molecular human reproduction

ORIGINAL RESEARCH

A predicted deleterious allele of the essential meiosis gene *MND1*, present in ∼ 3% of East Asians, does not disrupt reproduction in mice

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ABSTRACT: Infertility is a major health problem affecting ∼15% of couples worldwide. Except for cases involving readily detectable chromosome aberrations, confident identification of a causative genetic defect is problematic. Despite the advent of genome sequencing for diagnostic purposes, the preponderance of segregating genetic variants complicates identification of culprit genetic alleles or mutations. Many algorithms have been developed to predict the effects of 'variants of unknown significance', typically single nucleotide polymorphisms (SNPs), but these predictions are not sufficiently accurate for clinical action. As part of a project to identify population variants that impact fertility, we have been generating clustered regularly interspaced short palindromic repeats-Cas9 edited mouse models of suspect SNPs in genes that are known to be required for fertility in mice. Here, we present data on a non-synonymous (amino acid altering) SNP (rs140107488) in the meiosis gene *Mnd1*, which is predicted bioinformatically to be deleterious to protein function. We report that when modeled in mice, this allele ($MND1^{K85M}$), which is present at an allele frequency of ∼3% in East Asians, has no discernable effect upon fertility, fecundity or gametogenesis, although it may cause sex skewing of progeny from homozygous males. In sum, assuming the mouse model accurately reflects the impact of this variant in humans, rs140107488 appears to be a benign allele that can be eliminated or de-prioritized in clinical genomic analyses of infertility patients.

Key words: infertility / meiosis / mouse modeling / CRISPR / variants of unknown significance / recombination

Introduction

Meiosis begins with programmed formation of double stranded breaks (DSBs) induced by the SPO11 protein (Gray and Cohen, 2016). Processing and repair of these DSBs via recombination is crucial for proper pairing and segregation of homologous chromosomes at the first meiotic division. The recombination repair process involves several steps, including resection of the 5′ ends, loading of proteins (including the replication protein A (RPA) complex) that protect the 3′ overhangs, replacement of RPA by the RecA-related recombinases RAD51 and DMC1, then invasion of the single stranded nucleoprotein filament into the homologous chromosome locus to form recombination intermediates called D-loops. Subsequent resolution of the recombination intermediates occurs via non-crossover and crossover recombination. The recombinase loading and D-loop formation depend upon a heterodimer of the meiotic nuclear divisions 1 (MND1) and HOP2 accessory proteins (Bugreev *et al.*, 2014; Chen *et al.*, 2004; Petukhova *et al.*, 2005).

Mnd1 knockout mice of both sexes are sterile, causing an absence of oocytes and postmeiotic spermatids, respectively (Pezza *et al.*, 2014). Most mutant spermatocytes are defective in homologous chromosome synapsis and DSB repair, leading to apoptosis. Nevertheless, 23% of spermatocytes exhibited extensive or complete synapsis and underwent HOP2-dependent DSB repair, although they were deficient in crossovers (chiasmata) that are crucial for normal chromosome disjunction (and thus, aneuploidy prevention) (Pezza *et al.*, 2014).

We have been conducting a project to identify segregating infertility variants in human populations, using a strategy (Singh and Schimenti, 2015) that does not depend upon genome-wide association studies. Our approach begins with a list of genes known to be required for fertility, most of which (∼700) have been identified by mutagenesis in mice (Schimenti and Handel, 2018). Next, the genes are scanned

for the existence of non-synonymous single nucleotide polymorphisms (nsSNPs) that are predicted computationally to be deleterious to protein function. We focus on nsSNPs rather than those that cause frameshifts or premature stop codons because the latter two are clearly disruptive, whereas predicting the functional impact of nsSNPs depends upon inexact bioinformatic predictions. Third, an amino acid altered by an nsSNP must be conserved between humans and mice, thus allowing for modeling. Fourth, we select nsSNPs that are present at a substantial frequency in at least one population but not exceeding 2% since higher frequency infertility variants are unlikely to persist in populations. We typically use a range of 0.001–2%. Finally, we model the human amino acid change in mice by clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) genome editing. Our ultimate goal is to produce a database of human variants that have been functionally evaluated. We anticipate such a database to be useful for accurately diagnosing genetic causes of infertility in individual patients, and thus to better inform potential clinical interventions or genetic counseling.

Here, we report here on the creation and analysis of a mouse model containing a predicted deleterious nsSNP (rs140107488) in *MND1*. This SNP is present at an allele frequency of 1.6% in East Asians (meaning that ~3.2% are carriers) and 0.14% in South Asians (gnomAD database). We find that mice bearing this SNP are fertile, and show no signs of defective meiosis.

Materials and Methods

Mouse generation and genotyping

The DNA template for making single guide RNA (sgRNA) was generated as described via overlap PCR (Varshney *et al.*, 2015). The guide RNA sequence (all 5′ to 3′) corresponding to *Mnd1* was AGCCTC-CAACTTGCGCTTCC, embedded within the forward overlap primer GAAATTAATACGACTCACTATAGG*AGCCTCCAACTTGCGCTTCC*GT TTTAGAGCTAGAAATAGC. This was matched with a universal reverse primer: CAAAATCTCGATCTTTATCGTTCAATTTTATTCC GATCAGGCAATAGTTGAACTTTTTCACCGTGGCTCAGCCACG AAAA.

The DNA template produced by the overlap PCR was reverse-transcribed into RNA using Ambion MEGAshortscript T7 Transcription Kit (cat#AM1354), and purified using Qiagen MinElute columns (cat#28004). For pronuclear injection, the sgRNA (50 ng/μl), single stranded homologous recombination template (ssODN; 50 ng/μl, IDT Ultramer Service) and Cas9 mRNA (25 ng/μl, TriLink) were co-injected into zygotes (F1 hybrids between strains FVB/NJ and B6(Cg)-Tyr^c-2J/J) then transferred into the oviducts of pseudopregnant females. The ssODN sequence was as follows: AAAGCA CATCTCTTCATAAGGCGGTGACTTACCTGAGAGTTCAGGGCC TCTAACATGCGCTTCCTTGCATGAAGAGCCTTGCTCGGAAAA GCCCAATAGTAATTGGACGTCCCGA. Any founders potentially carrying a copy of the desired alteration were identified by PCR using primers flanking the targeting site, followed by Sanger sequencing. This typically generates a chromatogram reflecting mosaicism in founders, so each founder mouse with an apparent desired allele was mated to a wild-type FVB/NJ mouse, and the offspring genotyped for inheritance of the desired clean allele. One such F1 animal was used to establish the colony of *Mnd1^K85M* mice used in this study.

Crude lysates for PCR were made from small tissue biopsies (tail, toe or ear punches), as described (Truett *et al.*, 2000). Genotyping primers were as follows: 5′-GCGTTGAGCCCAAATAAGAA-3′ and 5′-GTGGATGACGGTATGGTTGA-3′. Cycling conditions were initial denaturation at 95° for 5 min, then 30 cycles of 95° for 30 s, 58° for 30 s, 72° for 30 s, and final elongation at 72° for 5 min. For identification of *Mnd1* mutants, the 233 bp amplimer was digested with *Hae*III, and run on an agarose gel. The mutant, but not (WT) product was cleaved into two fragments of 133 and 100 bp.

All animal use was conducted under protocol (2004–0038) to J.C.S. and approved by Cornell University's Institutional Animal Use and Care Committee.

Immunocytochemistry of spermatocyte chromosomes

This was performed essentially as described previously, but summarized as follows (McNairn *et al.*, 2017). To prepare surface spreads, testes were isolated from 8–12-week-old males, detunicated and minced in minimum essential medium. Spermatocytes were hypotonically swollen in 4.5% sucrose solution, lysed in 0.1% Triton X-100, 0.02% sodium dodecyl sulphate and 2% formalin. Slides were blocked for 1 h at room temperature in 5% goat serum in PBS, 0.1% Tween20. Primary antibodies and dilutions used were anti-SYCP3 (1:600, Abcam, Cambridge, MA, USA #ab15093), anti-DMC1 (1:100, Abcam, #ab11054) and anti-MLH1 (1:100, BD Pharmingen, Franklin Lakes, NJ, USA #554073). Immunolabeling was carried out overnight at 4°C. Secondary antibodies used were goat anti-mouse IgG AlexaFluor 488 (1:1000, ThermoFisher Scientific, Waltham, MA, USA #R37120) and goat anti-rabbit IgG AlexaFluor 594 (1:800, ThermoFisher Scientific, Waltham, MA, USA #R37117). Secondary antibodies were incubated for 1 h at room temperature. Foci were quantified using ImageJ with plugins Cell Counter (Kurt De Vos) and Nucleus Counter.

Sperm counts and histology

For sperm counts, cauda epididymides (both sides) were collected from 8-week-old males, minced in PBS, then incubated at 37°C for 10 min to allow sperm to swim out. Sperm solutions were diluted and counted on a hemocytometer.

For histological sections, testes were fixed in Bouin's for 24 h, washed in 70% ethanol for 24 h, then embedded in paraffin. Sections were made at 6 μM thickness. Slides were stained with hematoxylin and eosin (H&E).

Statistical analysis

All plotting of data and Student's *t*-test calculations were performed using Prism 7 (GraphPad Software, San Diego, CA, USA.).

Results

Selection of the *MND1^K85M*, encoded by SNP variant rs140107488, as a potential infertility allele

In our ongoing project to identify and functionally validate segregating infertility alleles in human populations by mouse modeling, we select

candidate nsSNPs in genes that are known to be required for fertility in mice, and which are computationally predicted to be pathogenic. rs140107488 encodes a lysine-to-methionine change at amino acid (AA) position 85 of human and mouse MND1. The human and mouse proteins are both 205 amino acids in length, being 90% identical and 94% similar. The SIFT, PolyPhen-2 and Mutation Assessor algorithms, which predict deleteriousness of an nsSNP based on physico-chemical properties of the substituted AA, potential structural impact and evolutionary conservation (Adzhubei *et al.*, 2013; Kumar *et al.*, 2009; Reva *et al.*, 2007), all predict this variant to be highly deleterious to protein function. The SIFT, PolyPhen-2 and Mutation Assessor algorithm scores for rs140107488 are, respectively, 0 (the 'worst' possible), 0.963 ('probably damaging') and 0.933 ('high' likelihood of being damaging). Given these predictions, in conjunction with the knowledge that MND1 is essential for meiosis and exists as a significant minor allele in East Asians, we selected it to model in mice and assess the consequences *in vivo*.

Mnd1[K85M/K85M] mice are fertile and produce normal numbers of sperm

To generate mice modeling the human *MND1*[K85M] allele, CRISPR/Cas9-mediated genome editing in single-celled zygotes was performed. An ssODN was used as a repair template to introduce a nucleotide change into codon 85 via homologous recombination, causing this codon to encode methionine instead of lysine (Fig. 1A). Other silent changes were also introduced to facilitate genotyping (creation of a novel restriction enzyme site) and to reduce deletion mutations (mutation of the protospacer adjacent motif (PAM) site required for Cas9:sgRNA recognition) (Singh *et al.*, 2015). A founder mouse transmitting the correct mutation was identified (Fig. 1B), the allele was backcrossed into FVB/NJ for at least two generations, then intercrossed to generate homozygous mice for phenotypic analysis.

Homozygous mutant mice were normal in gross appearance, consistent with the lack of somatic phenotypes reported for null mice (Pezza *et al.*, 2014). To determine if the *Mnd1*[K85M] allele impacts fertility, homozygotes and wild-type (WT; +/+ or heterozygotes from the same genetic background) adults between 2 and 7 months of age were bred to WT mates, and litter sizes were recorded. The average litter sizes produced by *Mnd1*[K85M/K85M] males and females (Fig. 2A) were 8.2 ($n = 123$ pups) and 8.6 ($n = 95$ pups), respectively, compared to 8.3 for WT intercrosses ($n = 58$ pups). Thus, fecundity of mutants was normal ($P = 0.78$ and 0.93 for females and males, respectively by Student's *t*-test). Interestingly, however, male (but not female) homozygotes produced more male (82) than female (41) offspring ($P = 0.000073$ by Student's *t*-test), a 2:1 ratio (Fig. 2B). The basis for this is unknown.
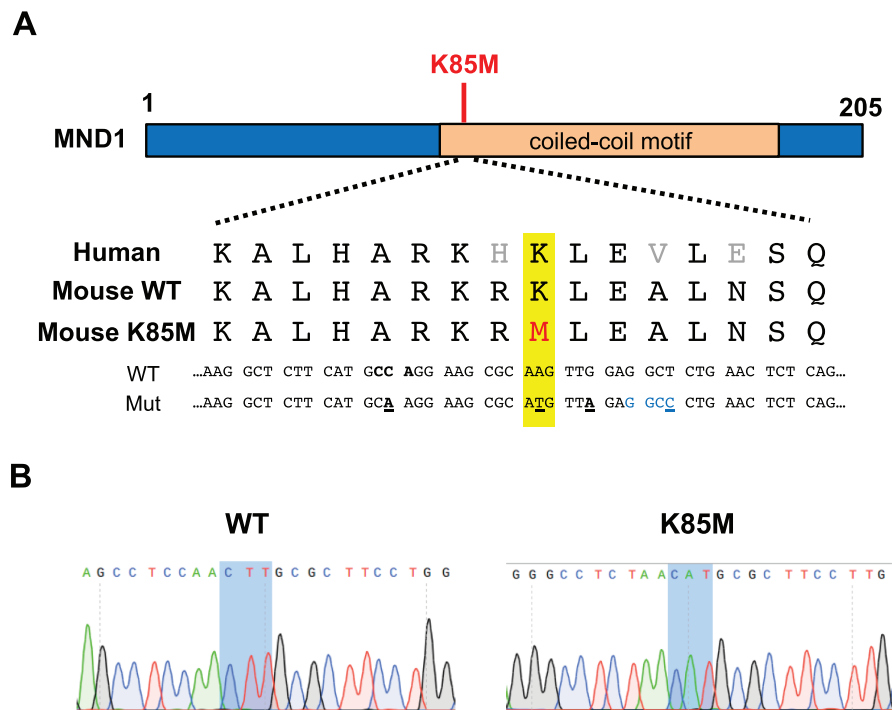
Figure 1 CRISPR/Cas9 genome editing strategy to generate mice *Mnd1*[K85M]. (**A**) Schematic of the human meiotic nuclear divisions 1 (MND1) protein and amino acid and nucleotide sequences surrounding the target single nucleotide polymorphism (SNP) in the human and mouse genomes. Point changes introduced by the single stranded homologous recombination (ssODN/HR) template are indicated as follows: yellow highlighted area contains the altered amino acid (M) in red, and the nucleotide that was mutated text/(underlined) to cause the amino acid change that models the human SNP variant; black text/underline = silent nucleotide changes to prevent Cas9 re-cleavage, including the PAM site (also bolded); blue text = *Hae*III restriction enzyme site used for genotyping. K85M, MND1[K85M]. (**B**) Chromatograms of wild-type (WT) and *Mnd1*[K85M/K85M] mice (sequence is reverse complement of the sequences above).
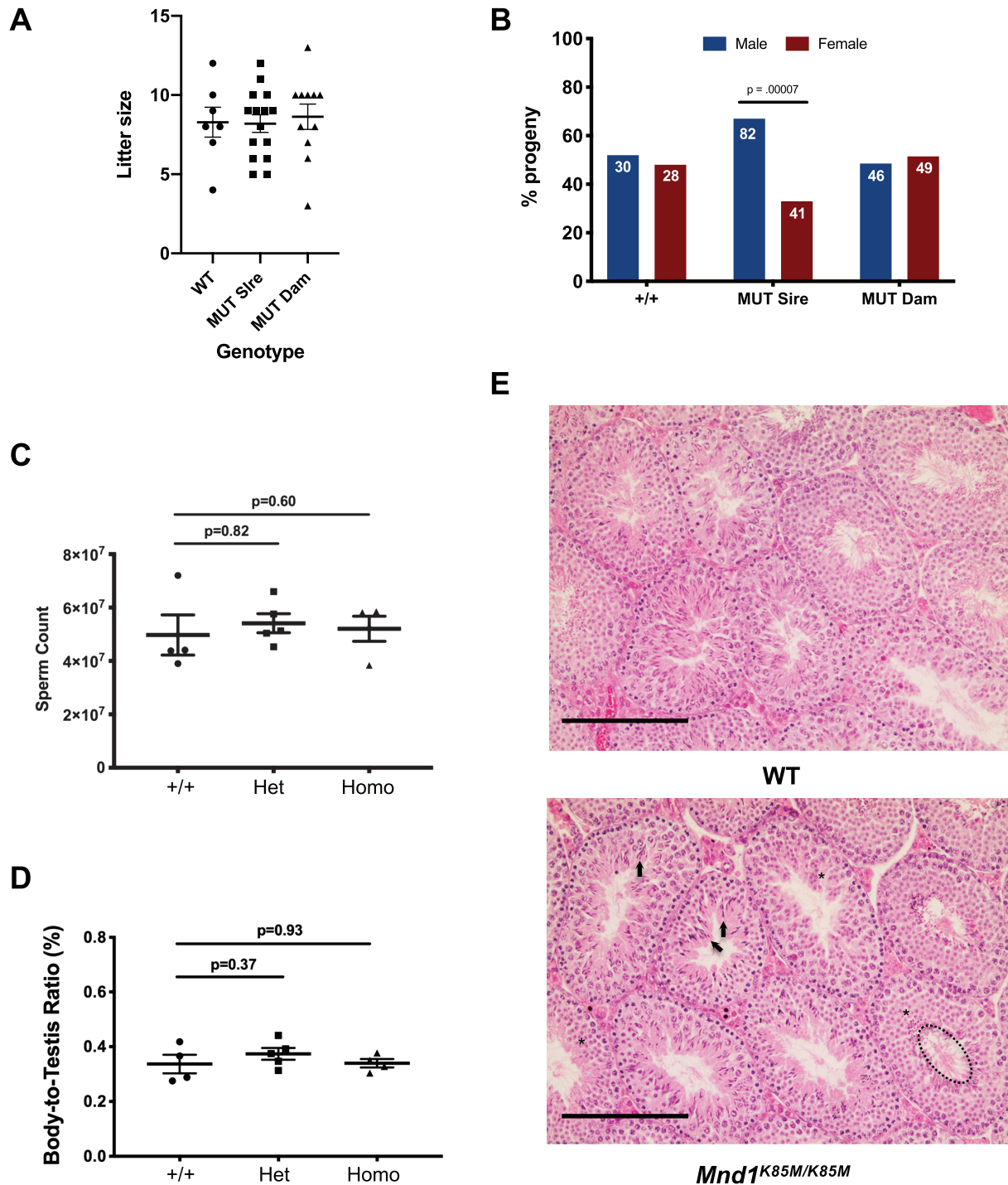
Figure 2 $Mnd1^{K85M/K85M}$ **males have normal sperm counts and spermatogenesis, but exhibit sex distortion.** (**A**) Litter sizes from matings of indicated genotypes of males or females to WT mates. WT mice were intercrossed, so no sex is indicated. MUT = $Mnd1^{K85M/K85M}$. Each point represents a litter (males and females combined), and horizontal bars mark the average and SEM. (**B**) Progeny separated by sex of WT and $Mnd1^{K85M/K85M}$ (MUT) mice mated to WT animals. Note that mutant males (sires) produce a 2:1 ratio of male:female offspring. The P value is from Student's t-test. (**C**) Total sperm quantified from both epididymides of each animal. $N = 3–5$ per genotype. P values are from Student's t-test. Het: heterozygous, Homo: homozygous. (**D**) Relative body:testis masses. $N = 4–5$ per genotype. P values are from Student's t-test. (**E**) Testis histology. Hematoxylin and eosin stained testis cross-sections of 8-week-old WT and mutant males. Highlighted in selected seminiferous tubules of the mutant sample are normal examples of elongated spermatids (arrows), round spermatids (asterisks) and flagellated, spermiated spermatozoa (dashed ellipse in lower right tubule). Size bar = 75 µM.

To determine if there were potential subclinical effects in sperm production or spermatogenesis in mutants, we performed gross and histological analyses of testes and sperm. Eight-week-old *Mnd1*[K85M/K85M] males had testis weights and epididymal sperm numbers that were similar to WT (Fig. 2C and D). Consistent with these observations, testis histological cross-sections appeared normal in the mutant; all spermatogenic stages were present, and no notable pathologies or abnormal cellular phenotypes were observed (Fig. 2E).

Meiosis and recombination appears to be normal in *Mnd1*[K85M/K85M] mice

MND1 is an important binding partner of HOP2, and this complex stabilizes DMC1 and RAD51 binding to ssDNA to promote strand invasion into, and recombination with, the homologous chromosome (Tourtellotte *et al.*, 1999). To assess if MND1[K85M] affects DMC1 recruitment and displacement, spermatocyte chromosome surface spreads were immunolabeled with DMC1 and the synaptonemal complex axial element protein SYCP3 (Fig. 3A). In response to meiotically programmed DSBs, DMC1 loading was observed in foci that were present at normal levels (compared to WT; ~200 foci/nucleus) in leptotene spermatocytes (cells in which homologous chromosome pairing and synapsis has yet to occur). Focus numbers also disappeared normally as meiotic prophase I progressed into pachynema (when chromosome synapsis is complete), indicating that homologous recombination-mediated DSB repair was occurring without defects in the mutant (Fig. 3A). Unlike *Mnd1* null spermatocytes, we did not observe defects in chromosome synapsis (Pezza *et al.*, 2014). To assess
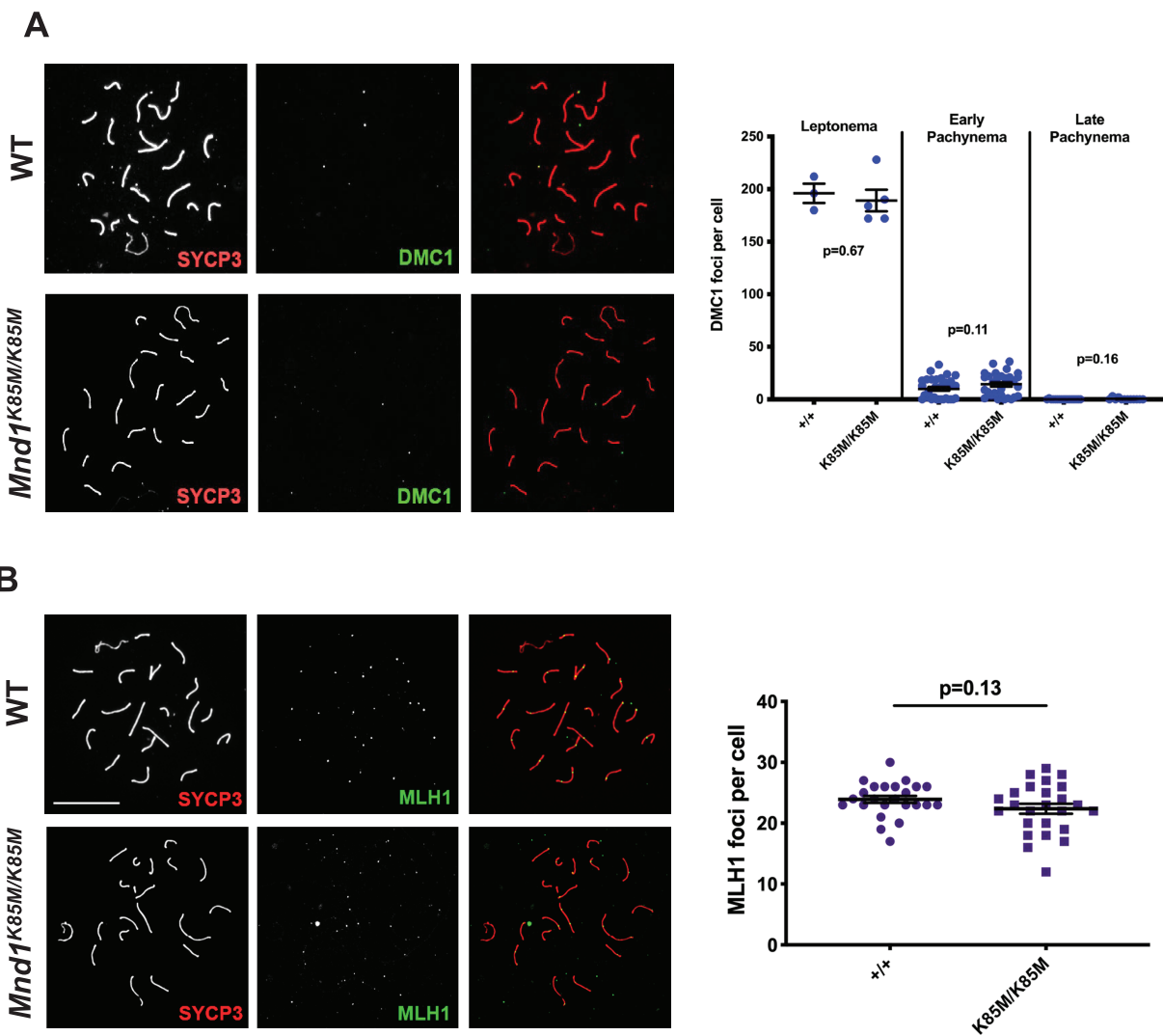
Figure 3 Cytological data indicating that *Mnd1*[K85M/K85M] is not defective in meiotic recombination. (**A**) Late pachytene spermatocyte chromosome surface spreads immunolabeled with synaptonemal complex axial element protein (SYCP3) and DMC1 (merged in rightmost subpanels). To the right of each panel are plots of DMC1 focus counts at indicated substages. $N = 2$ per genotype. *P* values are from Student's *t*-test. (**B**) Spreads immunolabeled with MLH1 (a chiasmata marker protein) and SYCP3 (merged in rightmost subpanels). Size bar in WT panel of B = 20 μm, and applies to all panels. To the right of each panel are plots of the MLH1 focus counts at indicated substages. $N = 3$ per genotype. DMC1: dosage suppressor of mck1 homolog.

crossing-over, late pachytene spreads were immunolabeled with the chiasmata marker MLH1. In contrast to null spermatocytes that have decreased crossing-over in surviving spermatocytes (Pezza *et al.*, 2014), there was no significant difference in MLH1 focus numbers between WT and *Mnd1*$^{K85M/K85M}$ ($P = 0.13$) (Fig. 3B), indicating a normal level of crossing over. Aberrations in crossover number can lead to aneuploidy and death of offspring, typically resulting in reduced litter sizes, which is not the case with *Mnd1*^{K85M} mutants, as indicated above.

Discussion

In summary, our studies indicate that *MND1*^{K85M} is a benign allele that has no discernable effect upon fertility. Homozygous mutants of both sexes were normal in both reproductive and molecular phenotypes. By eliminating this as a candidate infertility allele, these finding may be useful in prioritizing candidate genetic variants that may underlie reproductive infertility in people bearing this SNP. While it is conceivable that the allele is pathogenic in humans but not mice, the consequences are likely to be subtle or subclinical, especially since the allele is present at a relatively high frequency in East Asians. One remaining question is the unusual sex distortion ratio exhibited by allele transmission from homozygous males. At present, it is unclear whether this is a consequence of the mutant allele itself, a statistical rarity, or perhaps an unrelated closely linked allele. Further studies of this phenomenon are under way.

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Authors' roles

T.T. conducted most of the experiments, wrote a draft of the text and figures and supervised J.M., who contributed to the genotyping and immunohistochemistry analyses. J.S. supervised the work and the aforementioned students, and prepared the manuscript.

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Conflict of interest

None declared.

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