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Interallelic and Intergenic Incompatibilities of the Prdm9 (Hst1) Gene in Mouse Hybrid Sterility

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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.Prdm9)F1, 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.

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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 Hst1 allele (e.g., the PWD strain) and laboratory strains bearing the Hst1 allele [11]. Strains with the Hst1 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 Hst1 strains with PWD females, e.g., (PWD × B6)F1, are azoospermic, the males from the reciprocal crosses using PWD males [(B6 × PWD)F1] can sire offspring (Figure 1, [6,12]). Other laboratory strains (C3H, the congenic B6.C3H-Hst1, etc.) harbor the Hst1 allele and produce sperm-carrying F1 males in crosses with PWD females [13]. The male offspring of B6 with C3H, as well as F1 females in all PWD, B6, and C3H crosses are fertile.

The Hst1 gene is the first mammalian candidate for a speciation gene. Hst1 was identified by consecutive mapping of the Hst1 alleles [14–16], expression profiling [17], allelic sequencing [18,19], and transgenic rescue [6]. The best candidate was confirmed by comparing the phenotypes of its null allele with the
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 (C57BL/6J etc.) mice. Hst1, the first mammalian candidate for a speciation gene, was identified as a meiotic PR/SET-domain gene, Prdm9, but the mechanism causing sterility has remained unknown. While the F1 male offspring of C57BL/6J males and PWD females produce no sperm, the males from the reciprocal cross using PWD males and C57BL/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 C57BL/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 (Meiotic gene with SET/PR domain and Zinc fingers). The product of this gene trimethylates histone 3 on lysine 4 (H3K4me3; [20]). Spermatocytes of Prdm9+/− animals and sterile hybrids arrest at the pachytene stage of meiotic prophase I, displaying defects in chromatid pairing and sex body formation, as well as downregulation of meiotic genes. The downregulation correlates with a lower level of H3K4me3 at promoters, at least in the case of the Morc2b gene (4932411A10Rik; [6,20]). The Morc2b mRNA is elevated in (PWD×B6-C3H).Hst1+/−F1, compared to (PWD×B6).F1 prepubertal testes, while the levels of all known Prdm9 transcripts are similar [6].

The chromatin of mouse meiotic recombination hotspots is marked by H3K4me3 at the start of meiosis [21]. Genetic mapping of a gene acting in trans 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 Hst1 and Hst1′, 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 Mmrn laboratory strains [19,23]. The ZnF-encoding region of the PWD allele differs from both C3H and B6 [6], but whether Prdm9PWD is identical to Hst1′ 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 Prdm9 allele is necessary but not sufficient for hybrid sterility. The Hst1 gene from Mmrn is also located on chr17 [12]. In the sterile (PWD×B6).F1 males, chr17 carries the combination Hst1′/Prdm9+/−, 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 Hst1′/Prdm9+/− is the only combination resulting in a complete meiotic arrest in (PWD×B6).F1 hybrid males, because both Prdm9+/−/Prdm9+/− and Hst1′/Hst1′ homozygotes on the same background were fertile [12]. However, even on the F1 background, the Hst1′/Prdm9+/− 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 Prdm9+/− 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 Prdm9+/− incompatibility(ies), 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 Prdm9PWD+/− allele in either type of reciprocal hybrid that results into a more sterile phenotype than the corresponding hybrid harboring only one Prdm9PWD allele. This exception indicates an F1 hybrid-specific dominant-negative interaction(s) of Prdm9+/−.

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 Prdm9+/−-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 (BAC5 transgene) in Prdm9PWD/−/− (PWD×B6).F1 intersubspecific hybrid males ([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 Prdm9PWD allele was utilized. The C57BL/6J-Tg(RP23-159N6)75Bdm 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 B6 females, all the F1 hybrid males were fertile (Figure 1; [27]).

Male hemizygous for these alleles on (129 * B6) as well as on B6 background display similar fertility parameters as their littermates, most of the hybrids having a normal male genotype (Table S2). To distinguish the effect of the null alleles on intersubspecific F1 hybrids, the hemizygous males were outcrossed to PWD. Unlike their azoospermic Prdm9PWD/−/− 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 Prdm9PWD/− 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×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×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×B6)F1 hybrids that is not present in the parental strains.

Incompatibility of Prdm9B6 in (PWD×B6)F1 males

The Prdm9C3H allele rescues the fertility phenotype of the (PWD×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 strain B6-Prdm9C3H, and then crossing the preselected Prdm9C3H males to PWD females, the resulting Prdm9PWD/C3H hybrids had a significantly lower TW and SC than those of 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/C3H 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×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×B6)F1 hybrid displays a complete male-specific arrest of gametogenesis that is at least partially alleviated in reciprocal (B6×PWD)F1 and in 94% of
the reciprocal, sperm-carrying (B6 × PWD) F1 males were inspected. The increased fecundity of a subset of these males was statistically significant relative to the testicles in mg; SC, average sperm count (millions) in paired caput epididymides; 

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 Prdm9C3H 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 Prdm9F1 hybrids were superior to those of their (B6 × PWD) F1 littermates (Table 2). The parameters of the Prdm9F1 transgensics carrying BAC3 were also better than those of the Prdm9F1 control (Table 2). In contrast, BAC21 overlapping most of BAC5 but carrying truncated Prdm9 [6] did not improve the fertility of Prdm9EcPWD hybrids (Table S3). To discern whether a single copy of Prdm9C3H can improve the fertility of reciprocal hybrids, males from the cross (B6 × B6-Prdm9C3H × PWD) were inspected. The increased fecundity of a subset of these males could be ascribed to the presence of Prdm9C3H (Table 2, pTW < 0.001, pSC < 0.003). The reciprocal Prdm9F1:PWD F1 males displayed superior fertility parameters than the Prdm9F1:PWD/C3H hybrids (Table S4; pTW = 0.004, pSC = 0.03, pOFM = 0.04).

To determine whether the fertility rescue of the reciprocal hybrids is limited to the Prdm9C3H allele and the PWD Mmm strain, we again used the C57BL/6J-Tg(RP23-159N675Bdm male heterozygous for a Prdm9 transgene; Prdm9, 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; *significantly higher than in PWD/Prdm9 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).

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

| Cross (female first) | Prdm9 | n | TW | SC |
|----------------------|-------|---|----|----|
| PWD × B6-BACB6 | PWD/B6 | 9 | 55 | 0.00 |
| PWD × B6-BACB6 | PWD/B6+2B6 | 3 | 167 | 18 |
| PWD × B6-KO | PWD/B6 | 10 | 59 | 0.00 |
| PWD × B6-KO | PWD/− | 12 | 82 | 0.06 |
| PWD × (B6-Prdm9C3H × KO) | PWD/− | 10 | 94 | 0.10 |
| PWD × (B6-Prdm9C3H × KO) | PWD/C3H | 8 | 140 | 1.06 |
| PWD × B6-Prdm9C3H | PWD/C3H | 8 | 109 | 0.2 |
| PWD × (KO × BAC5) | PWD/− | 13 | 85 | 0.07 |
| PWD × (KO × BAC5) | PWD/− + 2C3H | 15 | 168 | 1.6 |
| PWD × (KO × BAC24) | PWD/− | 9 | 77 | 0.03 |
| PWD × (KO × BAC24) | PWD/− + 6C3H | 5 | 235 | 3.9 |

6-BAC6, C57BL/6J-Tg(RP23-159N675Bdm mouse heterozygous for a Prdm9 transgene; BAC5, transgenic strain with two copies of Prdm9C3H; BAC24, strain carrying six transgenic copies of Prdm9C3H. KO, B6-KO, heterozygote for the Prdm9 knockout; Prdm9, genotype at the Prdm9 locus (maternal/paternal); ∼, null; +, transgenic Prdm9 alleles; n, number of males; TW, mean weight of paired testicles in mg; SC, average sperm count (millions) in paired caput epididymides.

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

| Cross (female first) | Prdm9 | n | BW | TW | SC |
|----------------------|-------|---|----|----|----|
| B6-KO × PWD | B6/PWD | 15 | 25 | 105 | 0.5 |
| B6-KO × PWD | −/PWD | 11 | 27 | 174* | 4.1* |
| (B6 × B6-Prdm9C3H × PWD) | B6/PWD | 7 | 24 | 97 | 0.3 |
| (B6 × B6-Prdm9C3H × PWD) | C3H/PWD | 5 | 25 | 171* | 2.2* |
| BAC5/BAC5 × PWD | B6+/C3H/PWD | 7 | 25 | 181* | 3.1* |
| B6 × 17 × PWD | B6+/PWD* | 7 | 24 | 220* | 4.2* |
| B6 × B6 | B6/B6 | 3 | 28 | 195* | 3.2* |
| PWD × PWD | PWD/PWD | 4 | 20 | 119* | 1.9* |

B6-KO, heterozygote for the Prdm9 knockout; B6-BACB6, C57BL/6J-Tg(RP23-159N675Bdm mouse heterozygous for a Prdm9 transgene; Prdm9, 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; *significantly higher relative testis weight (TW per body weight) compared to animals in rows 1 and 3 (p < 0.002).

Incompatibilities of Hst1 in Hybrid Sterility

The overall fertility phenotypes correlate with the strength of meiotic arrest

The Prdm9f1/PWD, Prdm9f1/f1, and Prdm9f1/PWD/C3H F1 hybrids show a progressive increase in overall fertility, yet even the
Table 3. Overview of male reproductive phenotypes.

| Prdm9 | −/− | PWD/B6 | PWD/− | B6/PWD | PWD/C3H | PWD/PWD | B6/B6 |
|-------|-----|--------|-------|--------|---------|---------|-------|
| Background | B6 | F1 | F1 | F1 | F1 | PWD | B6 |
| Sex body | 21% | 31% | 67% | 71% | 88% | 96% | 99% |
| Diplotene | 0.3% | 5% | 16% | 17% | 16% | 18% | 21% |
| Spermatids | <1% | <2% | 30% | 45% | 45% | 80% | 74% |
| SC | 0.000 | 0.000 | 0.06 | 0.4 | 0.4 | 1.9 | 3.2 |
| TW | 54 | 61 | 85 | 105 | 110 | 119* | 195 |
| OFM | 0.00 | 0.00 | 0.3 | 3.6 | 3.4 | 6.3 | 6 |

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; *significantly higher relative TW (TW per body weight) than for males in columns 1 through 5. See Table S4 for more details and statistics.

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Prdm9PWD/C3H 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 nuclear data presented in Table S4), the chromosome spreads revealed the presence of spermatids in (PWD x B6) Prdm9PWD/B6 Prdm9PWD/− males. The relative number of round spermatids in these Prdm9PWD/− tests did not reach the number observed in Prdm9PWD/C3H hybrids (Table 3 and Table S4, p<0.001). Due to an arrest at pachynema, the relative number of the four stages of primary spermatocytes in Prdm9PWD/B6 was different from Prdm9PWD/− and Prdm9PWD/C3H F1 intersubspecific hybrids (Table 3 and Table S4, Figure S1). A sex body was formed in 67% of pachytene spermatocytes of Prdm9PWD/− hybrids (Figure 2), a higher proportion in comparison to Prdm9PWD/B6 (p<0.001) but lower compared to 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/− than in the Prdm9PWD/B6 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.

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

| Class | Sterile | Semisterile | Semifertile | Fertile | Fertile |
|-------|---------|-------------|-------------|---------|---------|
| TW (mg) | 45 to 70 | 70 to 90 | 90 to 140 | above 140 | above 180 |
| SC (x10⁵) | 0.00 | 0.01 to 0.2 | 0.2 to 1.1 | above 1.1 | above 3.5 |
| OFM | 0.00 | 0.1 to 1 | 3 to 4 | above 4 | above 5 |
| Background: Prdm9wt; Prdm9a; Prdm9a; Prdm9a; Prdm9a; Prdm9a | PWD/B6 | PWD/C3H | PWD/B6+2C3H | PWD/B6+6C3H | PWD/B6+12C3H |
| PWD x B6 | PWD/B6 | PWD/− | PWD/B6+2C3H | PWD/B6+6C3H | PWD/B6+12C3H |
| PWD x B6 | PWD/C3H | PWD/− | PWD/C3H | PWD/B6+2C3H | PWD/B6+6C3H |
| B6 x PWD | B6/PWD | −/PWD | B6/PWD | B6+2C3H/PWD |
| B6 x STUS | B6/STUS | STUS/B6+286 | STUS/B6+286 | STUS/B6+286 |
| B6 x B6 | −/− | B6/B6 | B6/B6 | B6/B6 |

TW, mean testicular weight; SC, mean sperm count in paired caput epididymides; OFM, offspring per female per month; *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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Incompatibilities of Hst1 in Hybrid Sterility
To determine whether the partial arrest of spermatogenesis of the semifertile reciprocal \((B_6\times PWD)F_1\) hybrids involves meiosis I, spermatocyte chromosome spreads were analyzed. The relative number of pachytene cells carrying a sex body in the \(Prdm9^{PWD/B6}\) hybrid was lower than in \(B_6\) and \(PWD\) (Table 3 and Table S4) and it was elevated by removing the \(Prdm9^{B6}\) allele from the \(Prdm9^{B6/PWD}\) hybrid \((p = 0.03, \text{Table 3 and Table S4})\). The number was higher in the \(Prdm9^{C3H/PWD}\) intersubspecific male in comparison to the \(Prdm9^{B6/PWD}\) \(F_1\) hybrid \((p = 0.03, \text{Table S4})\), and increased in the \(BAC_5\)-carrying reciprocal hybrid compared to the same hybrid harboring \(BAC_21\) \((p = 0.003, \text{Table S5})\). Analysis of MLH1 recombination nodules indicated that \(Prdm9^{B6/PWD}\) hybrid testes carried less pachytene spermatocytes with completed recombination than \(B_6\) or \(PWD\) (Table S4). The relative number of the four stages of primary spermatocytes in \(Prdm9^{B6/PWD}\) 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 \(F_1\) mouse hybrid sterility.

**Hybrid incompatibility(ies) of \(Prdm9^{B6}\) is not due to a change in \(Prdm9\) transcript levels**

The fertility of the azoospermic \(Prdm9^{PWD/B6}\) \(F_1\) hybrids can be rescued by \(Prdm9\) overexpression [6]; however, the amounts of the \(Prdm9\) mRNAs are similar in prepubertal \(Prdm9^{PWD/B6}\) and \(Prdm9^{PWD/C3H}\) 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 \(F_1\) hybrid testes carrying \(Prdm9^{PWD/B6}\), \(Prdm9^{PWD/C3H}\), \(Prdm9^{B6/PWD}\), and \(Prdm9^{C3H/PWD}\), but were significantly decreased to 52.9±2.3% in the prospectively sperm-carrying \(Prdm9^{PWD/B6}\) \(F_1\) hybrids compared to the prospectively azoospermic \(Prdm9^{PWD/B6}\) 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 \((PWD\times B6)F_1\) 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 \(Prdm9^{B6}\) on multiple \(F_1\) intersubspecific hybrid backgrounds is one of the causes of reduced fertility, but a rescue can be achieved with a transgene carrying multiple copies of \(Prdm9^{B6}\) or \(Prdm9^{C3H}\). The replacement of \(Prdm9^{B6}\) with \(Prdm9^{C3H}\) in \((PWD\times B6)F_1\) males significantly improves fecundity, but it nevertheless leads to semifertility 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 \(F_1\) 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 \(Prdm9^{B6}\) and \(Prdm9^{C3H}\) alleles improved the fertility of \(F_1\) hybrids, the variation of effects among the \(Prdm9\) alleles when in one or two copies suggests either
variation in the strength of the same interaction(s) or specific DMIs for each Prdm9 allele. Prdm9Prdm9 was the only allele that resulted in a worse phenotype when one copy was added to either type of F1 reciprocal hybrids carrying one Prdm9P WD allele, indicating a dominant-negative effect of Prdm9P WD specific for (PWD × B6)F1 and (B6 × PWD)F1. The beneficial effect of the increased copy number of Prdm9 transgenes irrespective of the transgenic allele suggests that the “toxic” effect of the Prdm9P WD incomparability can be diluted by the overabundance of Prdm9P WD.

The fertility of F1 hybrid males harboring chrX PWD was always better than that of the comparable reciprocal chrXB6 -carrying males, suggesting a chrXP WD DMI(s) occur(s) in intersubspecific hybrids. Although theoretical options also include the interactions of chrY, mitochondri al genome or genomic imprinting, the interaction of chrXP WD and chr17P WD/B6 was revealed by mapping hybrid sterility loci in (PWD × B6)B6 backcross, as well as in F1 using chrX subcomics [12]. The decreased fertility of the reciprocal hybrid males Prdm9P WD/P WD compared to Prdm9P WD/P WD and Prdm9P WD/P WD could be explained by the incomparability of Prdm9P WD-Hstws with chrXP WD or autosomal loci.

Another DMI(s) not involving Prdm9P WD probably also acts in F1 hybrids, since the null Prdm9 alleles do not restore complete fertility. As the fertility of the reciprocal Prdm9P WD/P WD hybrids was superior to that of the Prdm9P WD/- F1 males, this DMI (or one of these DMIs) independent of Prdm9P WD could involve chrXP WD. Both the elimination of Prdm9P WD and Prdm9 overexpression rescued fecundity in the reciprocal (B6 × PWD)F1 hybrids, suggesting that Prdm9P WD also participate(s) in a DMI(s) not involving chrXP WD.

Supposing the same DMIs also work in the (PWD × B6)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: Prdm9P WD with chrXP WD, Prdm9P WD with an unknown autosomal locus or loci, and chrXP WD 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; chrXP WD with B6 autosomes; Prdm9P WD with PWD and/or with 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, Hstws on chr17P WD.

The Hst1 -Hstws - (Prdm9P WD-chr17P WD) incompatibility in F1 hybrids is alleviated by deletion of Prdm9P WD and substitution of chr17B6 with chr17P WD leading to increased fertility. An epistatic interaction of chr17P WD/B6 with chrXP WD is necessary, albeit not sufficient for sterility of (PWD × B6) × B6BC1 males [12]. However, at the moment we cannot distinguish between the effects of intergenic and interallelic interactions, also because the impact of Prdm9P WD 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 Prdm9P WD/- 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 Prdm9P WD deletions in the (PWD × B6)F1 hybrid.

Figure 3. Expression of Prdm9 and Morc2b in prepubertal hybrid testis. Real-time qRT-PCR was performed using RNAs of 14-day-old F1 intersubspecific hybrids of the indicated Prdm9 genotypes (maternal/paternal); −, null. The animals carrying genotypes labeled by the same color were littermates. The columns indicate mRNA expression (mean ± standard deviation) relative to β-actin mRNA for five amplificans in Prdm9 (arranged from the 5’ to 3’ of the gene) and one in Morc2b (orange); the asterisks mark significantly different values (*, p < 0.05; **, p < 0.01); the expression of Prdm9 was similar in all except the Prdm9P WD−/− F1 hybrids.

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can be interpreted as the consequence of \textit{Prdm9}\textsubscript{PWD} haploinsufficiency in the context of the F\textsubscript{1} hybrid background, because \textit{Prdm9}\textsubscript{PWD}/\textsubscript{C3H} F\textsubscript{1} males were more affected than \textit{Prdm9}\textsubscript{C3H}/\textsubscript{B6} backcross males.

The hybrid sterility phenotype shows the features of spermato-genes in the \textit{Prdm9}\textsubscript{C3H}/\textsubscript{B6} male and it can be alleviated by \textit{Prdm9}\textsubscript{C3H} transgenics [6]. \textit{Prdm9}\textsubscript{C3H} thus appears to participate in a loss-of-function DMI [32]. However, hybrid sterility can also be partially corrected by \textit{Prdm9} null alleles, suggesting a gain-of-function DMI. Thus \textit{Prdm9}\textsubscript{B6} might take part in multiple DMIs, both gain- and loss-of-function. Alternatively, the increased dosage of \textit{Prdm9} could leave its part not participating in a gain-of-function DMI(s) for the normal function.

The viability of certain Drosophila hybrids is affected by the gene dosage of \textit{Hst1} (Hybrid male rescue) gene [33] and by the DNA-regulatory divergence of the \textit{Lhr} (Lethal hybrid rescue) gene [34]. Although the fertility of mouse hybrids can be rescued by the increased dosage of \textit{Prdm9}, we excluded that the key difference between the \textit{Prdm9}\textsubscript{C3H} and \textit{Prdm9}\textsubscript{B6} alleles lies in increased transcription, because the expression of \textit{Prdm9} mRNAs in \textit{Prdm9}\textsubscript{B6/\textsubscript{C3H}}, \textit{Prdm9}\textsubscript{B6/\textsubscript{PWD}}, and \textit{Prdm9}\textsubscript{C3H/\textsubscript{PWD}} 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 \textit{Prdm9}\textsubscript{C3H} and \textit{Prdm9}\textsubscript{B6} 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 \textit{Prdm9} allele, one must control the number of copies in experiments designed to discern the functional sequence differences in the \textit{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 \textit{Prdm9}; no difference in the distribution was seen in the control \textit{Prdm9}\textsubscript{B6} BAC transgenics. It might seem that the correction of hybrid sterility caused by the same \textit{Prdm9}\textsubscript{B6} BAC could be caused by a different mechanism than redistribution of recombination hotspots. However, the increased dosage of \textit{Prdm9} in F\textsubscript{1} hybrids may overcome the DMIs and change the localization of hotspots. Nevertheless, it is unknown how a changed distribution of hotspots could lead to sterility, especially when considering that \textit{Prdm9} function is dispensable for fertility in the dog [35,36]. Thus (an)other function(s) of \textit{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 \textit{F1}-like animals in the zone [37,38]. \textit{F1} 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].

\textit{Prdm9}\textsubscript{B6} plays a role in the complete meiotic arrest of \textit{PWD}×\textit{B6}\textsubscript{F1} 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 \textit{Prdm9} also participates in the sterility of the reciprocal (\textit{B6}×\textit{STUS})\textsubscript{F1} and in the partial meiotic failure of the (\textit{B6}×\textit{PWD})\textsubscript{F1} males. The meiosis of \textit{PWD}×\textit{B6}\textsubscript{Prdm9}\textsubscript{C3H}\textsubscript{F1} hybrids harboring another \textit{Mmd} \textit{Prdm9} allele is adversely affected by a DMI that can be alleviated by an increased \textit{Prdm9} dosage or using the reciprocal cross, (\textit{B6}\textsubscript{Prdm9}\textsubscript{C3H}×\textit{PWD})\textsubscript{F1}. The reciprocal crosses of \textit{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 (\textit{WSB}×\textit{PWD})\textsubscript{F2} intercross males, heterozygosity in a region overlapping the genomic position of \textit{Prdm9} decreases SC and relative TW; regions associated with fertility were also found on chrX\textsubscript{PWD} [40]. The \textit{Prdm9} allele of \textit{WSB} is similar to \textit{C3H}, being the same in the ZnF domain [25], yet WSB differs from \textit{C3H} in other parts of \textit{Prdm9} [19,41]. Therefore, the semisterility of (\textit{PWD}×\textit{WSB})\textsubscript{F1} males seems to involve \textit{Prdm9}. Admittedly, the degree of importance of \textit{Prdm9} for mouse specification also depends on the frequency of alleles causing reduced fertility near the hybrid zone that is currently unknown; however, the relevance of \textit{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 Střečov. 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 86/609/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, \textit{Sod2}\textsubscript{df14J}, were generated through embryonic stem cells [28] harboring \textit{Prdm9}\textsubscript{B6} on (129×\textit{B6})\textsubscript{F1} background and were transfected with BAC5. The deletion causes lethality when homozygous, it is several Mbp in length, and it includes the \textit{Hst1} region [6]. The BAC5, BAC21, and BAC24 \textit{C3H}/\textit{HeJ} 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 \textit{Prdm9}\textsubscript{C3H}. BAC21 carries two copies of truncated \textit{Prdm9} (only the last, ZnF-encoding exon). BAC5 and BAC24 transgenes rescue fertility in \textit{Prdm9}\textsubscript{C3H} (data not shown). All three transgenic lines were transferred to \textit{B6} background through 10 generations of backcrossing. The \textit{B6}\textsubscript{Prdm9}\textsubscript{C3H} (\textit{B6}\textsubscript{B10.1.C3H/Hst1} congenic carries the C3H polymorphisms at \textit{Prdm9} and the differential segment is 3.5 Mbp (position in the mm9 genome assembly 12.5 to 15.9 Mbp) to 6.4 Mbp (10.2 to 16.5 Mbp) in length. The knock-out line \textit{Prdm9}\textsubscript{nullin} was generated in 129P2/OlaHsd ES cells by replacement of the first five coding exons with \textit{LacZ} [20] and maintained on mixed 129P2/OlaHsd * C57BL/6 background. The C57BL/6\textsubscript{Tg}(RP23-159N6)75Bdm strain (transgene Accession ID: MGl5311012) was generated by injection of a circular BAC DNA into zygotic male pronucleus; it carries four to five copies of RP23-159N6 BAC harboring \textit{Prdm9}\textsubscript{B6} (\textit{Hst1}) on \textit{B6} background, and the \textit{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 \textit{Prdm9} copies are expressed from the transgene.

Genotyping, phenotyping, and statistics

See Text SI 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], except for the experiments using the Prdm9\textsuperscript{mTmut} 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. Semi-quantitative 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 qRT-PCR values 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 significant 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-SYCP1-Y H2AX staining of spread nuclei of adult testicular cells. \( \sim /\sim, \) \( \sim /\sim^{Prdm9}\textsuperscript{mTmut} \) on B6 background; PWD/B6, \( \text{PWD}/\text{B6}\text{\textsuperscript{F1}}; \) PWD/\( \sim, \) hemizygous \( \text{PWD}/\text{B6}\text{\textsuperscript{Prdm9}\textsuperscript{mTmut}} \); B6/PWD, \( \text{B6}/\text{B6}\text{\textsuperscript{F1}}; \) PWD/C3H, \( \text{PWD}/\text{B6}\text{\textsuperscript{Prdm9}\textsuperscript{C3H}} \); Fert, fertile males (pooled data from B6, B6\textsuperscript{Prdm9}\textsuperscript{mTmut}; and BAC5\times PWD\textsuperscript{F1}). Diplo, diplo-tetra-; Pachy, 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): * \( p<0.05; ** p<0.01; *** p<0.001. \)

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### Table S1

| Description | References |
|-------------|------------|
| The effect of Prdm9 dosage on hybrids of the STUS strain. | (DOC) |
| The fertility of males hemizygous for the Prdm9\textsuperscript{mTmut} knock-out. | (DOC) |
| The effect of the Sdyn\textsuperscript{Df} deletion and Prdm9\textsuperscript{mTmut} knock-out (KO) on hybrid sterility. | (DOC) |
| Details of reproductive phenotypes of various males. | (DOC) |
| Details of reproductive phenotypes. | (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 BD MF JG SG. Wrote the paper: ZT JF BD MF JG SB. Interpreted the data analyses: ZT JF BD MF JG SG. Commented and approved the manuscript: PF OM PS SG YM JP.

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Incompatibilities of Hst1 in Hybrid Sterility

Table S1: The effect of Prdm9 dosage on hybrids of the STUS strain. (DOC)

Table S2: The fertility of males hemizygous for the Prdm9\textsuperscript{mTmut} knock-out. (DOC)

Table S3: The effect of the Sdyn\textsuperscript{Df} deletion and Prdm9\textsuperscript{mTmut} 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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