11 X-linked germ cell-specific genes are expected to play a role in meiosis (Table 1). To date no X-linked meiosis-specific factors have been identified by gene disruption in mice. However, the 11 X-linked germ cell-specific genes found are expressed in early spermatocytes during the leptotene and zygotene stages of meiotic prophase I, suggesting that they may have meiotic roles (Wang *et al*. 2005).

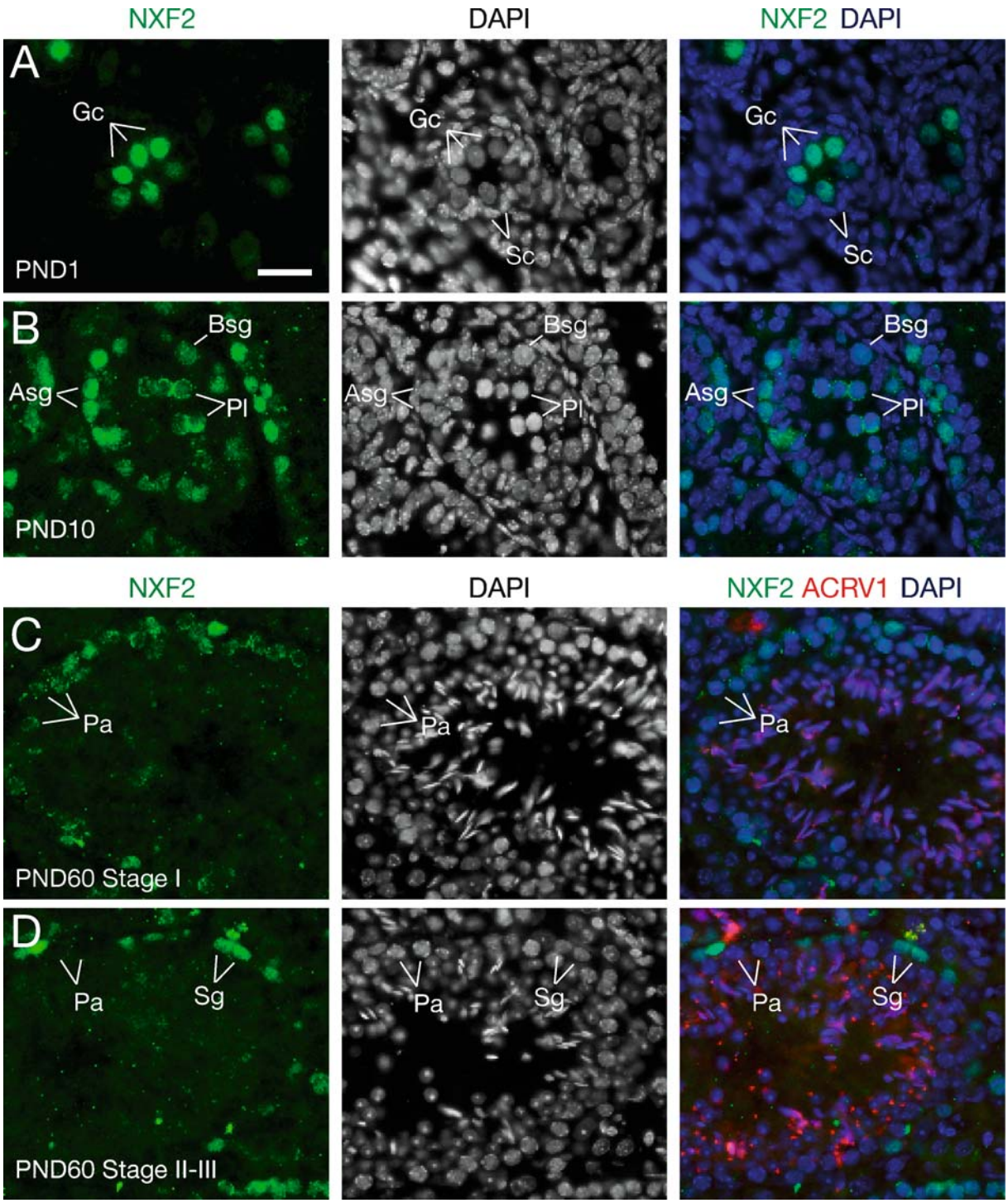
Genetic studies in diverse organisms have shown that critical meiotic processes are initiated or specified during the leptotene and zygotene stages, including homologous recombination, chromosome pairing, and chromosomal synapsis (Zickler & Kleckner 1999, Page & Hawley 2004). Strikingly, nearly all the spermatogonially expressed gene mutant mice with meiotic impairment exhibit zygotene arrest or severe defects prior to the pachytene stage (*Sycp1*, *Sycp2*, *Sycp3*, *Mvh*, *Piwil2*, *Dazl*, *Tex14,* and *Stra8*) (Table 1), underlining the importance of leptotene and zygotene stages. In contrast with the differential transcriptional status between sex chromosomes and autosomes during the pachytene stage, there is little evidence that sex chromosomes and autosomes differ in their transcriptional activity prior to the pachytene stage. Therefore, we reason that the early meiotic function obtained from disruption of autosomal genes can be extrapolated to the role of not only autosomal but also X-linked genes identified from spermatogonia in the same genomic screen (Table 1).

One of these X-linked genes encodes TAF7L, a TATA-binding protein (TBP) associated factor that is abundant in meiotic and post-meiotic germ cells (Pointud *et al*. 2003). As a component of the basal transcription factor TFIID complex, TAF7L might specify a germ cell-specific transcription program. Disruption of *Taf7l* leads to reduced testis weight, decreased sperm production, and defects in sperm motility but no meiotic arrest (Cheng *et al*. 2007). However, TAF7L might play a non-essential role in meiosis. To further test the role of X-linked genes in meiotic regulation, we examined the expression of a novel X-encoded factor, NXF2, during spermatogenesis (Table 1).

Differential localization of NXF2 in spermatogonia and spermatocytes

The cytoplasm and the nucleus are separate compartments in eukaryotic cells. Thus, bulk mRNA must be transported from the nucleus into the cytoplasm through the nuclear pore complex before translation can occur. Active mRNA export is mostly carried out by evolutionarily conserved nuclear mRNA export factors (NXF). In mammals the *Nxf* gene family consists of several members including *Nxf1* and

Nxf2. *Nxf1*, the founding member of this family, is conserved from yeast to humans and ubiquitously expressed (Katahira *et al*. 1999). By contrast, *Nxf2* expression is restricted to testis and brain (Sasaki *et al*. 2005). Interestingly, a recent study shows that NXF2 interacts with the fragile X mental retardation



protein (FMRP) and is restricted exclusively to spermatogonia in the testis (Lai *et al*. 2006).

However, our study demonstrated that NXF2 is also expressed in early spermatocytes and exhibited two distinct subcellular localization patterns in germ cells. We examined the localization of NXF2 in testes from juvenile and adult mice by immunofluorescent analysis. The anti-NXF2 polyclonal antibodies that we generated did not exhibit cross-reactivity with NXF1, as tested by Western blot analysis (data not shown). Consistent with previous reports, NXF2 was expressed in germ cells but not in Sertoli cells (Figure 1). Notably, NXF2 was exclusively nuclear in gonocytes and type A spermatogonia (Figure 1A and B). In type B spermatogonia, NXF2 appeared to accumulate around the nuclear periphery (Figure 1B). Furthermore, NXF2 localized predominantly to the nuclear periphery in a punctate pattern in preleptotene (Figure 1B), leptotene, and zygotene spermatocytes. Such a punctate localization pattern persisted in early pachytene spermatocytes in Stage I seminiferous tubules (Figure 1C). However, NXF2 was not observed in pachytene spermatocytes from stage II–III tubules and thereafter (Figure 1D). The nuclear peripheral localization of NXF2 might be relevant to its nuclear mRNA export activity, since NXF2 is known to interact with components of nuclear pore complex (Sasaki *et al*. 2005). The differential localization patterns of NXF2 in germ cells suggest that its functions may differ between spermatogonia (gonocytes and type A spermatogonia) and early spermatocytes (preleptotene through early pachytene). Importantly, our data suggest that NXF2 might be implicated in the regulation of male meiosis.

Implications of X-linked meiosis factors

Based on the genetic studies of 13 genes, we entertain the possibility that the majority of germ cell-specific genes identified in the genomic screen might play a role in meiosis (Table 1) (Wang *et al*. 2001). Such a possibility has enormous implications for the X-linked genes in the aetiology of male infertility in humans. Since males have only one X chromosome, mutations in the X-linked germ-cell-specific genes might cause or predispose to male sterility.

In several genetic studies, two X-linked germ cell-specific genes (*TAF7L* and *USP26*) were screened for mutations in infertile men (Paduch *et al*. 2005, Stouffs *et al*. 2005, 2006b). Mutation screening of *TAF7L* in 25 men with non-obstructive azoospermia did not reveal mutations that are associated with infertility (Stouffs *et al*. 2006b). Two studies indicated that a haplotype in *USP26* (371insACA, 494T>C and 1423C>T) might be a risk factor for male infertility (Paduch *et al*. 2005, Stouffs *et al*. 2005). In contrast, other studies showed that this *USP26* haplotype is a polymorphism in non-Caucasian populations (Ravel *et al*. 2006, Stouffs *et al*. 2006a). However, these studies focused on azoospermic males. The fact that no causative mutations in *TAF7L* and *USP26* have been identified so far does not exclude a role for these genes in meiosis or spermatogenesis, since mutations in these genes might cause oligospermia (low sperm count) rather than azoospermia. Indeed, our recent study has shown that mice lacking the *Taf7l* gene exhibit reduced sperm count (Cheng *et al*. 2007). In addition, causative point mutations in a single gene are extremely rare in infertile men, given that hundreds if not thousands of genes are specifically involved in regulation of male fertility (Matzuk & Lamb 2002). Studies of mice lacking *Usp26* or other X-linked germ cell-specific genes (Table 1) will shed light on their role in spermatogenesis and facilitate mutation screening in infertile males.

The putative X-linked meiosis factors might be implicated in hybrid sterility in mammalian species. Hybrid sterility represents one of the early events in speciation and follows Haldane's rule: the fertility

Figure 1. Distinct subcellular localization patterns of NXF2 in spermatogonia and early spermatocytes. Two rabbits were immunized with the 6xHis-NXF2 (230–431 aa) recombinant protein, resulting in antisera UP1988 and UP1989 (Cocalico Biologicals, Inc.). Specific anti-NXF2 antibodies were affinity-purified using the immunoblot method (Harlow & Lane 1998). Frozen sections of testes from mice at different postnatal days (PND) were immunostained with anti-NXF2 antibodies. Adult testicular sections were also immunostained with guinea pig anti-ACRV1 antibodies (1:500) to visualize the morphology of acrosomes for precise staging of seminiferous tubules as previously described (Yang *et al*. 2007). Texas red or FITC-conjugated secondary antibodies were used (Vector Laboratories). Nuclear DNA was stained with DAPI. Gc, gonocytes; Sc, Sertoli cells; Asg, type A spermatogonia (that contain little heterochromatin); Bsg, type B spermatogonia (that contain heterochromatin distributed around the nuclear periphery); Sg, spermatogonia; Pl, preleptotene spermatocytes; Pa, pachytene spermatocytes. Scale bar, 25 μm.

or viability of heterogametic sex is preferentially affected (Haldane 1922). Genetic studies in *Drosophila* demonstrated an excess of hybrid sterility loci on the X chromosome, a phenomenon referred to as 'large X-effect' (Orr & Coyne 1989, Tao *et al.* 2003). Several X-linked hybrid sterility loci were also reported in mice (Oka *et al.* 2004, Storchova *et al.* 2004). These genetic studies suggest that hybrid sterility might be caused by incompatibility of multiple loci (or genes). The X-linked factors (Table 1), if involved in meiosis, might interact genetically with each other and with autosomal meiosis-specific factors. These X-linked germ cell-specific genes are highly divergent between species, suggesting that they undergo rapid evolution. Therefore, we posit that some of these X-linked factors might be involved in reproductive isolation during mammalian speciation.

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