Interallelic and intergenic incompatibilities of the prdm9 (hst1) gene in mouse hybrid sterility


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Introduction

Hybrid sterility is a condition in which two fertile parental forms produce progeny with disturbed gametogenesis. In mammals and Drosophila, it affects spermatogenesis more often than oogenesis [1]. Hybrid sterility acts as a reproductive barrier between species [2]. Although its molecular mechanism is of great interest, only five animal genes involved in hybrid sterility have been cloned and characterized, four of them from Drosophila [3–9]. The Dobzhansky-Muller model of incompatibilities of genes [10] explains the reproductive isolation between species by their incorrect epistatic interactions. These interactions (or lack of the correct ones) result in hybrid fitness reduction, probably because the combination of the diverged alleles of the interactors did not pass through natural selection.

The mouse Hybrid sterility 1 gene (Hst1) is one of the major genes causing meiotic arrest in F1 male hybrids between Mus m. musculus (Mmm) mice harboring the Hstw allele (e.g., the PWD strain) and laboratory strains bearing the Hstf allele [11]. Strains with the Hstf allele include Mus m. domesticus (Mmd)-derived C57BL/6J (henceforth B6) and various substrains of the 129 strain. While the male offspring from the crosses of Hstf strains with PWD females, e.g., (PWD×B6)F1, are azoospermic, the males from the reciprocal cross enhanced fertility of other sperm-carrying male hybrids, (PWD×B6-C3H)Prdm9F2, harboring another Prdm9 allele of M. m. domesticus origin. The levels of Prdm9 mRNA isoforms were similar in the prepubertal testes of all types of F1 hybrids of PWD with B6 and B6-C3H. Prdm9 despite their different prospective fertility, but decreased to 53% after removal of Prdm9. Therefore, the Prdm9 allele probably takes part in posttranscriptional dominant-negative hybrid interaction(s) absent in the parental strains.

Abstract

The Dobzhansky-Muller model of incompatibilities explains reproductive isolation between species by incorrect epistatic interactions. Although the mechanisms of speciation are of great interest, no incompatibility has been characterized at the gene level in mammals. The Hybrid sterility 1 gene (Hst1) participates in the arrest of meiosis in F1 males of certain strains from two Mus musculus subspecies, e.g., PWD from M. m. musculus and C57BL/6J (henceforth B6) from M. m. domesticus. Hst1 has been identified as a meiotic PR-domain gene (Prdm9) encoding histone 3 methyltransferase in the male offspring of PWD females and B6 males, (PWD×B6)F1. To characterize the incompatibilities underlying hybrid sterility, we phenotyped reproductive and meiotic markers in males with altered copy numbers of Prdm9. A partial rescue of fertility was observed upon removal of the B6 allele of Prdm9 from the azoospermic (PWD×B6)F1 hybrids, whereas removing one of the two Prdm9 copies in PWD or B6 background had no effect on male reproduction. Incompatibility(ies) not involving Prdm9 also acts in the (PWD×B6)F1 hybrids, since the correction of hybrid sterility by Prdm9 deletion was not complete. Additions and subtractions of Prdm9 copies, as well as allelic replacements, improved meiotic progression and fecundity also in the progeny-producing reciprocal (B6×PWD)F1 males. Moreover, an increased dosage of Prdm9 and reciprocal cross enhanced fertility of other sperm-carrying male hybrids, (PWD×B6-C3H)Prdm9F2, harboring another Prdm9 allele of M. m. domesticus origin. The levels of Prdm9 mRNA isoforms were similar in the prepubertal testes of all types of F1 hybrids of PWD with B6 and B6-C3H. Prdm9 despite their different prospective fertility, but decreased to 53% after removal of Prdm9. Therefore, the Prdm9 allele probably takes part in posttranscriptional dominant-negative hybrid interaction(s) absent in the parental strains.
Author Summary

Disturbed gametogenesis in the progeny of two fertile parental forms is called hybrid sterility; it is an important part of reproductive barriers between species. The Dobzhansky-Muller model of incompatibilities explains reproductive isolation between species by incorrect interactions between genes. Hybrid sterility 1 (Hst1) is one of the genes causing meiotic arrest in F1 male hybrids between certain Mus musculus musculus (e.g., the PWD strain) and M. m. domesticus (CS7BL/6J etc.) mice. Hst1, the first mammalian candidate for a speciation gene, Prdm9, but the mechanism causing sterility has remained unknown. While the F1 male offspring of CS7BL/6J males and PWD females produce no sperm, the males from the reciprocal cross using PWD males and CS7BL/6J females yield progeny. Here we show that the meiotic progress and fertility of hybrid males from both F1 crosses improved by removal as well as overexpression of the CS7BL/6J allele of Prdm9, suggesting that Prdm9 interactions not present in the parental species (incompatibilities) play a role in hybrid sterility. Furthermore, the Prdm9 dosage also controlled fecundity in other F1 hybrids, indicating that this gene is an important regulator of mouse hybrid fertility.

phenotypes of the sterile hybrids [6]. Hst1 is also called Prdm9 (PR-domain containing 9) or Meisetz (Mammalian with SET/PR domain and Zinc fingers). The product of this gene trimethylates histone 3 on lysine 4 (H3K4m3; [20]). Spermatocytes of ZPLOS Genetics | www.plosgenetics.org 2 November 2012 | Volume 8 | Issue 11 | e1003044

evolution of the minisatellite-like ZnF-encoding region of Prdm9 contributes to hybrid sterility is not known. Although accelerated Meisetz domain containing 9) or Hst1s in a complete meiotic arrest in (PWD and genetic manipulation of ZnFs changes the localization of Hstws, respectively), display many polymorphisms [6]. The Hst1f mRNA is elevated in (PWD×B6-C3H.Hst1fF1, compared to (PWD×B6)F1 prepubertal testis, while the levels of all known Prdm9 transcripts are similar [6].

The chromatin of mouse meiotic recombination hotspots is marked by H3K4m3 at the start of meiosis [21]. Genetic mapping of a gene acting in a trans background to influence the activity of recombination hotspots also led to the identification of Prdm9 [22,23]. PRDM9 binds DNA at recombination hotspots via its zinc-fingers (ZnFs) in vitro, and genetic manipulation of ZnFs changes the localization of the hotspots [22,24].

The Prdm9 genes from C3H and B6 strains (alleles Hst1f and Hst1t, respectively) display many polymorphisms [6]. The difference that may underlie hybrid sterility is the number of C-terminal ZnFs [6]. The number of ZnFs corresponds to Hst1 alleles in other classical Mmd laboratory strains [19,25]. The ZnF-encoding region of the PWD allele differs from both C3H and B6 [6], but whether Prdm9PWD is identical to Hst1t and whether it contributes to hybrid sterility is not known. Although accelerated evolution of the minisatellite-like ZnF-encoding region of PRDM9 was manifested in human and animals [26], it remains to be shown whether PRDM9 has a more general role in speciation.

The Prdm9iso allele is necessary but not sufficient for hybrid sterility. The Hst1 gene from Mmm is also located on chr17 [12]. In the sterile (PWD×B6)F1 males, chr17 carries the combination Hst1t/Prdm9iso, but the same genotype in the B6 background of (B6.PWD-Chr17×B6)F1; yields fertile males (Figure 1; [27]). It was shown recently that Hst1t/Prdm9iso is the only combination resulting in a complete meiotic arrest in (PWD×B6)F1 hybrid males, because both Prdm9iso/Prdm9iso and Hst1t/Prdm9iso homozygotes on the same background were fertile [12]. However, even on the F1 background, the Hst1t/Prdm9iso combination does not always lead to azoospermic males, as the reciprocal (B6×PWD)F1 males carry sperm. Thus, mouse hybrid sterility reflects incompatibilities among multiple hybrid sterility loci, one of them being the Prdm9 gene [12].

Here we manipulated the dosage and allelic combinations of Prdm9 in an attempt to characterize the role of this mouse hybrid sterility gene in the incompatibilities. If Prdm9PWD has a dominant-negative effect(s), its deletion should alleviate the meiotic arrest and rescue the fertility of hybrids. Moreover, a Prdm9-overexpressing transgene might dilute the Prdm9iso incompatibility(s), which should rescue fertility regardless of the Prdm9 allele origin. We show that the fertility of all F1 intersubspecific hybrids tested is proportional to the dosage of Prdm9 regardless of its allele; the only exception is the combination of one Prdm9 allele with one Prdm9iso allele in either type of reciprocal hybrid that results into a more sterile phenotype than the corresponding hybrid harboring only one Prdm9iso allele. This exception indicates an F1 hybrid-specific dominant-negative interaction(s) of Prdm9iso.

Results

Dosage-dependent, allele-independent rescue of fertility in the (PWD×B6)F1 hybrid

The fertility of azoospermic intersubspecific (PWD×B6)F1 hybrid males is rescued by Prdm9iso-carrying BACs [6]. Moreover, six copies of the Prdm9C3H allele in a transgene (BAC24) increased reproductive fitness compared to two copies of the same allele (BAC3 transgene) in Prdm9PWD/B6 (PWD×B6)F1 intersubspecific male hybrids ([6] and Table 1), although no effect of increased Prdm9 dosage appeared on the intrasubspecific background (a mix of 129 and B6 genomes, henceforth B6*129 [6]). There are three possible explanations for the fertility rescue by the Prdm9C3H BACs first, by allelic replacement; second, by increased dosage regardless of allelic origin; third, both. To distinguish among these hypotheses, a transgene harboring the Prdm9iso allele was utilized. The C57BL/6J-Tg(RP23-159N6)7Scm strain carries two Prdm9-expressing copies of a B6 BAC transgene on B6 background [24]. In this background, the transgene has no effect on fertility (Table S1). After outcrossing the heterozygous transgenic males to PWD, transgenic F1 hybrid males were azoospermic (as expected), while their transgenic littermates had an increased testicular weight (TW) and carried sperm (Table 1). The increased copy number of the Prdm9iso allele also rescued fertility of azoospermic hybrids produced by another Mmm strain, STUS (Table S1). Thus the improvement of fertility by increased Prdm9 dosage is not restricted to the C3H allele.

To further investigate the effect of Prdm9 dosage on hybrid sterility, we used two null alleles of Prdm9, a large deletion of proximal chr17 (Sod2attO) [28] and a knock-out of Prdm9 that removes the first five coding exons (Prdm9m1attO) [20]. Males hemizygous for these alleles on (129* B6 as well as on B6 background display similar fertility parameters as their littermates ([20,29], and Table S2). To determine the effect of the null alleles on intersubspecific F1 hybrids, the hemizygous males were outcrossed to PWD. Unlike their azoospermic Prdm9PWD/B6 F1 littermates, most of the Prdm9PWD/- intersubspecific hybrids were semisterile with TW, sperm count (SC), and offspring production (offspring per female per month, OFM) significantly higher than in the (PWD×B6)F1 littermates (Table 1 and Table S3). All Prdm9PWD/- hybrid males resulting from the cross of PWD females with Prdm9iso/- on B6 background carried a low but detectable amount of sperm and most of them produced offspring (0.3±0.2 OFM). As an additional control, we introduced the
null allele into the PWD background. Here, one copy of Prdm9PWD was sufficient to maintain the fertility parameters of the Prdm9PWD/PWD littermates (Table S2). Therefore, the (PWD x B6)F1 hybrid genetic background appears to be more sensitive to low Prdm9 dosage than that of either parent. Both addition and removal of Prdm9B6 improves the phenotype of (PWD x B6)F1 males. The fertility rescue of azoospermic hybrid males by the Prdm9 null alleles, albeit partial, suggests that an aberrant interaction(s) of Prdm9B6 occurs in (PWD x B6)F1 hybrids that is not present in the parental strains.

Incompatibility of Prdm9C3H in (PWD x B6)F1 males

The Prdm9C3H allele rescues the fertility phenotype of the (PWD x B6)F1 hybrid in a dosage-dependent manner. To compare the effect of a single C3H allele and a null allele on hybrid males resulting from a cross segregating these alleles, we utilized the congenic strain B6-Prdm9C3H. After outcrossing animals hemizygous for Prdm9 to this congenic and then crossing the preselected Prdm9C3H+/ males, the resulting Prdm9PWD/C3H F1 females, the resulting Prdm9PWD/C3H hybrids had a significantly lower TW and SC than their Prdm9PWD/C3H littermates (Table 1). The Prdm9PWD/C3H hybrids produced markedly less progeny (0.3 ± 0.2 OFM) in comparison with the Prdm9PWD/C3H hybrids (3.6 ± 0.5 OFM, Table S1). Thus the intersubspecific F1 hybrid males carrying Prdm9PWD/- were not just more fertile than Prdm9PWD/B6, but they were also less fertile than Prdm9PWD/C3H.

To analyze the sensitivity of Prdm9PWD/- F1 background, the dosage of Prdm9C3H was increased by utilizing the BAC5 (two copies of Prdm9C3H) or the BAC24 (six copies of Prdm9C3H) transgenes. Again, the fertility parameters of the F1 transgenic hybrids improved with Prdm9C3H dosage (Table 1; pSC = 0.006, pTW = 0.008, but no significant difference in relative testes weight: 6.6 versus 7.3, p = 0.33). The Prdm9 dosage effect is therefore observed in both Prdm9PWD/B6 and Prdm9PWD/C3H F1 hybrids. In conclusion, the B6 allele displays different properties than the C3H allele when present in one copy in the Prdm9PWD/B6 F1 hybrid male, because it decreases the fertility compared to Prdm9PWD/C3H hybrid. This could be the result of a dominant-negative interaction of Prdm9B6 with specific loci in (PWD x B6)F1 and/or with the Prdm9PWD allele.

The semisterility of Prdm9PWD/- maintains characteristics of hybrid sterility

Hybrid sterility is most often sex-specific [1] and dependent on the origin of parents. The (PWD x B6)F1 hybrid displays a complete male-specific arrest of gametogenesis that is at least partially alleviated in reciprocal (B6 x PWD)F1 and in 94% of

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**Figure 1. Fertility of male offspring resulting from various crosses.** The female parents are shown from left to right and male parents from top to bottom. The circles symbolize genomes (chrX sticking out), intersubspecific hybrids have the circles split in halves; the pictures of sperm cells within the circles indicate the degree of fertility. Except for the (PWD x B6)F1, hybrids, all males carry sperm; female offspring from all indicated crosses are fertile.

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Prdm9 dosage and alleles affect spermatogenesis of reciprocal (B6×PWD)F1 hybrids

Previously, the fertility of the azoospermic (PWD×B6)F1 males resulting from the cross of PWD females with B6 males was rescued by Prdm9<sup>Chi</sup> overexpression [6], but Prdm9 dosage has not been studied in the reciprocal, sperm-carrying (B6×PWD)F1 hybrids, although these males do not reach the reproductive fitness of fully fertile males (Table 2 and Table 3). To analyze the Prdm9 dosage effect in the reciprocal hybrids, we crossed PWD males with females carrying a variable number of four different Prdm9 alleles on B6 background. The fertility parameters of the Prdm9<sup>Chi/PWD</sup> F1 hybrid were superior to those of their (B6×PWD)F1, Prdm9<sup>B6/PWD</sup> littermates (Table 2). The parameters of the Prdm9<sup>B6/PWD</sup> transgenics carrying BAC5 were also better than those of the Prdm9<sup>Chi/PWD</sup> control (Table 2). In contrast, BAC21 overlapping most of BAC5 but carrying truncated Prdm9 [6] did not improve the fertility of Prdm9<sup>B6/PWD</sup> hybrids (Table S3). To discern whether a single copy of Prdm9<sup>Chi</sup> can improve the fertility of reciprocal hybrids, males from the cross (B6×B6-Prdm9<sup>Chi/PWD</sup>) were inspected. The increased fecundity of a subset of these males could be ascribed to the presence of Prdm9<sup>Chi</sup> (Table 2, p<sub>F1</sub><sub>0.001</sub>, p<sub>SC</sub><sub>0.003</sub>). The reciprocal Prdm9<sup>Chi/PWD</sup> F1 males displayed superior fertility parameters than the Prdm9<sup>PWD/Chi</sup> hybrids (Table S4; p<sub>F1</sub><sub>0.004</sub>, p<sub>SC</sub><sub>0.03</sub>, p<sub>TC</sub><sub>0.04</sub>).

To determine whether the fertility rescue of the reciprocal hybrids is limited to the Prdm9<sup>Chi</sup> allele and the PWD Mmm strain, we again used the C57BL/6J-Tg(RP23-159N675Bdm mouse heterozygous for a Prdm9 transgene; Prdm9<sup>B6</sup>, genotype at the Prdm9 locus (maternal/paternal); --, null, +, transgenic Prdm9 alleles; n, number of males; BW, body weight (g); TW, mean weight of paired testicles in mg; SC, average sperm count (millions) in paired caput epididymides; or

In the left epididymis by a different method;

*significantly higher (p<0.01) compared to littermates (in the row above).

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**Table 1.** The effect of Prdm9 dosage on hybrid sterility.

<table>
<thead>
<tr>
<th>Cross (female first)</th>
<th>Prdm9</th>
<th>n</th>
<th>TW</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWD×B6-BACB6</td>
<td>PWD/B6</td>
<td>9</td>
<td>55</td>
<td>0.00*</td>
</tr>
<tr>
<td>PWD×B6-BACB6</td>
<td>PWD/B6+Z6B6</td>
<td>3</td>
<td>167+</td>
<td>18**</td>
</tr>
<tr>
<td>PWD×B6-KO</td>
<td>PWD/B6</td>
<td>10</td>
<td>59</td>
<td>0.00</td>
</tr>
<tr>
<td>PWD×B6-KO</td>
<td>PWD/−</td>
<td>12</td>
<td>82*</td>
<td>0.06*</td>
</tr>
<tr>
<td>PWD×(B6-Prdm9&lt;sup&gt;Chi&lt;/sup&gt;/KO)</td>
<td>PWD/−</td>
<td>10</td>
<td>94</td>
<td>0.10</td>
</tr>
<tr>
<td>PWD×(B6-Prdm9&lt;sup&gt;Chi&lt;/sup&gt;/KO)</td>
<td>PWD/C3H</td>
<td>8</td>
<td>140*</td>
<td>1.06*</td>
</tr>
<tr>
<td>PWD×(B6-Prdm9&lt;sup&gt;Chi&lt;/sup&gt;/KO)</td>
<td>PWD/C3H</td>
<td>8</td>
<td>109</td>
<td>0.2</td>
</tr>
<tr>
<td>PWD×(KO×BAC5)</td>
<td>PWD/−</td>
<td>13</td>
<td>85</td>
<td>0.07</td>
</tr>
<tr>
<td>PWD×(KO×BAC5)</td>
<td>PWD/−+2C3H</td>
<td>15</td>
<td>168*</td>
<td>1.6*</td>
</tr>
<tr>
<td>PWD×(KO×BAC24)</td>
<td>PWD/−</td>
<td>9</td>
<td>77</td>
<td>0.03</td>
</tr>
<tr>
<td>PWD×(KO×BAC24)</td>
<td>PWD/−+6C3H</td>
<td>5</td>
<td>235*</td>
<td>3.9*</td>
</tr>
<tr>
<td>PWD×(KO×BAC24)</td>
<td>PWD/−</td>
<td>9</td>
<td>77</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*6-BACB6, C57BL/6J-Tg(RP23-159N675Bdm mouse heterozygous for a Prdm9<sup>Chi</sup> transgene; BAC5, transgenic strain with two copies of Prdm9<sup>Chi</sup>; BAC24, strain carrying six transgenic copies of Prdm9<sup>Chi</sup>; KO, B6-KO, heterozygote for the Prdm9 knockout; PWD, genotype at the Prdm9 locus (maternal/paternal); --, null, +, transgenic Prdm9 alleles; n, number of males; BW, body weight (g); TW, mean weight of paired testicles in mg; SC, average sperm count (millions) in paired caput epididymides or

In the left epididymis by a different method;

*significantly higher (p<0.01) compared to littermates (in the row above).

DOI:10.1371/journal.pgen.1003044.t002

**Table 2.** Effects of Prdm9 alleles and dosage on reciprocal hybrids.

<table>
<thead>
<tr>
<th>Cross (female first)</th>
<th>Prdm9</th>
<th>n</th>
<th>BW</th>
<th>TW</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6-KO×PWD</td>
<td>B6/PWD</td>
<td>15</td>
<td>25</td>
<td>105</td>
<td>0.5</td>
</tr>
<tr>
<td>B6-KO×PWD</td>
<td>−/PWD</td>
<td>11</td>
<td>27</td>
<td>174*</td>
<td>4.1*</td>
</tr>
<tr>
<td>(B6×B6-Prdm9&lt;sup&gt;Chi&lt;/sup&gt;)×PWD</td>
<td>B6/PWD</td>
<td>7</td>
<td>24</td>
<td>97</td>
<td>0.3</td>
</tr>
<tr>
<td>(B6×B6-Prdm9&lt;sup&gt;Chi&lt;/sup&gt;)×PWD</td>
<td>PWD/−</td>
<td>5</td>
<td>25</td>
<td>171*</td>
<td>2.2*</td>
</tr>
<tr>
<td>B6C5×BAC5/PWD</td>
<td>B6C5×BAC5/PWD</td>
<td>7</td>
<td>25</td>
<td>181*</td>
<td>3.1*</td>
</tr>
<tr>
<td>B6C5×BAC5/PWD</td>
<td>PWD/PWD</td>
<td>7</td>
<td>24</td>
<td>220*</td>
<td>4.2*</td>
</tr>
<tr>
<td>B6×B6</td>
<td>B6/B6</td>
<td>3</td>
<td>28</td>
<td>195*</td>
<td>3.2*</td>
</tr>
<tr>
<td>PWD×PWD</td>
<td>PWD/PWD</td>
<td>4</td>
<td>20</td>
<td>119*</td>
<td>1.9*</td>
</tr>
</tbody>
</table>

B6-KO, heterozygote for the Prdm9 knock-out; B6C5×B6, C57BL/6J-Tg(RP23-159N675Bdm male heterozygous for a Prdm9<sup>Chi</sup> transgene; Prdm9<sup>B6</sup>, genotype at the Prdm9 locus; B6-PWD-Chr17, reciprocal hybrids (rows 1 and/or 3); *significantly higher relative testis weight (TW per body weight) compared to animals in rows 1 and 3 (p<0.002).

DOI:10.1371/journal.pgen.1003044.t0002

The overall fertility phenotypes correlate with the strength of meiotic arrest

The Prdm9<sup>PWD/Chi</sup>, Prdm9<sup>PWD/−</sup>, and Prdm9<sup>PWD/Chi</sup> F1 hybrids show a progressive increase in overall fertility, yet even the...
Table 3. Overview of male reproductive phenotypes.

<table>
<thead>
<tr>
<th>Prdm9</th>
<th>−/−</th>
<th>PWD/B6</th>
<th>PWD/−</th>
<th>B6/PWD</th>
<th>PWD/C3H</th>
<th>PWD/PWD</th>
<th>B6/B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>B6</td>
<td>F1</td>
<td>F1</td>
<td>F1</td>
<td>F1</td>
<td>PWD</td>
<td>B6</td>
</tr>
<tr>
<td>Sex body</td>
<td>21%</td>
<td>31%</td>
<td>67%</td>
<td>71%</td>
<td>88%</td>
<td>96%</td>
<td>99%</td>
</tr>
<tr>
<td>Diplotene</td>
<td>0.3%</td>
<td>5%</td>
<td>16%</td>
<td>17%</td>
<td>16%</td>
<td>18%</td>
<td>21%</td>
</tr>
<tr>
<td>Spermatids</td>
<td>&lt;1%</td>
<td>&lt;2%</td>
<td>30%</td>
<td>45%</td>
<td>45%</td>
<td>80%</td>
<td>74%</td>
</tr>
<tr>
<td>SC</td>
<td>0.00</td>
<td>0.00</td>
<td>0.06</td>
<td>0.4</td>
<td>0.4</td>
<td>1.9</td>
<td>3.2</td>
</tr>
<tr>
<td>TW</td>
<td>54</td>
<td>61</td>
<td>85</td>
<td>105</td>
<td>110</td>
<td>119*</td>
<td>195</td>
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<td>OFM</td>
<td>0.00</td>
<td>0.00</td>
<td>0.3</td>
<td>3.6</td>
<td>3.4</td>
<td>6.3</td>
<td>6</td>
</tr>
</tbody>
</table>

Prdm9, genotype at Prdm9 (maternal/paternal); Sex body, % pachytene spermatocytes that form a sex body; Diplotene, % diplotene of all primary spermatocytes; Spermatids, % round spermatids counted from the total of round spermatids and primary spermatocytes; SC, sperm count in paired caputs (millions); TW, testicular weight (mg); OFM, offspring per female per month; TW-SC-OFM data; the TW-SC-OFM data; the PWD/B6 hybrid does not reach the parameters of other fertile males (Table 1). The fecundity defects in these hybrids could either represent different degrees of the same arrest or multiple breakdowns affecting different stages of spermatogenesis. To compare the progress of spermatogenesis in these hybrids, indirect immunofluorescence microscopy was performed on surface-spread nuclei of adult testicular cells (chromosome spreads, Table 3 and Table S4). In agreement with the SC data but in contrast to sterile nuclei of adult testicular cells (chromosome spreads, Table 3 and Table S4). The meiotic phenotypes thus correlate with the chromosome spreads with MLH1 and SYCP1 revealed that the proportion of nuclei with fully synapsed pachytene chromosomes carrying over 20 recombination nodules is higher in the Prdm9PWD/B6 hybrids than in the Prdm9PWD/C3H hybrids (p = 0.001). The Prdm9PWD/C3H hybrid carried a lower ratio of pachytene spermatocytes displaying a sex body than the B6 (p = 0.004) and PWD (p = 0.03) fertile controls. The staining of spermatocyte chromosome spreads with MLH1 and SYCP1 revealed that the proportion of nuclei with fully synapsed pachytene chromosomes carrying over 20 recombination nodules is higher in the Prdm9PWD/B6 hybrids than in the Prdm9PWD/C3H hybrids (p = 0.001). The Prdm9PWD/C3H hybrids were similar in this respect to Prdm9PWD/B6, but both carried less pachytene spermatocytes with completed recombination than B6 and PWD (Table S4). The meiotic phenotypes thus correlate with the TW-SC-OFM data; the Prdm9PWD/B6, Prdm9PWD/−, and Prdm9PWD/C3H F1 hybrids display a gradual increase in meiotic progress, yet even the Prdm9PWD/C3H F1 hybrid does not reach the parameters of B6 or PWD. The F1 hybrid does not reach the parameters of other fertile males (Table 1). The fecundity defects in these hybrids could either represent different degrees of the same arrest or multiple breakdowns affecting different stages of spermatogenesis. To compare the progress of spermatogenesis in these hybrids, indirect immunofluorescence microscopy was performed on surface-spread nuclei of adult testicular cells (chromosome spreads, Table 3 and Table S4). In agreement with the SC data but in contrast to sterile nuclei of adult testicular cells (chromosome spreads, Table 3 and Table S4). The meiotic phenotypes thus correlate with the chromosome spreads with MLH1 and SYCP1 revealed that the proportion of nuclei with fully synapsed pachytene chromosomes carrying over 20 recombination nodules is higher in the Prdm9PWD/B6 hybrids than in the Prdm9PWD/C3H hybrids (p = 0.001). The Prdm9PWD/C3H hybrid carried a lower ratio of pachytene spermatocytes displaying a sex body than the B6 (p = 0.004) and PWD (p = 0.03) fertile controls. The staining of spermatocyte chromosome spreads with MLH1 and SYCP1 revealed that the proportion of nuclei with fully synapsed pachytene chromosomes carrying over 20 recombination nodules is higher in the Prdm9PWD/B6 hybrids than in the Prdm9PWD/C3H hybrids (p = 0.001). The Prdm9PWD/C3H hybrids were similar in this respect to Prdm9PWD/B6, but both carried less pachytene spermatocytes with completed recombination than B6 and PWD (Table S4). The meiotic phenotypes thus correlate with the TW-SC-OFM data; the Prdm9PWD/B6, Prdm9PWD/−, and Prdm9PWD/C3H F1 hybrids display a gradual increase in meiotic progress, yet even the Prdm9PWD/C3H F1 hybrid does not reach the parameters of B6 or PWD. Prdm9a, added transgenic copies of Prdm9, allele, its dosage, or background divided into classes according to fertility.

Table 4. Males differing by the Prdm9 allele, its dosage, or background divided into classes according to fertility.

<table>
<thead>
<tr>
<th>Class</th>
<th>Sterile</th>
<th>Semisterile</th>
<th>Semifertile</th>
<th>Fertile</th>
<th>Fertile</th>
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<tr>
<td>TW (mg)</td>
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<td>70 to 90</td>
<td>90 to 140</td>
<td>above 140</td>
<td>above 180</td>
</tr>
<tr>
<td>SC (×10⁶)</td>
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<td>0.01 to 0.2</td>
<td>0.2 to 1.1</td>
<td>above 1.1</td>
<td>above 3.5</td>
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<tr>
<td>OFM</td>
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<td>0.1 to 1</td>
<td>3 to 4</td>
<td>above 4</td>
<td>above 5</td>
</tr>
<tr>
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<td>Prdm9wt</td>
<td>Prdm9wt</td>
<td>Prdm9wt</td>
</tr>
<tr>
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<td>PWD/B6</td>
<td>PWD/−</td>
<td>PWD/B6+2B6</td>
<td>PWD/PWD</td>
<td></td>
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<tr>
<td>PWD×B6</td>
<td>PWD/C3H</td>
<td>PWD/−×2C3H</td>
<td>PWD/−×6C3H</td>
<td></td>
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<tr>
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<td>PWD/B6+6C3H</td>
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<tr>
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<td>−/PWD</td>
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<td>PWD/B6</td>
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<tr>
<td>B6×B6</td>
<td>B6+2C3H/B6</td>
<td></td>
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</table>

TW, mean testicular weight; SC, mean sperm count in paired caput epididymides; OFM, offspring per female per month; Prdm9, genotype at Prdm9 (maternal/paternal); −, null; +, added transgenic copies of Prdm9; Background: maternal × paternal background. Note that the two fertile classes display overlapping parameters.

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To determine whether the partial arrest of spermatogenesis of the semifertile reciprocal (B6×PWD)F1 hybrids involves meiosis I, spermatocyte chromosome spreads were analyzed. The relative number of pachytene cells carrying a sex body in the Prdm9PWD/B6 hybrid was lower than in B6 and PWD (Table 3 and Table S4) and it was elevated by removing the Prdm9B6 allele from the Prdm9B6/PWD hybrid (p = 0.03, Table 3 and Table S4). The number was higher in the Prdm9C3H/PWD intersubspecific male in comparison to the Prdm9B6/PWD F1 hybrid (p = 0.03, Table S4), and increased in the BAC5-carrying reciprocal hybrid compared to the same hybrid harboring BAC21 (p = 0.003, Table S5). Analysis of MLH1 recombination nodules indicated that Prdm9B6/PWD hybrid testes carried less pachytene spermatocytes with completed recombination than B6 or PWD (Table S4). The relative number of the four stages of primary spermatocytes in Prdm9B6/PWD differed from that in fertile males (Table S4, Figure S1). The number of offspring (OFM) correlated with meiotic phenotypes in all investigated hybrids (Table S4), thus postmeiotic incompatibilities may play a minor role in our model of F1 mouse hybrid sterility.

Hybrid incompatibility(ies) of Prdm9B6 is not due to a change in Prdm9 transcript levels

The fertility of the azoospermic Prdm9PWD/B6 F1 hybrids can be rescued by Prdm9 overexpression [6]; however, the amounts of the Prdm9 mRNAs are similar in prepubertal Prdm9PWD/B6 and Prdm9PWD/C3H hybrids that differ in prospective fertility but not in Prdm9 dosage [6]. To understand the mechanism of the partial fertility rescue inflicted by the Prdm9 null alleles in PWD hybrids, the transcript levels of Prdm9 were investigated in prepubertal hybrid testes. The expression of Prdm9 was analyzed using five qRT-PCR amplicons along the gene to account for all alternative transcripts [6]. The mRNA levels of Prdm9 were similar in four investigated types of 14-day-old F1 hybrid testes carrying Prdm9PWD/B6, Prdm9PWD/C3H, Prdm9B6/PWD, and Prdm9C3H/PWD, but were significantly decreased to 52.9±2.3% in the prospectively sperm-carrying Prdm9PWD/F1 hybrids compared to the prospectively azoospermic Prdm9PWD/B6 littermate controls (Figure 3). In other words, the transcription from the PWD, C3H, and B6 alleles seems to be similar and dosage-dependent. Therefore, the dominant-negative interaction(s) of the Prdm9 allele contributing to sterility in the (PWD×B6)F1 hybrid is most likely not a consequence of a change in the Prdm9 transcript level.

Discussion

Our “digital genetics” approach (additions and subtractions of Prdm9 copies) brings a new insight into the interactions of Prdm9 and other hybrid sterility genes participating in the genetic Dobzhansky-Muller incompatibilities (DMIs) and controlling the reproductive fitness of intersubspecific mouse hybrids.

The phenotype of intersubspecific hybrid males is affected by Prdm9 allelic combination and dosage (summarized in Table 4). One copy of Prdm9B6 on multiple F1 intersubspecific hybrid backgrounds is one of the causes of reduced fertility, but a rescue can be achieved with a transgene carrying multiple copies of Prdm9B6 or Prdm9C3H. The replacement of Prdm9B6 with Prdm9C3H in (PWD×B6)F1 males significantly improves fecundity, but it nevertheless leads to sem fertility that can be improved by an increased Prdm9 dosage. The fertility rescue of hybrids by Prdm9 transgenes is also dependent on the Prdm9 copy number.

The F1 background is sensitive to Prdm9 dosage, indicating DMIs between PWD and B6 genomes. These DMIs could involve genetic interactions between Prdm9 and other loci, but they might also be explained by interactions between Prdm9-independent loci appearing as the consequence of sensitization by the Prdm9 dosage. Although the overexpression of both Prdm9B6 and Prdm9C3H alleles improved the fertility of F1 hybrids, the variation of effects among the Prdm9 alleles when in one or two copies suggests either

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**Figure 2. Sex body formation in the Prdm9PWD/F1 hybrid male.** Surface-spread nuclei of adult testicular cells treated with a hypotonic solution were indirectly labeled using antibodies marking the synaptonemal complex (anti-SYCP1 and anti-SYCP3) to discern the stage of primary spermatocytes and the phosphorylated form of the histone variant H2AX (anti-γH2AX) to visualize the sex body and then observed under a fluorescent microscope. Left, pachytene carrying a sex body (67 cases found per total of 100 nuclei from four biological replicates); right, pachytene without a sex body (33/100 nuclei).

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variation in the strength of the same interaction(s) or specific DMIs for each Prdm9 allele. Prdm9\\textsuperscript{B6} was the only allele that resulted in a worse phenotype when one copy was added to either type of F1 reciprocal hybrids carrying one Prdm9\\textsuperscript{PWD} allele, indicating a dominant-negative effect of Prdm9\\textsuperscript{B6} specific for (PWD×B6)\textsubscript{F1} and (B6×PWD)\textsubscript{F1}. The beneficial effect of the increased copy number of Prdm9 transgenes irrespective of the transgenic allele suggests that the “toxic” effect of the Prdm9\\textsuperscript{B6} incompatibility can be diluted by the overabundance of Prdm9\\textsuperscript{PWD}.

The fertility of F1 hybrid males harboring chrX\textsuperscript{B6} was always better than that of the comparable reciprocal chrX\textsuperscript{PWD}-carrying males, suggesting a chrX\textsuperscript{PWD} DMI(s) occur(s) in intersubspecific hybrids. Although theoretical options also include the interactions of chrY, mitochondrial genome or genomic imprinting, the interaction of chrX\textsuperscript{PWD} and chr17\textsuperscript{PWD/B6} was revealed by mapping hybrid sterility loci in (PWD×B6)×B6 backcross, as well as in F1 using chrX subchromosomes [12]. The decreased fertility of the reciprocal hybrid males Prdm9\textsuperscript{B6/PWD} compared to Prdm9\textsuperscript{B6/PWD} and Prdm9\textsuperscript{B6/chr17/PWD} could be explained by the incompatibility of Prdm9\textsuperscript{B6-Hstws} with chrX\textsuperscript{B6} or autosomal loci.

Another DMI(s) not involving Prdm9\textsuperscript{B6} probably also acts in F1 hybrids, since the null Prdm9 alleles do not restore complete fertility. As the fertility of the reciprocal Prdm9\textsuperscript{B6-PWD} hybrids was superior to that of the Prdm9\textsuperscript{B6/PWD} F1 males, this DMI (or one of these DMIs) independent of Prdm9\textsuperscript{B6} could involve chrX\textsuperscript{PWD}. Both the elimination of Prdm9\textsuperscript{B6} and Prdm9 overexpression rescued fecundity in the reciprocal (B6×PWD)\textsubscript{F1} hybrids, suggesting that Prdm9\textsuperscript{B6} also participate(s) in a DMI(s) not involving chrX\textsuperscript{PWD}. Supposing the same DMIs also work in the (PWD×B6)\textsubscript{F1} male, a hypothesis supported by its complete sterility, there seems to be at least three sets of incompatibilities affecting the meiotic arrest in this male: Prdm9\textsuperscript{B6} with chrX\textsuperscript{PWD}; Prdm9\textsuperscript{B6} with an unknown autosomal locus or loci, and chrX\textsuperscript{PWD} with an unknown autosomal locus or loci. Alternatively, the sets of incompatibilities could be: interautosomal B6 versus PWD sensitive to the dosage of any Prdm9 allele; chrX\textsuperscript{PWD} with B6 autosomes; Prdm9\textsuperscript{B6} with or without PWD autosomal loci. Although backcrosses using the Prdm9 null alleles could reveal the number and map positions of the unknown autosomal loci, we already have a good candidate for one of these loci, Hst\textsuperscript{a} on chr1\textsuperscript{PWD}.

The Hst1-Hstws (Prdm9\textsuperscript{B6-chr17\textsuperscript{PWD}}) incompatibility in F1 hybrids is alleviated by deletion of Prdm9\textsuperscript{B6} and substitution of chr17\textsuperscript{B6} with chr17\textsuperscript{PWD} leading to increased fertility. An epistatic interaction of chr17\textsuperscript{PWD/B6} with chrX\textsuperscript{PWD} is necessary, albeit not sufficient for sterility of (PWD×B6)×B6BC\textsubscript{I} males [12]. However, at the moment we cannot distinguish between the effects of intergenic and interallelic interactions, also because the impact of Prdm9\textsuperscript{B6} on hybrid sterility has not been directly investigated. While there is strong evidence that Prdm9 is identical with Hst1 in Mmm [6], we are unable to exclude that the Hstws locus in Mmm is linked to but different from Prdm9. On the other hand, Prdm9 carries the fastest evolving ZnF domain in metazoans [26] and Prdm9\textsuperscript{B6-PWD} F1 hybrids display a reduced number of pachytene spermatocytes harboring sex bodies, as well as other features of partial meiotic arrest. Therefore, the incompleteness of the rescue of hybrid fertility by Prdm9\textsuperscript{B6} deletions in the (PWD×B6)\textsubscript{F1} hybrid

![Figure 3. Expression of Prdm9 and Morc2b in prepubertal hybrid testis](Image)
can be interpreted as the consequence of Prdm9<sup>PWD</sup> haploinsufficiency in the context of the F<sub>1</sub> hybrid background, because Prdm9<sup>PWD/</sup><sup>B6</sup> males were more affected than Prdm9<sup>PWD/B6</sup> backcross males.

The hybrid sterility phenotype shows the features of spermatogenesis seen in the Prdm9<sup>S/J</sup> male and it can be alleviated by Prdm9<sup>B6</sup> transgenics [6]. It might seem that the correction of hybrid sterility can be overcome by the increased dosage of Prdm9, we excluded that the key difference between the Prdm9<sup>C3H</sup> and Prdm9<sup>B6</sup> alleles lies in increased transcription, because the expression of Prdm9 mRNAs in Prdm9<sup>PWD/B6</sup>, Prdm9<sup>PWD/C3H</sup>, Prdm9<sup>B6/PWD</sup>, and Prdm9<sup>C3H/PWD</sup> prepubertal hybrid testes of the same age were similar despite the different prospective fertility (Figure 3). Increased translational efficiency remains a possibility for the key allelic difference, as Prdm9<sup>C3H</sup> and Prdm9<sup>B6</sup> differ in the 5′-untranslated region [6]. However, the polymorphism in the ZnF region of the protein products could also provide an explanation for the functional allelic difference.

As hybrid sterility can be overcome by the increased dosage of any Prdm9 allele, one must control the number of copies in experiments designed to discern the functional sequence differences in the Hst1 alleles. Grey et al [24] successfully used transgenesis to learn that the distribution of meiotic recombination hotspots is affected by the ZnF domain allele of Prdm9; no difference in the distribution was seen in the control Prdm9<sup>B6</sup> BAC transgenics. It might seem that the correction of hybrid sterility caused by the same Prdm9<sup>B6</sup> BAC could be caused by a different mechanism than redistribution of recombination hotspots. However, the increased dosage of Prdm9 in F<sub>1</sub> hybrids may overcome the DMI and change the localization of hotspots. Nevertheless, it is unknown how a changed distribution of hotspots could lead to sterility, especially when considering that Prdm9 function is dispensable for fertility in the dog [35,36]. Thus (an)other function(s) of Prdm9 may be involved in hybrid sterility, e.g., transactivation of meiotic genes.

While azoospermia was rare or absent, fertility reduced below the range found in pure species was found in one third of males in the Bavarian part of the natural house mouse hybrid zone [37]. The lack of azoospermic males can be explained by the absence of F<sub>1</sub>-like animals in the zone [37,38]. F<sub>1</sub> male sterility may thus be more important for establishing than for maintaining the hybrid zone. Although most of the fertility differences detected in our study were robust enough to affect the number of offspring, they are likely to have even greater impact in nature considering the sperm competition during multiple mating [39].

Prdm9<sup>B6</sup> plays a role in the complete meiotic arrest of (PWD×B6)<sub>F<sub>1</sub></sub> hybrids [6]. The importance of this finding for mouse speciation could be somewhat limited considering that only males carrying a certain allele resulting from one direction of a cross between two subspecies are affected. In this report, we demonstrated that Prdm9 also participates in the sterility of the reciprocal (B6×STUS)<sub>F<sub>1</sub></sub> and in the partial meiotic failure of the (B6×PWD)<sub>F<sub>1</sub></sub> males. The meiosis of (PWD×B6)<sub>Pwdm9<sup>C3H</sup></sub><sub>F</sub> hybrids harboring another Mmd Prdm9 allele is adversely affected by a DMI that can be alleviated by an increased Prdm9 dosage or using the reciprocal cross, (B6×Prdm9<sup>C3H</sup>×PWD)<sub>F<sub>1</sub></sub>. The reciprocal crosses of PWD and of the wild-Mmd-derived strain WSB/Ei also display differences in hybrid sterility [40]. Although many quantitative trait loci were detected in (WSB×PWD)<sub>F<sub>2</sub></sub> intercross males, heterozygosity in a region overlapping the genomic position of Prdm9 decreases SC and relative TW; regions associated with fertility were also found on chrX<sup>PWD</sup> [40]. The Prdm9 allele of WSB is similar to C3H, being the same in the ZnF domain [25], yet WSB differs from C3H in other parts of Prdm9 [19,41]. Therefore, the semisterility of (PWD×WSBF)<sub>F<sub>1</sub></sub> males seems to involve Prdm9. Admittedly, the degree of importance of Prdm9 for mouse speciation also depends on the frequency of alleles causing reduced fertility near the hybrid zone that is currently unknown; however, the relevance of Prdm9 for hybrid sterility now appears to be greater than shown previously.

**Materials and Methods**

**Ethics statement**

The mice were kept at the Specific Pathogen-Free Facility of the Institute of Molecular Genetics, Prague, and in a conventional breeding facility of the Institute of Vertebrate Biology in Studenec. Principles of laboratory animal care obeyed the Czech Republic Act for Experimental Work with Animals (Decree No. 207/2004 Sb, and the Acts Nos. 246/92 Sb, and 77/2004 Sb) fully compatible with the corresponding EU regulations and standards, namely Council Directive 806/609/EEC and Appendix A of the Council of Europe Convention ETS123.

**Mice**

The STUS and PWD/Ph strains are derived from wild mice of Mmm subspecies [30,42]. Mice carrying a deletion of chr17, Sod2<sup>−/−</sup>, were generated through embryonic stem cells [28] harboring Prdm9<sup>B6</sup> on (129×B6)<sub>F<sub>1</sub></sub> background and were trans-fected with BAC5. The deletion causes lethality when homozygous, it is several Mb in length, and it includes the Hst1 region [6]. The BAC5, BAC21, and BAC24 C3H/H<sub>E</sub> transgenes have no effect on fertility in non-intersubspecific hybrid males, but BAC5 and BAC24 rescue fertility of sterile hybrids [6]. The results of quantitative PCR [6] indicate that BAC24 line contains six and BAC5 two copies of Prdm9<sup>C3H</sup>; BAC21 carries two copies of truncated Prdm9 (only the last, ZnF-encoding exon). BAC5 and BAC24 transgenes rescue fertility in Prdm9<sup>B6</sup> (data not shown). All three transgenic lines were transferred to B6 background through 10 generations of backcrossing. The B6×Prdm9<sup>C3H</sup>(B6×B10.C3H/Hst1<sup>F</sub>) congenic carries the C3H polymorphisms at Prdm9 and the differential segment is 3.5 Mb (position in the mm9 genome assembly 12.5 to 15.9 Mb) to 6.4 Mb (10.2 to 16.5 Mb) in length. The knock-out line Prdm9<sup>−/−</sup> was generated in 129P2/OlaHsd ES cells by replacement of the first five coding exons with Lacz [20] and maintained on mixed 129P2/OlaHsd * C57BL/6 background. The C57BL/6J-Tg(RP23-159N6)5Bdm strain (transgene Accession ID: MGI:5311012) was generated by injection of a circular BAC DNA into zygotic male pronuclei; it carries four to five copies of RP23-159N6 BAC harboring Prdm9<sup>B6</sup>(Hst1<sup>F</sub>) on B6 background, and the Prdm9 steady-state mRNA level in primary spermatocytes is about 1.3- to 2.5-times increased compared to non-transgenic animals [24], suggesting that two Prdm9 copies are expressed from the transgene.

**Genotyping, phenotyping, and statistics**

See Text S1 for the PCR primers and conditions used for genotyping. Body weight (BW) and testicular weight (TW, from
paired testicles) were determined in adult males (9 to 12-week old). Sperm count (SC) was obtained from paired caput epididymides at room temperature [6]. For the experiments using the Prdm9<sup>B6</sup> transgene, where the entire left epididymis was extracted at 37°C [43]. Multiple biological replicates of each genotype were also analyzed for cellular phenotypes and RNA expression. Slides with surface-spread nuclei (chromosome spreads) were obtained from adult testicular cells using isotonic [44] or hypotonic [45] treatment; see Text S1 for the antibodies. Semiquantitative real-time RT-PCR was performed using total testicular RNAs of 14-day-old F1 intersubspecific hybrids exactly as described previously [6]. The significance of BW, TW, and testicular RNAs of 14-day-old F1 intersubspecific hybrids was analyzed using Welch's t-test, SC and OFM with Wilcoxon rank sum test, and cellular phenotypes with $\chi^2$ test. Unless stated otherwise, the comparison significance for TW was also significant for relative TW (TW/BW).

**Supporting Information**

**Figure S1** The proportions of four stages of primary spermatocytes determined by SYCP3-SYP1-hH2AX staining of spread nuclei of adult testicular cells. $\rightarrow$, Prdm9<sup>B6</sup> on B6 background; PWD/B6, [PWDxB6]<sup>F1</sup>; PWD/<sup>B6</sup>, hemizygous [PWDxB6-Prdm9<sup>B6</sup>]F<sub>1</sub>; B6/PWD, (B6 x PWD)<sup>F1</sup>; PWD/C3H, (PWDxB6-Prdm9<sup>C3H</sup>)F<sub>1</sub>; Fert, fertile males (pooled data from B6, B6-Prdm9<sup>B6</sup>/B6, and (BAC5 x PWD)<sup>F1</sup>). Diplo, diploptene; Pach, pachytene; Zygo, zygotene; Lept, leptotene spermatocytes. The number above each column designates the total number of counted cells (average of 3.6 males per column); the asterisks indicate significant differences ($\chi^2$ test); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (TIF)

**References**


**Table S1** The effect of <i>Prdm9</i> dosage on hybrids of the STUS strain. (DOC)

**Table S2** The fertility of males hemizygous for the <i>Prdm9<sup>B6<sup>mut</sup></sup></i> knock-out. (DOC)

**Table S3** The effect of the <i>Sod2</i><sup>df14J</sup> deletion and <i>Prdm9<sup>B6<sup>mut</sup></sup></i> knock-out (KO) on hybrid sterility. (DOC)

**Table S4** Details of reproductive phenotypes of various males. (DOC)

**Table S5** Details of reproductive phenotypes. (DOC)

**Text S1** Supporting Materials and Methods. Antibodies, primers, PCR conditions. (DOC)

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**Author Contributions**

Conceived and designed the experiments: ZT. Performed the experiments: PF ZT OM JP. Analyzed the data: ZT PS. Contributed reagents/materials/analysis tools: JCS YM FB BdB MF SG. Wrote the paper: ZT JF BdB MF JCS. Interpreted the data analyses: ZT JF BdB MF JCS GP. Commented and approved the manuscript: PF OM PS SG YM JP.