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A quantitative assay for crossover and noncrossover molecular events at individual recombination hotspots in both male and female gametes

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ABSTRACT

Meiotic recombination is a fundamental process in all eukaryotes. Among organisms in which recombination initiates prior to synapsis, recombination preferentially occurs in short 1-to 2-kb regions, known as recombination hotspots. Among mammals, genotyping sperm DNA has provided a means of monitoring recombination events at specific hotspots in male meiosis. To complement these current techniques, we developed an assay for amplifying all copies of a hotspot from the DNA of male and female germ cells, cloning the products into *Escherichia coli,* and SNP genotyping the resulting colonies using fluorescence technology. This approach examines the molecular details of crossover and noncrossover events of individual meioses directly at active hotspots while retaining the simplicity of using pooled DNA. Using this technique, we analyzed recombination events at the *Hlx1* hotspot located on mouse chromosome 1, finding that the results agree well with a prior genetic characterization of 3026 male and 3002 female meioses.

The location and intensity of meiotic recombination events provide the substrate for evolutionary selection and underlie linkage gene mapping and population genetics. Recombination begins during meiosis I with a double-strand break (DSB) that is eventually repaired to produce either a crossover, with the exchange of flanking parental DNA sequences across the site of DSB, or a noncrossover, in which the localized region surrounding the DSB acquires the DNA sequence of its partner chromatid without an exchange of flanking parental sequences (for a review of recombination pathways see Neale and Keeney [1]). In organisms such as yeast, higher plants, and mammals, in which meiotic recombination initiates prior to synapsis, both outcomes of recombination are concentrated at preferred 1-to 2-kb regions, known as recombination hotspots [2]. These hotspots account for the majority of the crossover events and are surrounded by long regions with diminished crossover activity. Although all recombination hotspots are similar in length, their recombining activity can differ appreciably between the sexes and vary by several orders of magnitude [3]. Currently, although the protein components of the recombination machinery have been extensively characterized in yeast [1], we know relatively little about the factors controlling DSB locations and their recombination frequencies, particularly in mammalian systems. For a review of mammalian meiotic recombination hotspots, see Arnheim et al. [4]. Accordingly, the ability to monitor mammalian crossover and noncrossover events at the molecular level is a matter of considerable importance.

Characterizing the behavior of individual hotspots by their genetic outcomes requires substantial numbers of progeny, something that has been difficult for our own species and is limited among experimental mammals by the costs of generating and typing large genetic crosses. To overcome this limitation, sperm genotyping has become the technique of choice as each individual sperm represents the haploid product of a single meiotic event.

Sperm genotyping assays rely on PCR amplification from either single-sperm DNA or pooled-sperm DNA. In the single-sperm assay first developed by Li et al. [5], individual sperm are separated, lysed, and subjected to an initial round of whole-genome amplification followed by a round of allele-specific amplification to enrich crossover molecules, which are then genotyped by gel electrophoresis. An adaptation by Cullen et al. [6] added one extra PCR amplification step with radioactive labeling to analyze recombination activity among ~ 21,000 individual sperm. In single-sperm assays, recombination events over large distances (> 10kb) can be examined, but studying noncrossover events is not feasible [7].

To overcome the need for examining large numbers of single sperm and allow for finer mapping of hotspots, pooled-sperm DNA genotyping was developed [8]. In pooled-sperm analysis, the number of sperm examined is adjusted such that on average each aliquot is likely to contain a single crossover molecule. Positive detection of recombinant molecules by PCR using allele-specific oligonucleotides, followed by dot-blot hybridization, provides a quantitative estimate of

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the recombination rate at a chosen hotspot, and the crossover molecules obtained can be sequenced to provide molecular details of the recombination process. This technique was used to characterize several human recombination hotspots, including TAP2 [9], the MHC region [10], and the pseudoautosomal pairing region [11]. Carrington and Cullen [12] have provided a comprehensive review of sperm genotyping techniques.

Although pooled-sperm samples overcome the need for single-sperm preparations and have been widely applied with considerable success, they do entail some limitations. The multistep DNA enrichment process requires allele-specific probes, and radioactive hybridization probes are typically required for genotyping [8]. Moreover, there are an appreciable number of aliquots containing multiple crossover molecules. When sperm pools contain an average of 0.3 crossover molecule each, as the procedure is commonly carried out, the Poisson distribution indicates that 74% of samples will not contain a crossover molecule, 22.2% of samples will contain one crossover molecule and 3.7% of the samples will contain two or more crossover molecules, resulting in a positive PCR amplification for 25.9% of the samples. However, 14.2% of these positive samples contain amplified products derived from multiple recombinant clones [8]. The genotyping and/or sequencing of these samples cannot definitively reveal the molecular details of the individual recombinants, and these samples must be recognized and removed from the analysis. Reducing the probability of amplifying multiple recombinants to less than 5% requires analyzing pooled-sperm DNA samples with an average of only 0.1 recombinant molecule, increasing the amount of necessary work.

As the term "sperm genotyping" implies, both single-and pooled-sperm genotyping are limited to detecting male recombination events. It is now known that the location and frequency of recombination events are substantially influenced by sex at both the hotspot and the regional levels [13,14]. An adaptation of the pooled-sperm genotyping approach for female oocytes has been described [15]; however, this technique results in a mixture of primordial germ

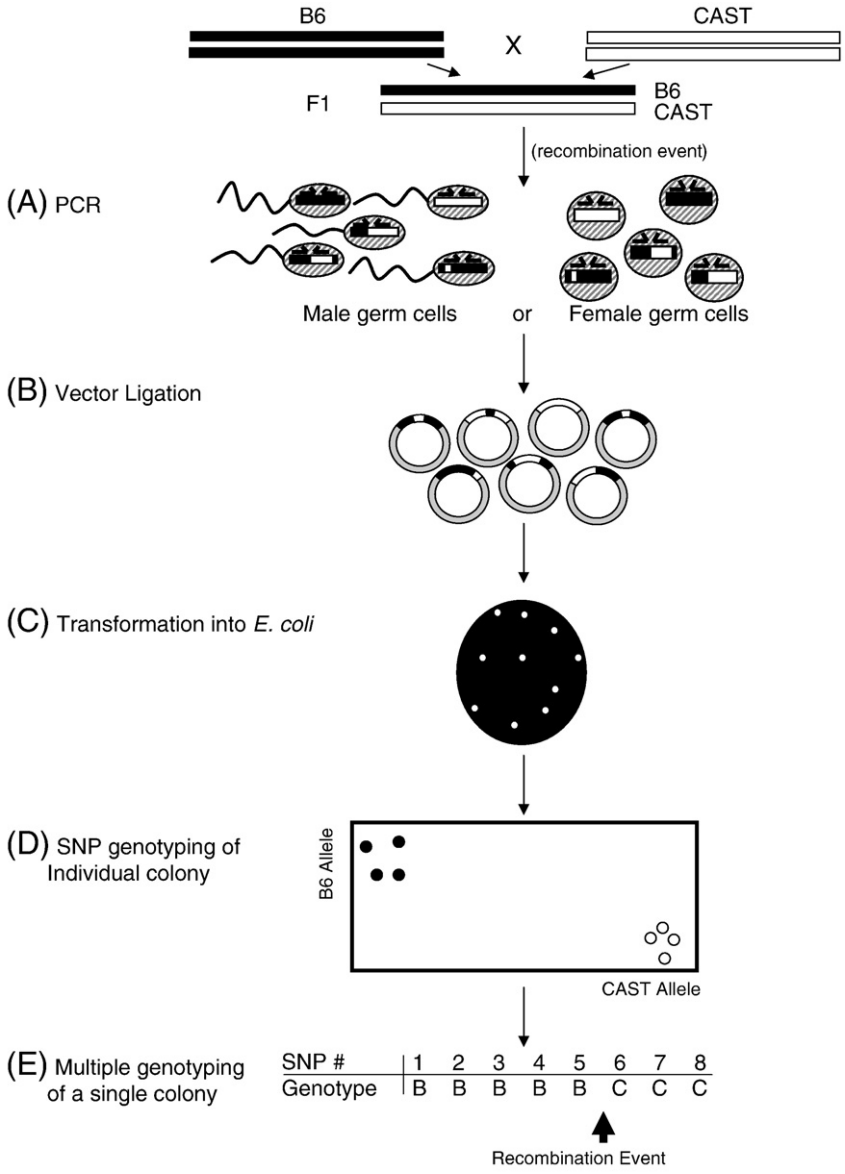

Fig. 1. A diagram representation of the *E. coli* cloning assay for monitoring recombination events in gametic cells. (A) Common primers are designed in the region flanking the recombination hotspot of interest to amplify all recombinant and nonrecombinant DNA from either male or female pooled-DNA samples. (B) The subsequent pool of DNA is ligated into a vector and (C) individual clones are recovered from *E. coli*. (D) The recovered clones are genotyped using fluorescence at polymorphic sites within the hotspot to reconstruct the recombination events. (E) Detection of recombination events from multiple SNP genotyping of a single *E. coli* colony.

cells and somatic cells and requires the determination of the germ cell proportion by immunofluorescence.

Pooled-sperm genotyping can test for the presence of crossovers among upward of 100,000 sperm at a time and can detect recombination activity at less than 0.01cM. However, an important experimental challenge is to understand hotspots of higher activity, as they account for the majority of mammalian recombination. In a recent analysis of approximately 1400 recombination events across a 24.7-Mb region of mouse chromosome 1, 15 hotspots accounted for almost 50% of all crossovers [16]. The recombination rate at each of these hotspots was greater than 0.4cM and hotspots whose activity was below 0.1cM accounted for only a small fraction of all recombination events. It is likely that the majority of recombination events in the mouse genome, and probably in the human genome, are concentrated in hotspots with high activity.

Here we present a simple assay for monitoring the molecular details of both crossover and noncrossover outcomes of individual meioses at these more active hotspots that is applicable to both male and female gametes. It is based on an *Escherichia coli* cloning strategy that combines the specificity of PCR amplification, DNA cloning, and single-nucleotide polymorphism (SNP) genotyping to examine gametic cells in mammalian species with a very low incidence of duplicate clones. In this way, it provides both the advantages of pooled-DNA samples and the sensitivity of assaying the details from individual meioses. Additionally, this technique is readily adapted to high-throughput analyses of crossover and noncrossover events in a mammalian genome.

The potential of this assay is illustrated by its application to a hotspot named *Hlx1* located on mouse chromosome 1 and a comparison of the results with those obtained by characterizing this hotspot from a large-scale genetic cross.

Results

Recovery of individual haplotypes from pooled DNA

In our genotyping assay, DNA extracted from either male or female germ cells served as the starting material. Beginning with pooled DNA samples, both recombinant and nonrecombinant copies of the targeted hotspot were PCR amplified using primers common to both parental haplotypes (Fig. 1A). To recover the products of meioses from the PCR, the amplified fragments were cloned, ligated, and transformed into *E. coli* such that each colony would represent a single DNA strand from the initial meiotic event (Figs. 1B and 1C). SNP genotyping was carried out directly on an aliquot of *E. coli* cultures grown from each colony in 96-well plates; prior plasmid purification proved unnecessary (Fig. 1D). The result is a simple PCR and cloning procedure that can be applied to either male or female germ cells.

Hlx1 hotspot

As a proof of concept for the *E. coli* genotyping technique, the results of applying this assay to the hotspot *Hlx1* were compared with the genetic data obtained from 3026 male and 3002 female meioses occurring in B6 × CAST F1 hybrids [16]. Hotspot *Hlx1* is located at 186.318Mb (NCBI build 36) on chromosome 1. A 2.7-kb fragment was amplified and cloned and eight internal polymorphic sites were optimized for the Amplifluor genotyping system.

Control library and proper PCR amplification conditions

For all gametic assay procedures, PCR amplification is an essential step for obtaining sufficient DNA material for subsequent analysis. The importance of appropriate PCR conditions during this step cannot be overemphasized as improper PCR conditions can generate artifactual recombinant molecules, known as jump-PCR products, as a result of template switching during successive rounds of amplification [3,17]. Using the generally accepted PCR condition of 1min per kilobase extension, “recombinant molecules” were detected in a 50:50 mixture of B6 and CAST sperm, where they should not be present. The sequence of the *Hlx1* hotspot contains a putative hairpin structure that may stall the Taq polymerase during elongation. The truncated product can then serve as the primer on a different allelic template in a subsequent round of amplification to generate artifactual recombinant molecules. To prevent template switching, we added dimethyl sulfoxide (DMSO) to reduce the secondary structures in the DNA template, increased the elongation time to minimize the production of truncated molecules, and shortened the annealing time to favor priming by added oligos. These steps prevented the generation of jump-PCR products. A control library prepared from a 50:50 mixture of B6 and CAST sperm provided equal frequencies of parental genotypes (221 B6:229 CAST), indicating unbiased amplification, and no crossover or noncrossover recombinants were detected in 450 clones. This validated the specificity of the PCR conditions for a recombination frequency of at least 0.2cM. For hotspots with lower

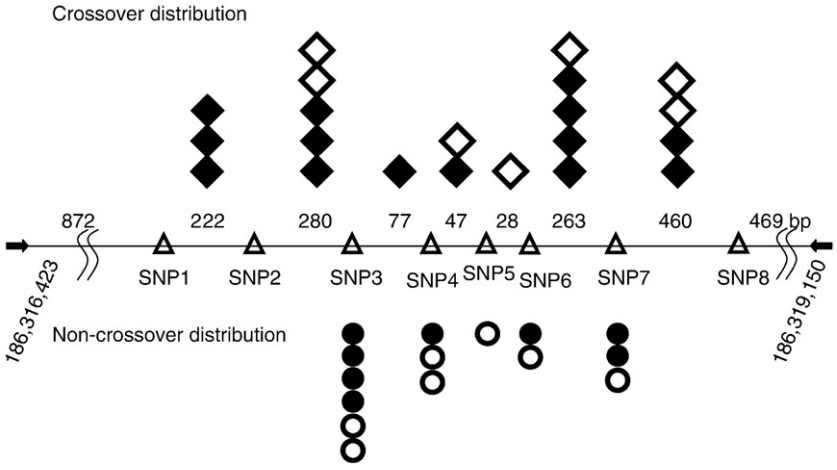

Fig. 2. The number of recombinant genotypes detected in the *Mus musculus Hlx1* hotspot in B6×CAST F1 hybrids. The horizontal line indicates the amplified PCR fragment (flanking primers indicated as arrows). Triangles represent the locations of the allelic SNPs between B6 and CAST, with the numbers between them denoting distances in base pairs. Recombinant molecules found in sperm are indicated in black and recombinant molecules from oocytes are in white. Diamonds represent crossover events, while circles depict conversion events.

Table 1
Comparison of the male and female crossover rates detected in the genetic cross and by the *E. coli* genotyping assay

	Genetic cross	*E. coli* genotyping assay
Males	91/3026 (3.0±0.3%)	14/500 (2.8±0.7%)
Females	16/3002 (0.5±0.1%)	7/830 (0.8±0.3%)

The recombination frequency detected in our *E. coli* genotyping assay is similar to that obtained from the genetic cross with a higher crossover activity in males than in females.

crossover activity, corresponding estimates of maximum detection sensitivity can be obtained by genotyping more control colonies.

Recombination activity of the Hlx1 hotspot

For the *Hlx1* hotspot, we analyzed a total of 500 clones obtained from sperm of F1 males and 830 clones from F1 female primordial follicles. In males, 14 crossovers and 8 noncrossovers were detected, while in females, 7 crossovers and 7 noncrossovers clones were obtained (Fig. 2A). We further validated our technique by sequencing the plasmids from 10 crossover and 7 noncrossover colonies and verified their genotypes.

The frequencies and distribution of crossing over for both males and females were similar to those of our genetic cross [16], quantitatively validating the *E. coli* assay and confirming that the recombination activity at this hotspot is influenced by sex, with crossover activity appreciably higher in males than in females (Table 1).

Statistical analysis of unique recombinant molecules

Cloning from a large pooled sperm sample that has been amplified carries with it the possibility that multiple *E. coli* clones were derived from the same initial recombinant molecule. However, application of the Poisson distribution indicates that this is highly unlikely, which is illustrated by an analysis of the male data for hotspot *Hlx1* (14 crossovers in 500 clones). We began with 11,000 genomic haploid equivalents (2.7pg per haploid mouse genome in 30ng of pooled sperm DNA). Each haploid DNA originates from a single sperm cell and represents a unique meiotic event. PCR amplification of the sperm DNA is unbiased (as shown by our control DNA sample) and each haploid DNA is amplified ~ 1×10^9-fold. The probability that the product of a given sperm is represented among the 500 selected clones is 500/11,000 = 0.045.

The probability that any 2 clones are duplicates arising from the same initial meiotic event can be estimated using the Poisson distribution $P(n) = (e^{-m} m^n)/n!$, where $n = 2$ and m is the likelihood that a given original sequence is represented in the aliquot (0.045). The probability of selecting 2 of the same molecules is then $P(2) = (e^{-0.045} \times 0.045^2)/2!$ or 0.00097. For males, we detected 14 crossovers in 500 clones, and the likelihood that 2 of these are derived from the same meiosis is $1 - p(\text{nonduplicates})^{13}$ or $1 - (1 - 0.00097)^{13} = 1.25\%$. That is, there is only a 1.25% probability that a set of 14 clones will contain a pair of clones duplicated from same initial molecule. This probability is further reduced by the fact that most recombinant molecules are readily distinguished by having distinct recombination sites. As such, recombinant molecules with different exchange points cannot be duplicate clones of each other. This further reduces the likelihood of analyzing two clones from the same initial molecule. For example, applying similar calculations to the 4 crossover clones with breakpoints between SNP6 and SNP7, the probability that 2 of these are duplicate clones is only 0.3%, and they cannot be duplicates of other recombinant clones. Thus, the probability of examining multiple representatives of the same initial recombinant molecule is minimal and can be further reduced by starting with a larger pool of sperm DNA.

Molecular details of crossover and noncrossover events

Examining the molecular details of the recombination products at the *Hlx1* loci, we found that males and females exhibited a similar distribution of crossover and noncrossover breakpoints (Fig. 2; the molecular details of each recombination event are in Supplementary Table 1). In both sexes, sites of noncrossover, detected by SNP marker conversions, were clustered closer than the sites of crossover exchange (Fig. 3). This tighter distribution of noncrossover events is similar to those previously observed and conforms to the current recombination model in which crossover and noncrossover recombination are processed by two different pathways [18].

The length of a mammalian noncrossover conversion tract can vary from 70 to 300bp [18]. For *Hlx1*, no noncrossover conversions spanning two SNP markers were found; thus the longest conversion involving a single marker is the distance between its two flanking markers. On this basis, 75% of the noncrossover conversion tracts could have spanned 300bp or longer, but some were definitely shorter. The three noncrossover recombinants at SNP4 must be less than 125bp (spanning from SNP3 to SNP5) and the single noncrossover molecule at SNP5 is less than 77bp (spanning from SNP4 to SNP6). In one recombinant molecule, both a conversion tract and a crossover were detected from a single meiotic event (data not shown). This molecule is likely to be a crossover-associated conversion tract as a result of the mismatch repair following resolution of the Holliday junction. The true number of short noncrossover recombinants and conversions

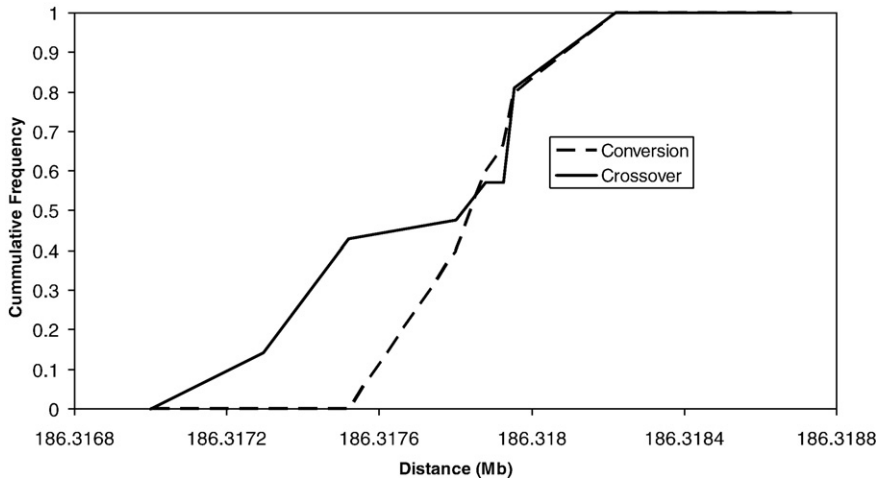

Fig. 3. The cumulative frequencies of crossover and noncrossover breakpoints. The crossovers are spread across 923 bp, while the conversion breakpoints are clustered within a 419-bp interval.

associated with a crossover is likely higher than what was observed as the detection of these short events is limited by the number and placement of informative markers between the parental strains.

Discussion

Sperm and oocytes contain haploid genomes derived from individual meiotic events. While sperm genotyping has been invaluable in monitoring male meiosis, adapting the technique to female meiosis has been less efficient, in part because of the difficulty of obtaining pure suspensions of primordial oocytes. We have devised a method to analyze both male and female recombination events in a mammalian system effectively by PCR amplification of the hotspot fragments from pooled DNA and recovery of individual recombinant molecules by cloning. In the case of females, the starting material was pure female primordial follicle preparations obtained using the simple technique of Eppig et al. [19]. We can now use pooled DNA from either male or female germ cells to map individual recombination events directly.

The cloning approach outlined here considerably facilitates the identification and characterization of noncrossover recombinants, as both crossover and noncrossover molecules are amplified, cloned, and SNP genotyped. In part, the power of the new approach also lies in the ease of genotyping it provides. Hotspot sequences from the initial sperm DNA are amplified and separated in high concentration as a result of the cloning step. SNP discrimination is robust and simplified, as each colony carries only one parental or recombinant allele, with no heterozygosity, and the cloned hotspot sequence is> 1000 times its concentration compared to bulk mammalian genomic DNA. SNPs located in repetitive elements that were difficult to analyze in genomic DNA were easily typed using *E. coli* colony DNA. The fluorescent SNP genotyping system is an additional improvement over traditional sperm genotyping because it avoids radioactive labeling and gel electrophoresis.

The cloning assay also considerably reduces the complication of amplifying and genotyping multiple recombinant molecules in the same DNA sample. Each *E. coli* clone corresponds to a single meiotic product and complex recombinant clones are easily genotyped. The possibility of assaying duplicate recombinants is very small and can be reduced even further using a larger pool of DNA in the initial sample. In the initial PCR amplification, the use of common primers is nondiscriminatory, capturing all recombinant (crossover and noncrossover molecules) as well as nonrecombinant genotypes within the targeted region. However, if desired, crossover recombinant molecules can be selectively amplified by using allele-specific primers (e.g., a B6-specific primer together with a CAST-specific primer) for PCR amplification. Cloning of this DNA fraction would be considerably enriched for fragments representing crossover meiotic events in the targeted hotspot. In addition, this technique can be easily automated using a colony picker and a liquid dispenser to scale the *E. coli* colony picking and genotyping into a high-throughput process for analyzing very large numbers of recombinant molecules, even at low-frequency recombination hotspots.

In summary, our technique extends and complements the current pooled-sperm genotyping procedures for investigating the molecular details of highly active mammalian recombination hotspots. Using this new approach, we have successfully mapped and characterized both male and female recombination events at the mouse *Hlx1* recombination hotspot. Although developed for mammals, the new technique should be applicable to a variety of organisms.

Materials and methods

Germ cell isolation

Live sperm were recovered from dissected vas deferens of 20-week-old males of the C57BL6/J (B6) and CAST/EiJ (CAST) mouse strains (The Jackson Laboratory, Bar Harbor, ME, USA) and their F1 hybrids. Dissected vas deferens were placed in PBS and sperm were extracted by squeezing the tissue. Incubation at 37°C for 10min allows any remaining live sperm to swim out of the vas deferens. Sperm samples were centrifuged at 9000*g* for 5min and resuspended in 100 μl of PBS with bovine serum albumin (BSA) (9.9mg/ml).

Female primordial follicles were purified from ovaries of B6 × CAST F1 animals using the method of Eppig et al. [19]. Briefly, four to six ovaries were dissected from 2-to 3-day-old female pups and placed in PBS with BSA (1mg/ml). The ovarian bursa was disrupted with a 30-gauge needle and a single-cell suspension was obtained with agitation of the ovaries incubated at 37°C in 3.0ml of digestion buffer (PBS with BSA, 0.05% trypsin, 0.53mM EDTA, and 0.02% DNase). Female germ cells were isolated by overnight incubation at 37°C; under these conditions somatic cells adhere to the culture dish while female germ cells remain unattached. To increase their purity, a second around of overnight incubation was done, followed by isolation of free-floating germ cells (~ 99% purity; personal communication; J. Eppig; August 2007, Bar Harbor, ME).

DNA extraction

DNA was extracted similarly from male sperm and female germ cells using the DNeasy DNA extraction kit (Qiagen, Valencia, CA, USA; Supplementary Protocol-DY03). The cells were incubated overnight at 55°C in Lysis Buffer X2 (20mM Tris-Cl, pH 8.0, 20mM EDTA, 200mM NaCl, 80mM DTT, 4% SDS, and 250μg/ml proteinase K). After purification through the spin column, DNA was eluted using 100μl of Buffer AE. An additional ethanol precipitation step was added to improve the quality of the final DNA sample.

PCR amplification, cloning, and transformation

The targeted hotspot sequence was amplified using flanking primers common to both parental haplotypes (Table 2). PCR was

Table 2
Primers used for PCR amplifying and SNP genotyping the *Hlx1* recombination hotspot on mouse chromosome 1

Primer	Sequence (5′–3′)	SNP
Hlx1 left flanking	CTCCAGCTCCAGAGAATTGC	
Hlx1 right flanking	GATTCACTGATGGGTGTTTG	
SNP1-B6	**GAAGGTCGGAGTCAACGGATT**CTGTGATAAGGAAGGGCTAGGA	T
SNP1-CAST	**GAAGGTGACCAAGTTCATGCT**TGTGATAAGGAAGGGCTAGGG	C
SNP1-reverse	CTCTATTTCCCCAAATCATTACCCT	
SNP2-B6	**GAAGGTCGGAGTCAACGGATT**TGCCCAGGCTATCTAGGTACG	G
SNP2-CAST	**GAAGGTGACCAAGTTCATGCT**GCCCAGGCTATCTAGGTACA	A
SNP2-reverse	CAACTAAACAAAAGCCCAAACTCAA	
SNP3-B6	**GAAGGTCGGAGTCAACGGATT**ATGGTTAGTGGAATGCGTAAAGA	T
SNP3-CAST	**GAAGGTGACCAAGTTCATGCT**GGTTAGTGGAATGCGTAAAGG	C
SNP3-reverse	GGAGGAGATGGTGGGTGAATA	
SNP4-B6	**GAAGGTGACCAAGTTCATGCT**TCGACCACTTAGACTCAAGGTTGC	G
SNP4-CAST	**GAAGGTCGGAGTCAACGGATT**CGACCACTTAGACTCAAGGTTGT	A
SNP4-reverse	ATGGGATGGAAGGTTCTGCCT	
SNP5-B6	**GAAGGTCGGAGTCAACGGATT**ACGTGAAGCTGAAGTTAATAAACC	G
SNP5-CAST	**GAAGGTGACCAAGTTCATGCT**TGACGTGAAGCTGAAGTTAATAAACT	A
SNP5-reverse	GTCTAAGTGGTCGGTGTGAGTAT	
SNP6-B6	**GAAGGTCGGAGTCAACGGATT**GTTTTTCCCTTTATACATCTCCTACT	A
SNP6-CAST	**GAAGGTGACCAAGTTCATGCT**GTTTTTCCCTTTATACATCTCCTACC	G
SNP6-reverse	GTCTAAGTGGTCGGTGTGAGTAT	
SNP7-B6	**GAAGGTGACCAAGTTCATGCT**AAGTGCTACCACGGCAAAACCG	G
SNP7-CAST	**GAAGGTCGGAGTCAACGGATT**AGTGCTACCACGGCAAAACCA	A
SNP7-reverse	CTGGGTGACATTGTAGACTCTT	
SNP8-B6	**GAAGGTGACCAAGTTCATGCTT**GCAGGCAGATCAAGTGGT	A
SNP8-CAST	**GAAGGTCGGAGTCAACGGATT**CCTGCAGGCAGATCAAGTGA	G
SNP8-reverse	GCTAGTTGCCATGTCTCTTTGTT	

Allele-specific primers are linked with either **GAAGGTGACCAAGTTCATGCTCA** or **GAAGGTCGGAGTCAACGGATT** as adaptor sequences for the Amplifluor SNP genotyping system.

performed on a Tetrad PCR system (Bio-Rad, Hercules, CA, USA) using approximately 30ng of DNA as template, 1 × PCR buffer [60mM Tris–SO_4, pH 8.9, 18mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.8μM each primer, 0.2mM each dNTP, 5% DMSO] and 0.5 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). Cycling conditions consisted of an initial denaturing at 95°C for 2min, followed by 30 cycles of 95°C for 1min, 54°C for 15s, and 72°C for 8min. In common with previous reports [3,17], we found it important to employ conditions that mitigate against jump-PCR products created when incomplete amplicons serve as primers in subsequent rounds of amplification and create artifactual crossovers. Optimization was achieved by incorporating DMSO into the PCR system to reduce DNA secondary structure, increasing the extension time to ensure full-length amplicons, and reducing the annealing time to enhance the preference for oligo primers over any incomplete fragments that might escape. The amplified products were ligated into a PCR2.1 vector using the TOPO T/A cloning kit and transformed into the TOP10 *E. coli* strain using the standard protocol (Invitrogen). Transformed cells were grown overnight on LB agar plates with 40μg/ml ampicillin and 20μl of X-gal (40mg/ml) spread on the surface.

E. coli culture and preparation

Individual, positive *E. coli* colonies from blue/white screening were grown in 80μl of LB medium using 96-well plates incubated at 37°C without shaking for 18h. Cell cultures were spun down while still in their plates at 3450*g* for 10min and resuspended in 80μl of water.

SNP genotyping

SNPs were genotyped using the Chemicon Amplifluor SNPs HT FAM-JOE system (Millipore, Billerica, MA, USA) and the alleles were discriminated on an ABI 7900HT real-time PCR system (Applied Biosystems, Framingham, MA, USA) by end-point reading. Reactions were carried out in 384-well plates using 5μl reaction volume consisting of a 2-μl sample of resuspended *E. coli* culture without plasmid purification, plus 0.5μl of 10 × Reaction Mix S Plus, 0.4μl 2.5mM each dNTP, 0.25μl 20 × FAM primer, 0.25μl 20 × JOE primer, 0.25μl SNP-specific primer mix (0.5μM green-tailed allele-specific primer, 0.5μM red-tailed complementary allele-specific primer, 7.5μM common reverse primer), and 0.05μl Titanium Taq DNA polymerase (Clontech, Mountain View, CA, USA) (for SNP primer sequences see Table 2). SNP genotyping was performed using the recommended protocol with an annealing temperature of 57°C in the initial 20 cycles.

SNP primer design

SNP primers were designed using the Amplifluor AssayArchitect software (https://apps.serologicals.com/AAA/).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2008.06.008.

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