Altering the binding properties of PRDM9 partially restores fertility across the species boundary

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Sterility or subfertility of male hybrid offspring is commonly observed. This phenomenon contributes to reproductive barriers between the parental populations, an early step in the process of speciation. One frequent cause of such infertility is a failure of proper chromosome pairing during male meiosis. In subspecies of the house mouse, the likelihood of successful chromosome synapsis is improved by the binding of the histone methyltransferase PRDM9 to both chromosome homologues at matching positions. Using genetic manipulation, we altered PRDM9 binding to occur more often at matched sites, and find that chromosome pairing defects can be rescued, not only in an inter-subspecific cross, but also between distinct species. Using different engineered variants, we demonstrate a quantitative link between the degree of matched homologue binding, chromosome synapsis and rescue of fertility in hybrids between Mus musculus and Mus spretus. The resulting partial restoration of fertility reveals additional mechanisms at play that act to lock-in the reproductive isolation between these two species.

Hybrid sterility is a commonly observed phenomenon in nature. It prevents exchange of genetic information between distinct populations, thereby ensuring their reproductive isolation (Abbott, et al. 2013). Hybrid sterility is mainly observed in the heterogametic sex (in mammals, males), according to Haldane’s rule (Haldane 1922), and frequently results from meiotic defects, often involving chromosome asynapsis (Bhattacharyya, et al. 2013). To better understand this critical evolutionary process, instances of hybrid sterility have been extensively studied (Maheshwari and Barbash 2011; Chen, et al. 2016). In mouse, work on inter-subspecific crosses between Mus musculus domesticus and Mus musculus (Forejt and Iványi 1974) has identified Prdm9 as the first and so far only speciation gene found in mammals (Mihola, et al. 2009).

Pдрm9 has two known roles in mammalian meiosis. The first identified role is that PRDM9 controls the positioning of double strand breaks (DSBs), which initiate recombination between homologous chromosomes during meiosis (Baudat, et al. 2010; Myers, et al. 2010; Parvanov, et al. 2010). This positioning is defined by the DNA-binding zinc finger array of PRDM9, which is highly variable between different individuals, populations, subspecies, and species, with different DNA sequences being recognized (Oliver, et al. 2009; Buard, et al. 2014). Recent work has established a second role for PRDM9, namely that while recombination is initiated by a DSB on one of the homologues, the binding of PRDM9 at the corresponding position on the other, uncut, homologue facilitates homology search, ensuring successful synapsis (Hinch, et al. 2019; Li, et al. 2019).
Although the mechanisms of this second role remain unknown, both functions underlie hybrid infertility between subspecies. The DNA sequences at recombination sites change over evolutionary time, partly due to chance mutation and partly as a consequence of DSB repair mechanisms (Pratto, et al. 2014). These changes are strongly biased in favour of mutations disrupting – eroding – the strongest binding sites for a particular Prdm9 allele, eliminating many PRDM9 binding sites from the genome (Myers, et al. 2010; Baker, et al. 2015). Recombination events move to weaker PRDM9 binding sites, maintaining successful chromosome synapsis and meiosis. Due to the first role of PRDM9, in two isolated populations A and B with two distinct Prdm9 alleles binding different sequences (say, Prdm9A and Prdm9B, respectively), distinct sites erode within each population. The best PRDM9A binding sites are lost from the genomes of individuals in population A but remain intact in the individuals in population B (Fig.1a), and conversely. In a hybrid individual, with one genome from each of the populations, and one copy of each Prdm9 allele, PRDM9A will preferentially bind to the uneroded, strong binding sites, which are retained mainly on the population “B” chromosomes they inherit. Because PRDM9 binding positions recombination-initiating DSBs, events positioned by PRDM9A tend to occur on the “B” chromosome. Importantly, the homologous sites on the “A” chromosome are mainly not bound by PRDM9, due to erosion of these sites in population A. The same will occur, mutatis mutandis, for the PRDM9B allele. This compromises successful chromosome synopsis, because of the second role of PRDM9 (Fig.1b).

Although the reason(s) this leads to asynapsis are not known, it seems likely that, following DSB formation, the homologous sequence is essential for recombinational repair of the DSB, and differences between homologues within PRDM9 binding motifs thus have the potential to disrupt this process. Indeed, it has been observed that at individual hotspots where one copy of the PRDM9 binding motif is eroded, DSBs occurring at the other, non-eroded copy are repaired more slowly, and are less likely to be recombinationally repaired using the homologue (Hinch, et al. 2019; Li, et al. 2019). This implies a disruption of homology search and/or homologous repair operating at individual hotspots. Recent work (Huang, et al. 2020; Mahgoub, et al. 2020; Wells, et al. 2020) has suggested that ZCWPW1, which is recruited to PRDM9-bound sites, likely by its recognition of the H3K4me3 and H3K36me3 histone modifications deposited upon PRDM9 binding, may play an important role in this process.

Our previous work used genetic manipulation to modify the binding pattern of Mus musculus domesticus PRDM9 (C57BL/6) to that of a common human PRDM9 sequence (hereafter, the humanized allele). When the modified allele was introduced into a sterile hybrid Mus musculus domesticus x Mus musculus musculus background, DSB sites moved to the binding sites of the humanized allele, and restoration of synapsis and fertility resulted (Davies, et al. 2016). Molecular
analysis that allows meiotic DSB sites to be mapped confirmed that the improved synapsis correlated
with increased levels of PRDM9 binding to both homologues at matching positions. Like the
humanized allele, new alleles arising in populations will likely recognize novel non-eroded DNA
sequences, present on both chromosome homologues, thus restoring chromosome pairing and
fertility (Fig.1c).

Recent work has examined the effect of the humanized Prdm9 allele in various inter-subspecific
hybrids, and its introduction was found to consistently improve the rate of chromosomal synapsis and
restore fertility (Mukaj, et al. 2020). To verify whether our previous finding that this restoration of
fertility is likely due to increased binding of PRDM9 at matching positions on both homologous
chromosomes in one of these additional humanized hybrids, we mapped the position of DSB sites in
the hybrid male generated by a mating between STUS/Jpia (Pialek, et al. 2008) (M. musculus musculus,
hereafter STUS) and C57BL/6J (M. musculus domesticus, hereafter B6) mice, heterozygous for the
humanized Prdm9 allele. This was achieved using DMC1 ssDNA sequencing, a modified ChIP-seq
protocol which allows the position of a resected DSB to be mapped (Khil, et al. 2012) (Supplementary
Table 1). We were able to establish which chromosome was being cut (Davies, et al. 2016) and which
Prdm9 allele was responsible (Hinch, et al. 2019) (Supplementary Fig.1). This allowed a metric to be
calculated, measuring the extent to which evolutionary erosion has impacted each hotspot, resulting
in PRDM9 binding and DSBs mainly on only one homologue (as defined by >75% of sequencing reads
resulting from one homologue).

In the infertile STUSB6F1 hybrid, a large proportion (~80% for both alleles) of the recombination sites
for each allele occurred from such eroded hotspots, with only ~20% resulting from positions where
both homologues were being bound and cut. Replacement of the B6 allele by the humanized allele
led to a substantial increase (to 66.5%) in the proportion of humanized PRDM9-controlled
recombination sites occurring at such positions (Supplementary Fig.2a). This increased binding of the
de novo humanized allele at matched positions on the two homologues correlated with substantially
increased levels of synapsis (P<0.0001; t-test) and restored sperm production (P<0.0001; t-test) to
levels comparable with the parental strains (P>0.05; t-test) (Supplementary Fig.2b-f). This establishes
reversal of evolutionary erosion as a likely cause of fertility rescue in these STUSB6F1 hybrids,
consistent with our previous results in PWDB6F1 hybrids harbouring the humanized Prdm9 allele
(Davies, et al. 2016).

Having confirmed that the introduction of a de novo Prdm9 allele rescues inter-subspecific hybrid
infertility, we next considered the impact of the de novo allele in a sterile hybrid generated from
different species. The Algerian mouse, Mus spretus, (SPRET/EiJ (Dejager, et al. 2009), hereafter SPRT)
is a distinct species of mouse inhabiting south-western Europe, Morocco, and the western
Mediterranean coast of Africa and male hybrids with *Mus musculus* strains are known to be sterile (Guénet and Bonhomme 1981). The hybrid sterility has previously been characterised in some detail, and these studies have revealed a number of different mechanisms at play. Cytogenetic analyses found significant anomalies in synapsis including frequent X-Y chromosome asynapsis (Matsuda, et al. 1991; Hale, et al. 1993), with the majority of cells undergoing arrest during the first meiotic division. The fertility of hybrid females allowed backcrossing and subsequent genetic mapping of the infertility traits, identifying an involvement of chromosome 17 (Hammer, et al. 1989; Pilder, et al. 1991) and regions of the X-chromosome (Elliott, et al. 2001). The X-Y asynapsis trait was strongly associated with loci within the pseudoautosomal region (Guénet, et al. 1990; Matsuda, et al. 1991; Hale, et al. 1993). Chromosome 17 harbours the *Hst1* locus, now known to be *Prdm9* (Mihola, et al. 2009), and X-chromosomal loci have long been associated with aspects of hybrid sterility (Storchová, et al. 2004; Lustyk, et al. 2019).

To assess the impact of *de novo Prdm9* alleles on these infertility phenomena, male SPRT mice were crossed with female B6 mice, heterozygous for either the humanized *Prdm9* allele or for an additional engineered allele in which the DNA-binding array of *Prdm9* is replaced with the orthologous sequence from the *Mus musculus castaneus* sub-species (hereafter CAST) (Baker, et al. 2014). Genetic modification of the DNA-binding array had no impact on *Prdm9* expression levels (Supplementary Fig.3).

Recombination hotspots were mapped by DMC1 ssDNA sequencing and the cut chromosome and the responsible *Prdm9* allele determined in each of the hybrids (Supplementary Table 1; Supplementary Fig.4). Only 15.0% of DMC1 sequence reads resulting from B6 PRDM9-controlled recombination sites in the wild-type B6SPRTF1 occurred at positions where both homologues were being appreciably bound and cut. In contrast, replacing the B6 allele with the humanized or CAST alleles improved this proportion over threefold, with 46.7% and 59.6% being observed for the *de novo* allele in the hybrids inheriting the humanized and the CAST alleles, respectively (Fig.2a). The hotspots occurring in the sterile B6SPRTF1 revealed a clear dominance of SPRT PRDM9 over B6. With the *de novo* alleles present, the dominance of the SPRT allele was reduced, with a stronger effect seen for the CAST allele (Fig.2b). A similar pattern of dominance was seen when assaying binding using the H3K4Me3 mark deposited by PRDM9 (Fig.2b).

Cytological analysis of meiotic chromosomes revealed that levels of chromosomal autosomal synapsis were significantly improved by the introduction of either the humanized (*P*<0.01; *t*-test) or CAST (*P*<0.001; *t*-test) *Prdm9* allele, consistent with the improved binding of these *de novo* alleles on both homologues (Fig.2c, Supplementary Fig.5). Introduction of the CAST allele resulted in a higher synapsis...
rate than the humanized allele, consistent with the relative levels of improved binding across the homologues. The rescue of the asynapsis phenotype, however, was not complete.

In agreement with previous work (Matsuda, et al. 1991; Matsuda, et al. 1992; Hale, et al. 1993), a high level of failure to synapse the X and Y chromosomes was evident in B6SPRTF1 hybrids relative to wild-type parental strains ($P<0.01$; t-test), and in contrast to the autosomes, the introduction of either of the de novo Prdm9 alleles had no impact on X-Y asynapsis levels ($P>0.05$; t-test; Supplementary Fig.6).

As expected, wild-type B6SPRTF1 hybrids were completely sterile, yet B6SPRTF1 hybrids harbouring either the humanized or the CAST Prdm9 allele showed significantly increased testes weight ($P<0.001$; t-test) (Fig.2d), and mature spermatozoa were recovered from the epididymis, albeit in much lower numbers than in the parental strains (Fig. 2e). Introduction of the CAST allele resulted in a more pronounced rescue than the humanized allele, as reflected by significantly higher testes weight and sperm count ($P<0.0001$; t-test), which also correlated with the larger impact on homologous binding seen for the CAST allele.

Our investigations of wild-type and genetically engineered hybrid mice reveal a spectrum of autosomal synapsis rates and fertility. By examining these parameters in individual mice, an association between synapsis rate and spermatozoa production was revealed which suggests a “cliff-edge” rather than a smoothly increasing relationship, with a certain threshold of synapsis – approximately 50% - being required for any significant level of sperm production to occur (Fig.2f). Below this threshold there is little or no sperm production. Above the threshold, sperm production is substantial. This observation is in accord with previous studies: a requirement of at least 50% synapsis of the sex chromosomes to achieve sperm production was revealed by a study examining mice expressing the Spo11β isoform with varying genetic background (Faisal and Kauppi 2016). Similarly, an approximately 50% threshold was also evident in studies of Mus musculus x Mus domesticus hybrids (Mukaj, et al. 2020). A possible explanation for this relationship lies in the ability of spermatocytes to exchange cytoplasmic products via cytoplasmic bridges, and indeed apoptotic spermatocytes have been shown to eliminate nearby unaffected cells, presumably because of these bridges (Royo, et al. 2010; ElNati, et al. 2017). Potentially this bystander effect, or non-independent cell death, explains the sudden dramatic effect on spermatozoa production when asynapsis reaches some critical level.

While appreciable levels of sperm production were restored in this interspecies hybrid, the rescued hybrids were still unable to sire litters in natural matings. Examination of the rescued sperm revealed an aberrant head morphology, with the characteristic spike, present in both parental strains, being absent (Fig.2g). In vitro fertilization (IVF) was performed successfully with sperm recovered from the humanized B6SPRTF1 males only when the zona pellucida of the oocytes was removed.
We infer that the aberrant sperm head was incompatible with the sperm’s ability to adhere or traverse the zona pellucida. Fertilized embryos were transferred into a recipient female and a litter of two pups resulted, genetic analysis of which confirmed that one pup had been derived from IVF with the humanized B6SPRTF1 sperm, confirming the viability of the rescued sperm (Supplementary Fig.7).

Despite the persistence of these additional reproductive blocks, the data establish for the first time that PRDM9 is, and remains, a key but reversible part of the hybrid infertility between these two species. Introduction of de novo Prdm9 alleles increases instances where PRDM9 binds at the same position on both chromosome homologues. This, in turn, increases the efficiency of homology search, increasing the rate of synapsis above a critical threshold, enabling the completion of meiosis and the partial restoration of sperm production. This partial restoration allows the two further reproductive blocks to be more clearly revealed.

Firstly, the introduction of de novo Prdm9 alleles ameliorates the autosomal asynapsis seen in the B6SPRTF1 hybrids, yet the asynapsis of the sex chromosomes remains evident and does not appear to be influenced by Prdm9. Indeed, early genetic mapping studies have implicated the distal region of the X-chromosome in this phenomenon, and not Hst1 (Prdm9), suggesting an incompatibility between the pseudoautosomal regions (PAR) of the two species is responsible (Guénet, et al. 1990; Matsuda, et al. 1991; Hale, et al. 1993). Structural constraints may underlie these genetic signals, as differences in the PAR boundary and the composition of the PAR sequences have been reported between Mus spretus and Mus musculus subspecies (Morgan, et al. 2019). PAR asynapsis being Prdm9-independent is consistent with previous findings that PRDM9-dependent recombination plays a relatively small role in PAR recombination in mice (Brick, et al. 2012; Hinch, et al. 2014).

Secondly, in the hybrids harbouring de novo Prdm9 alleles, sperm production occurs in the B6SPRTF1 hybrid, but the sperm show aberrant head morphology. Histological examination of B6SPRTF1 testes sections had previously revealed rare cases of spermatocytes which progress beyond the first metaphase developing abnormally with head shape anomalies (Matsuda, et al. 1991). The increased production of spermatozoa in hybrids harbouring de novo Prdm9 alleles, with appreciable numbers obtainable from the caudal epididymis, makes this phenotype abundantly clear. Aberrant sperm head morphology has been reported for interspecific crosses between Mus musculus and Mus macedonicus (Elliott, et al. 2004) and is a frequently observed phenomenon in inter-subspecific hybrids, with genetic mapping studies implicating several loci (Storchová, et al. 2004; White, et al. 2011; Campbell, et al. 2012; White, Stubbings, et al. 2012). Sperm head abnormalities have previously been shown to correlate with aberrant gene expression programmes during spermatogenesis resulting from perturbations in the processes which maintain transcriptional silencing of the sex chromosomes,
particularly through postmeiotic sperm development (Cocquet, et al. 2009; Larson, et al. 2017).

Matched expression of the Y-chromosomal multicopy gene *Sly*, and its related X-chromosomal parologue, *Slx*, are essential for this process (Ellis, et al. 2005). Interestingly, *Slx* and *Sly* copy numbers are usually well correlated within a species, but significant copy number variation exist between *Mus spretus* and *Mus musculus* (Morgan and Pardo-Manuel de Villena 2017). Furthermore, experimentally induced reduction in *Sly* has been shown to result in spermatozoa of abnormal morphology (Cocquet, et al. 2009), highlighting the need for balanced expression of these related genes. Whether the sperm abnormalities result from a breakdown in postmeiotic sex chromatin repression due to mismatched *Slx* and *Sly* expression in B6SPRTF1 or whether failure in these silencing mechanisms also arise due to the observed meiotic X-Y asynapsis will demand further investigation. Abnormal head morphology has previously been shown to be incompatible with oocyte penetration and fertilization (Krzanowska and Lorenc 1983), and our results confirm that fertilization with the abnormal sperm can only be achieved by IVF in oocytes stripped of their zona pellucida.

Besides PRDM9’s role in regulating the efficiency of autosomal synapsis, this study and others (Oka, et al. 2010) highlight multiple additional factors that may contribute to the sterility of *Mus spretus* and *Mus musculus* interspecies male hybrids. Our genetic approach of relaxing the constraint imposed by PRDM9 will allow further genetic dissection of reproductive barriers at play in these interspecific hybrids.

In summary, our results establish that the introduction of *de novo* alleles on the B6SPRTF1 background increases the binding of PRDM9 at the same position on both homologues, allowing more efficient homology search and rapid repair of DSBs. This increases the chromosome synopsis rate towards a critical threshold, allowing the completion of meiosis and the generation of mature sperm capable of generating offspring. With manipulation of a single gene, we are able to rescue aspects of the infertility that has arisen between two species separated by 1.5 million years of evolution (Boursot, et al. 1993).

**Materials and Methods**

**Animals.** Genetically modified mice harbouring the human PRDM9 B allele (*Prdm9<sup>tm1.1(PRDM9)Wthg</sup>*) were generated in house (Davies, et al. 2016), mice harbouring the *Mus musculus castaneus Prdm9* allele (Baker, et al. 2014) (*Prdm9<sup>tm1.1Kpgn</sup>*) were provided by Petko Petkov (Jackson Laboratories, Bar Harbor, USA). Wild-type STUS/Jpia mice were provided by Jaroslav Piálek, Institute of Vertebrate Biology, Brno and wild-type *Mus spretus* mice (SPRET/EiJ) mice were obtained from MRC Harwell. *Gt(ROSA)26Sor<sup>tm1(CAG-cas9)Wthg</sup>* were generated in house (Cebrian-Serrano, et al. 2017). The breeding of the hybrid mice was carried out in accordance with UK Home Office Animal [Scientific Procedures] Act 1986, with procedures reviewed by the Clinical Medicine Animal Welfare and Ethical Review Body at the University of Oxford, and conducted under project license PPL 30/3437 and 30/3085.
were housed in individually ventilated cages, provided with food and water *ad libitum* and maintained on a 12h light:12h dark cycle (150–200 lux). The only reported positives on FELASA health screening over the entire time course of these studies were for *Helicobacter hepaticus* and *Entamoeba* spp.

Experimental groups were determined by genotype and were therefore not randomized, with no animals excluded from the analysis. Sample sizes for fertility studies were selected on the basis of previously published studies (Davies, et al. 2016) and all phenotypic characterization was performed blind to experimental group.

Heterozygous mice harbouring the human allele were bred with STUS/Jpia in both directions (using females and males of each type) to generate the STUSB6F1 and B6STUSF1 hybrids. Heterozygous female mice harbouring either the human or the CAST *Prdm9* alleles were bred with male SPRET/Eij mice to generate the B6SPRTF1 hybrids. Male hybrid offspring were analysed at 10 weeks of age.

Paired testes weight from individual mice was recorded and normalized against lean body weight, as assessed using EchoMRI-100 Small Animal Body Composition Analyzer. Sperm count was obtained from individual mice by allowing caudal sperm to swim out in 1000 μl of warm PBS prior to counting with a haemocytometer.

**Hotspot determination and provenance.** Chromatin Immunoprecipitation (ChIP) with a rabbit polyclonal anti-DMC1 antibody (Santa Cruz Biotechnology, sc-22768) followed by single-stranded DNA sequencing (SSDS) was performed as previously described (Khil, et al. 2012) with some modifications (Hinch, et al. 2019). ChIP-seq with a rabbit polyclonal anti-H3K4me3 antibody (Abcam ab8580) was performed as previously described (Li, et al. 2019). Hotspot centres were localized by running a bioinformatic pipeline for identification of single-stranded sequences (Khil, et al. 2012) followed by de novo calling using a previously published peak calling algorithm (Davies, et al. 2016). Over 10,000 hotspots were called *de novo* in each of the DMC1 assays reported (Supplementary Table 1) and the analyses presented are based on the full set of autosomal hotspots. The PRDM9 allele responsible for activating specific hotspots was identified by comparison with mice harbouring a range of distinct alleles, as previously described (Hinch, et al. 2019). PRDM9 DNA motifs were inferred using a previously published Bayesian, *ab initio* motif finding algorithm (Altemose, et al. 2017). For each hotspot in the hybrid mouse, the fraction of reads that originated from the B6 and the SPRET (or STUS) chromosomes respectively, was inferred as previously described (Davies, et al. 2016). For mapping of reads to homologous chromosomes in B6SPRTF1, we leveraged previously published variant calls for SPRET/Eij by the Mouse Genomes Project (MGP Version 5 Release 1505) (Keane, et al. 2011). We restricted to SNPs that passed all MGP filters and we further filtered out SNPs that were heterozygous in SPRET/Eij. To perform this analysis in B6xSTUS hybrids, we first sequenced a male STUS mouse to
an average depth of 21X and used Platypus (Rimmer, et al. 2014) version 0.5.2 to call variants. We
restricted to SNPs that passed all quality filters and were homozygous in this animal.

**Immunocytochemistry.** Mouse testis chromosome spreads were prepared using surface spreading
and immunostained as previously described (Davies, et al. 2016). The following primary antibodies
were used: mouse anti-SYCP3 (Santa Cruz Biotechnology sc-74569, D-1) and rabbit anti-HORMAD2
(Santa Cruz Biotechnology sc-82192), and Alexa Fluor 594- or 488-conjugated secondary antibodies
against rabbit or mouse IgG, respectively (ThermoFisher Scientific). Images were acquired using either
a BX-51 upright wide-field microscope equipped with a JAI CVM4 B&W fluorescence CCD camera and
operated by the Leica Cytovision Genus software, or a Leica DM6B microscope for epifluorescence,
equipped with a DFC 9000Gt B&W fluorescence CCD camera, and operated via the Leica LASX
software. Image analysis was carried out using Fiji (ImageJ-win64). Synapsis rate was estimated by the
analysis of at least 50 meiotic nuclei. For synapsis analysis of the sex chromosomes, following
immunostaining of spermatocytes, fluorescent in situ hybridization identification of the PAR was
achieved with probes labelled with the Nick translation Kit (Abbott Molecular) as follows:
Chr X: RP23-119M14 + RP23-168A19 directly labelled with Gold dUTP (Enzo LifeScience); PAR region:
RP24-50014, directly labelled with Aqua dUTP (Enzo LifeScience); Chr Y: bMQ53i13, labelled with
digoxigenin dUTP (Sigma Aldrich), detected with sheep anti-digoxigenin (Sigma Aldrich), and anti-
sheep 633 (ThermoFisher Scientific).

**Assisted Reproduction.** In vitro fertilization was performed as previous described (Takeo and
Nakagata 2011). Briefly, sperm was dispersed in a modified Krebs-Ringer bicarbonate solution (TYH)
containing 1.0 mg/ml of polyvinyl alcohol and 0.75 mM Methyl-β-cyclodextrin (Sigma), before
transferring to fertilization drops containing cumulus masses of C57BL/6J oocytes in human tubal fluid
(HTF) medium (Millipore) supplemented with 0.25 mM reduced L-Glutathione (Sigma)and 4mg/ml
Bovine Serum Albumin. After 4 hours of incubation, presumptive zygotes were washed through drops
of HTF and incubated in vitro in KSOM media (Millipore), supplemented with 0.8 mg/ml Bovine Serum
Albumin (Takeo and Nakagata 2011). Where required, zona pellucida were removed by incubating
oocytes briefly in acidic Tyrode’s solution (Sigma Aldrich). The resulting zygotes were cultured to the
morula/blastocyst stage or were transferred at the 2-cell stage surgically into pseudopregnant CD1
recipients. Where required, IVF-derived embryos were supplemented with additional heterozygous
*B6.129S6-Gt(Rosa)26Sortm1(CAG-cas9)Wthg* embryos to allow a suitable number for embryo transfer.
Microsatellite analysis was performed by PCR using primer sets *D12Mit136* and *D12Mit14.*

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**Conflict of Interest:** The author declare no competing interests.

**Availability of materials and data:** The raw and processed sequencing files produced in this study are archived at the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number GEO: GSE180966. Analysis code is available from GitHub (https://github.com/anjali-hinch/hybrid-rescue). All data used to generate the figures is available as supplementary data tables.

**Authors’ contributions:**

BD, DB, SA and PP-H performed the animal husbandry and molecular biology. AC-S and CP performed the in vitro fertilization. DM performed the cytogenetics. GZ and PB performed the ChiP-seq. AGH and BD analysed the data. BD, AGH, SM and PD prepared the manuscript and all authors critically reviewed the manuscript.

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Figure 1 – A model of the impact of PRDM9 binding on chromosome synapsis

a) In two parental strains, A and B, different Prdm9 alleles are present, encoding PRDM9A and PRDM9B, which have distinct binding sites, with a spectrum of affinities (ranging from strong to weak). Due to the effects of mutation and recombination, these sites, preferentially the stronger sites, are frequently eroded. PRDM9 will still bind to its intact weaker binding sites and position DSBs to initiate the recombination process. PRDM9 is likely to bind at a similar level at these weaker sites at matched positions on the uncut homologue, enabling synapsis to occur and meiosis to proceed.

b) In the hybrid strain, two parental chromosomes and both PRDM9A and PRDM9B are present. The two PRDM9 variants now bind preferentially to the non-eroded stronger binding sites on the complementary chromosome, leading to a reduction in cut sites at which PRDM9 is bound to the matched position on the homologue. Chromosome synapsis is inefficient and meiosis arrests.

c) In the hybrid, the replacement of one of the Prdm9 alleles with a de novo allele, Prdm9C encoding PRDM9C whose distinct binding sites are not eroded on either parental chromosome, leads to strong binding to this non-eroded motif on both parental chromosomes, increasing the frequency of PRDM9 binding matched positions, favouring chromosome synapsis and rescuing the meiotic arrest.
Figure 2 – The introduction of de novo Prdm9 alleles partially rescues fertility across the species barrier

a) The proportion of DMC1 SSDS reads originating from matched positions on the two homologues (as defined by B6 contribution between 25-75%) for each of the alleles (SPRT: blue, B6: orange, Humanized: purple, CAST: green) present in B6SPRTF1 hybrids harbouring the wild-type Prdm9\textsuperscript{B6} allele (B6/SPRT) or the engineered Prdm9\textsuperscript{Hum} (Hum/SPRT) and Prdm9\textsuperscript{CAST} (CAST/SPRT) alleles. b) Overall hotspot attribution in these respective hybrids (bottom to top) using DMC1 ssDNA and H3K4Me3 ChIP sequencing peaks, coloured as a) and using grey for unattributed hotspots. c) Mean proportion of normal autosomal synapsis (n=4), d) testis weight (B6/SPRT, n=8; Hum/SPRT, n=10; CAST/SPRT, n=7) and e) total sperm count (B6/SPRT, n=8; Hum/SPRT, n=10; CAST/SPRT, n=7).
Hum/SPRT, n=10; CAST/SPRT, n=6) in the three hybrids and parental controls. Error bars show 1 s.d. 

The synapsis rate as determined by SYCP3/HORMAD2 staining, versus epididymal sperm count, are plotted for individual mice. PWDB6F1 and B6PWDF1 data is from (Davies, et al. 2016).

An arbitrary sperm count of 2 has been given to mice with no sperm to enable the plot. 

Morphology of representative sperm is shown for wild-type C57BL/6J mice, wild-type SPRET/EiJ mice and the humanized hybrid, B6SPRTF1.