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Site-specific transgenesis of the *Drosophila melanogaster* Y-chromosome using CRISPR/Cas9

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Abstract

Despite the importance of Y-chromosomes in evolution and sex determination, their heterochromatic, repeat-rich nature makes them difficult to sequence (due, in part, to ambiguities in sequence alignment and assembly) and to genetically manipulate. Therefore, they generally remain poorly understood. For example, the *Drosophila melanogaster* Y-chromosome, one of the most extensively studied Y-chromosomes, is widely heterochromatic and composed mainly of highly repetitive sequences, with only a handful of expressed genes scattered throughout its length. Efforts to insert transgenes on this chromosome have thus far relied on either random insertion of transposons (sometimes harbouring 'landing sites' for subsequent integrations) with limited success or on chromosomal translocations, thereby limiting the types of Y-chromosome-related questions that could be explored. Here, we describe a versatile approach to site-specifically insert transgenes on the Y-chromosome in *D. melanogaster* via CRISPR/Cas9-mediated homology-directed repair. We demonstrate the ability to insert, and detect expression from, fluorescently marked transgenes at two specific locations on the Y-chromosome, and we utilize these marked Y-chromosomes to detect and quantify rare chromosomal nondisjunction effects. Finally, we discuss how this Y-docking technique could be adapted to other insects to aid in the development of genetic control technologies for the management of insect disease vectors and pests.

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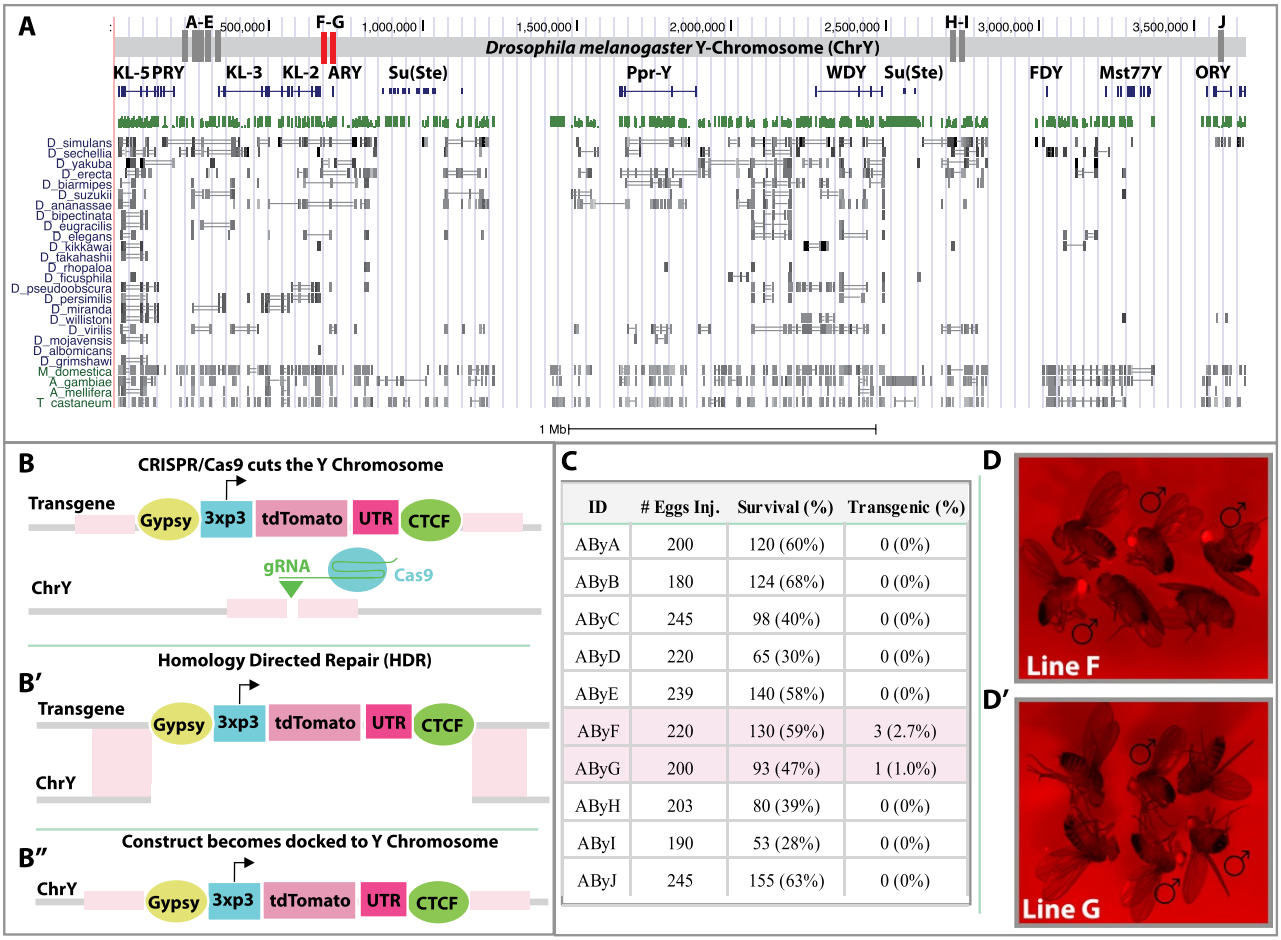
Introduction

The ability to perform site-specific transgenesis in insects and other organisms has greatly improved the ability to conduct controlled experiments in a precise way. In *Drosophila melanogaster*, for example, prior to the advent of site-specific transgene integration technologies, most transgenesis was carried out using transposon-based vectors that inserted embedded transgenes randomly throughout the genome, leaving them vulnerable to distinct position effects arising from surrounding *cis*-regulatory regions and chromatin structure (Wimmer, 2005). The development of site-specific transgenesis techniques, such as those that rely on the phage φC31-derived site-specific integrase (Groth *et al.*, 2004) or on recombinase-mediated cassette exchange (Oberstein *et al.*, 2005), represented a major advance (Venken & Bellen, 2005; Wimmer, 2005). This is because these techniques enabled more accurate and detailed transgene comparison and identification of insertion loci that are conducive to robust transgene expression levels and are fitness neutral, factors that can be especially important in practical applications of insect transgenesis, such as in vector control (Irvin *et al.*, 2004; Wimmer, 2005). However, the widely adopted site-specific recombinase-based transgenesis techniques (eg φC31) are limited by their requirement for pre-existing 'landing sites' within genomes of interest, which must first be generated by random transposon-mediated transgenesis, and unfortunately these sites are lacking in most non-model organisms (Wimmer, 2005).

The arrival of CRISPR technologies heralded a new era not only for traditional genome manipulation, such as mutagenesis, but also for precise, site-specific transgenesis (Gratz *et al.*, 2015; Bier *et al.*, 2018). By using a simplified two-component system consisting of a *Streptococcus pyogenes* Cas9 endonuclease (SpCas9) and a single chimeric guide RNA (sgRNA; Jinek *et al.*, 2012), one can generate DNA double-strand breaks (DSBs) in a location

of one's choosing, provided it contains a 3 bp (NGG) protospacer adjacent motif required for CRISPR/Cas9 function (where N is any nucleobase followed by two guanine nucleobases) (Jinek *et al.*, 2012). Then, as long as a donor template comprising homology-containing stretches (ie homology arms) flanking the target transgene is provided, the DSBs can be repaired via the cell's own homology-directed repair (HDR) pathway to integrate the transgene at the precise location of the DSB (Gratz *et al.*, 2015). This technique has been shown to be highly efficient in *D. melanogaster* (Gokcezade *et al.*, 2014; Gratz *et al.*, 2014, 2015), and given CRISPR's functionality in many insects (Gantz *et al.*, 2015; Hammond *et al.*, 2016; Kohno *et al.*, 2016; Li *et al.*, 2017a; Li *et al.*, 2017b; Li *et al.*, 2017c; Sun *et al.*, 2017; Dong *et al.*, 2018; Li *et al.*, 2018) it should be broadly applicable for insect transgenesis in general (eg Gantz *et al.*, 2015; Hammond *et al.*, 2016; Li *et al.*, 2017b).

The Y-chromosome of *D. melanogaster*, and of most other heteromorphic sex-chromosome-bearing organisms, has so far presented an elusive target for site-specific transgenesis applications. The Y-chromosome's degenerative, repetitive nature renders it difficult to sequence, assemble and analyse genomically (Carvalho, 2002; Piergentili, 2010; Hall *et al.*, 2016); additionally, it is almost entirely heterochromatic (Elgin & Reuter, 2013), which



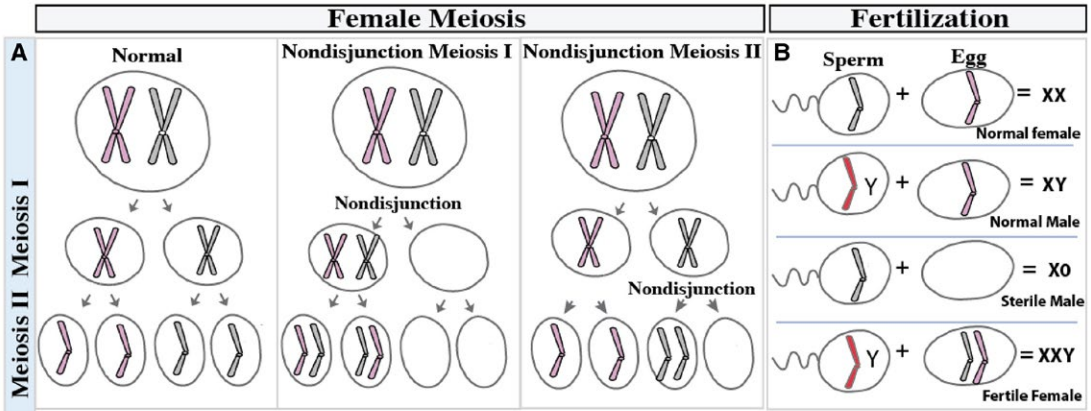
ID	# Eggs Inj.	Survival (%)	Transgenic (%)
AByA	200	120 (60%)	0 (0%)
AByB	180	124 (68%)	0 (0%)
AByC	245	98 (40%)	0 (0%)
AByD	220	65 (30%)	0 (0%)
AByE	239	140 (58%)	0 (0%)
AByF	220	130 (59%)	3 (2.7%)
AByG	200	93 (47%)	1 (1.0%)
AByH	203	80 (39%)	0 (0%)
AByI	190	53 (28%)	0 (0%)
AByJ	245	155 (63%)	0 (0%)

Figure 1. Targeted transgene insertion on the *Drosophila melanogaster* Y-chromosome. (A) A map of the *D. melanogaster* Y-chromosome (adapted from the UCSC Genome Browser) shows the position of attempted transgene insertions (vertical bars labelled A–J; red bars indicate successful insertions, while grey bars indicate insertion failure) relative to the location of major genes. Green lines indicate conservation of gene regions calculated via the PhastCons method, and grey bars indicate multiple alignment of gene regions, in 27 other species. (B) A schematic of the basic transgene utilized is shown. gRNA, guide RNA; UTR, untranslated region. The transgene contains a marker flanked by homology arms to a specific region of the Y-chromosome and is injected with a single chimeric guide RNA (sgRNA) that targets a sequence on the Y-chromosome between the homology arms, and a source of Cas9 (B). Following a Cas9-induced double-strand DNA break, the cell's homology-directed repair (HDR) pathway utilizes the transgene as a repair template to copy the marker between the regions of homology (B), generating a Y-chromosome with a marker gene at the precise sgRNA-targeted site (B). (C) The number of embryos injected for each of the Y-chromosome targeting transgenes, survival to larval stage of injected embryos and transgenesis rate (no. of independent transgenic individuals found/no. of embryos injected, half of which are predicted to be male) is shown. (D) All males (and none of the females) for each of the two recovered transgenic lines [AByF (D) and AByG (D)] express eye-specific red fluorescence.

likely makes it refractory to transgene integration and/or robust transgene expression (Bernardini *et al*., 2014). These contributing factors have made it difficult to manipulate the Y-chromosome in a precise fashion. For example, the only successful attempts at placing transgenes on the Y-chromosome in insects have relied on random (and rare) events, such as chromosomal rearrangements or low-frequency Y-specific transposon-mediated integrations, and subsequent manipulations (Zhang & Spradling, 1994; Zhang & Stankiewicz, 1998; Starz-Gaiano *et al*., 2001; Szabad *et al*., 2012; Bernardini *et al*., 2014, 2017).

Despite the challenges presented by the nature of the Y-chromosome, having the ability to engineer it in *D. melanogaster* and other insects would be of utility in a number of different applications. First, the ability to mark the Y-chromosome with an easily scorable, specific marker may in itself be useful. For example, it would allow for the facile identification of rare Y-bearing or Y-lacking individuals (eg XXY females and XO males arising from nondisjunction; Fig. 2A; Bridges, 1913; Bridges, 1916), which can be of experimental use (eg Hearn *et al*., 1991; Lemos *et al*., 2010; Yang *et al*., 2012; Brown & Bachtrog, 2017). A marked Y-chromosome would also enable interspecific Y-chromosome introgression studies, which can shed light on Y-chromosome evolution and function (Sackton *et al*., 2011; Araripe *et al*., 2016; Bernardini *et al*., 2017), and can also be useful for sexing embryos (Condon *et al*., 2007; Bernardini *et al*., 2014), although it would also mark XXY females and fail to identify XO males, which may be problematic for certain applications.

Additionally, the ability to express specific transgenes directly from the Y-chromosome would be useful in several different contexts. For example, it would enable general studies of Y-chromosome gene function, as well as analyses of chromosome loss and interaction (Szabad & Würgler, 1987; Szabad *et al*., 2012; Soós & Szabad, 2014). Perhaps more interestingly, it could aid in the development of genetic control mechanisms for insect vectors and pests, which offer promising solutions to significant health and agricultural problems (Sinkins & Gould, 2006; Esvelt *et al*., 2014; Champer *et al*., 2016). For example, distortion of the sex ratio in favour of males can lead to a gradual population reduction and eventual elimination of a target population (Hickey & Craig, 1966; Hamilton, 1967; Gould & Schliekelman, 2004; Papathanos *et al*., 2014), and natural so-called meiotic driving Y-chromosomes have been described (Newton *et al*.,1976; Sweeny & Barr, 1978; Wood & Newton, 1991). A system for sex-ratio distortion can also be engineered by designing transgenes that target the X-chromosome during spermatogenesis, and such an X-shredder element has already been developed in one species of mosquito (Windbichler *et al*., 2008;



C

X-Balancer Line (BS#; genotype)	XXY Female Frequency	XO Male Frequency
579; Df(1)JF5, f[1] car[1]/FM7a	6.5% (20/305)	27% (30/111)
727; Df(1)g, f[1] B[1]/In(1)AM	1.1% (6/516)	1.0% (2/182)
939; Df(1)dm75e19/FM7c	0% (0/419)	0.8% (1/122)
944; Df(1)JC70/FM7c, sn[+]	0.4% (2/430)	1.9% (3/153)

Figure 2. Using transgenic marked Y-chromosomes to track X-chromosome meiotic nondisjunction events. (A) In *Drosophila melanogaster*, chromosome nondisjunction can occur in meiosis I or meiosis II; in either case, nondisjunction of X-chromosomes leads to formation of two types of aberrant gametes: ones that have an extra copy of the X-chromosome, and ones that lack an X-chromosome entirely. (B) If nondisjunction occurs during meiosis in a female, she will produce four types of viable offspring when outcrossed to a normal male: normal (XX) females, normal (XY) males, sterile (XO) males and fertile (XXY) females. (C) Outcrosses of a marked Y-chromosome line to four distinct X-chromosome balancer lines (leftmost column) resulted in progeny arising from nondisjunction events. Frequencies of XXY females (females with marked Y-chromosome; middle column) and XO males (males with no Y-chromosome; rightmost column) are shown.

Galizi *et al*., 2014, 2016). However, to be maximally effective and self-perpetuating, such an element must be linked to the Y-chromosome (Deredec *et al*., 2008; Deredec *et al*., 2011; Galizi *et al*., 2016), a feature that the developed systems lacked but that could be achieved by an efficient method of targeted Y-chromosome transgenesis.

To overcome this technological gap, we describe here the development of a CRISPR/Cas9-based technique for site-specific engineering of the *D. melanogaster* Y-chromosome. Specifically, we demonstrate the ability to insert a fluorescently marked transgenic cassette at specific locations on the Y-chromosome, and to utilize the marked Y-chromosome to identify XXY females and XO males. The interspecies portability and limitations of this technique are also discussed.

Results and discussion

To generate transgenic elements that could be inserted in specific locations on the *D. melanogaster* Y-chromosome with CRISPR/Cas9-mediated HDR, we engineered a vector comprising a fluorescent marker (tdTomato) driven by the eye-specific 3xP3 promoter (Berghammer *et al*., 1999) and flanked by the *gypsy* (Holdridge & Dorsett, 1991) and CTCF (Kyrchanova *et al*., 2008) insulators, with unique restriction sites upstream and downstream for cloning specific homology arms (Fig. 1). We then selected 10 distinct intergenic regions spanning the Y-chromosome for targeting (Fig. 1A), reasoning that attempting transgene insertion at multiple different sites within gene-empty regions would increase our chances of a successful detectable integration. We identified a suitable sgRNA target site with no predicted off-target effects in each region, and cloned in homology arms, corresponding to ~800–1000 bp of sequence 5′ and 3′ of each selected target site, upstream and downstream of the insulator-flanked 3xP3-tdTomato element to generate 10 unique Y-chromosome targeting transgenes (AByA–J) (Fig. 1A).

Each transgene was then injected, along with the appropriate *in vitro* transcribed sgRNA and Cas9 protein, into a transgenic line expressing a germline source of Cas9 using standard procedures (Gratz *et al*., 2014), and G_1 progeny were screened for presence of eye-specific tdTomato fluorescence (Fig. 1B). Of the 10 distinct construct injections, only two (AByF and AByG) out of 10 yielded transgenic male individuals, despite the large number (>200 in most cases, 2142 total for all constructs) of G_0 embryos injected for each construct (Fig. 1C, D). This observation suggests that either the rate of Y-chromosome transgenesis is not particularly high (especially when compared with rates reported anecdotally and in other studies utilizing CRISPR/Cas9-based HDR in autosomal locations; eg Gratz *et al*., 2014), or that a number of tested Y-chromosome target sites were not located in regions conducive to somatic gene expression (although a low transgenesis rate not related to the Y-chromosome cannot be ruled out, and the fact that only injected male G_0 embryos can give rise to transgenics has to be considered). Neither of these explanations would be surprising, given the heterochromatic nature of the *D. melanogaster* Y-chromosome (Carvalho, 2002), and simply suggests that several target regions must be tested in order to identify those suitable for HDR and/or that allow robust expression. Following their recovery, we outcrossed transgenic males to white eye (w, w[1118]) females for several generations to confirm paternal inheritance of the tdTomato marker and saw expected inheritance patterns (ie all male progeny and no female progeny of a male inherited the fluorescent marker). We also verified that the transgene was correctly inserted by performing PCR across the transgene-insertion junction on genomic DNA of transgenic males and sequencing the products.

We then set out to determine whether a marked Y-chromosome could be useful in identifying the rare progeny resulting from meiotic sex chromosome nondisjunction events in females (Fig. 2A, B), as a clear means for identification of such individuals could be useful both for those that want to avoid them and those that wish to utilize them for addressing various research questions (eg O'Tousa, 1982; Gilliland *et al*., 2014; Brown & Bachtrog, 2017). To do this, we crossed males from the more strongly expressing of our two transgenic Y-chromosome lines, AByF, to a variety of X-chromosome balancer line virgins. These balancer lines contain genetic elements such as multiple inversions, rearrangements and deficiencies that are known to increase the probability of nondisjunction by causing abnormalities in recombination as well as unusual chromosomal pairing and segregation patterns (Forbes, 1962; Ramel, 1962; Xiang & Hawley, 2006; Ramel, 2009; Gilliland *et al*., 2014). For four of these crosses (all involving balancer lines with X-chromosome deficiencies), we identified progeny that appeared to be the result of meiotic nondisjunction events in the X-chromosome balancer females – XXY-bearing females (identified by presence of the fluorescent marker) and XO males (identified by the absence of the fluorescent marker) – at various frequencies ranging from 0 to 27% (Fig. 2C). These aberrant progeny were individually outcrossed to a common laboratory stock (w[1118]) to test for fertility. All putative XO males were sterile ($n = 36$), as expected (Griffiths, 2005). All XXY females were fertile ($n = 28$) and gave rise to progeny classes that indicated occurrence of secondary nondisjunction in XXY parents, which is in agreement with previous observations (Bridges, 1916; Carpenter, 1973; Xiang & Hawley, 2006). The presence or absence of the transgenic Y-chromosome in XXY females and XO males respectively was verified with PCR as described

earlier. Given the high rates of nondisjunction individuals obtained by crossing the marked Y-chromosome line with certain X-chromosome balancers (eg balancer line 579), future experiments requiring large quantities of XXY females and XO males could be conducted using this approach, which could accelerate basic research on deciphering key biological processes and gene networks involved in Y-chromosome functionality and evolution.

In short, here we describe the engineering of two Y-chromosome marked transgenic *D. melanogaster* lines using site-specific CRISPR/Cas9-mediated insertion. This may be useful for generating, identifying and tracking progeny arising from rare meiotic nondisjunction events in a more straightforward manner than is typically used (Hess & Meyer, 1968; Piergentili, 2010; Brown & Bachtrog, 2017). This may also be useful for detecting *in vivo* mosaic Y-chromosome loss (Szabad *et al*., 2012), although we did not observe evidence for such loss in assayed transgenic XY or XXY individuals. More broadly, however, we provide a proof of principle example of a CRISPR/Cas-based strategy for docking transgenes site specifically on the Y-chromosome that should, in principle, be applicable to many other species. Although the often-heterochromatic nature of a Y-chromosome may, as our observations possibly suggest, reduce the number of targeted insertion sites that are amenable to modification and/or robust transgene expression, the ease with which distinct Y-chromosome targeting transgene cassettes can be generated should allow for testing of many possible insertion sites, greatly increasing the chances of successful transgene integration with desired expression. The ability to mark, and express transgenes directly from, the Y-chromosome not only allows for facile identification of Y-chromosome-bearing individuals at various life stages and enables researchers to probe the enigmatic function of the Y-chromosome (eg (Bernardini *et al*., 2014; Brown & Bachtrog, 2017), but could also help pave the way for engineering genetic insect vector and pest control strategies, such as X-chromosome shredding (Hickey & Craig, 1966; Hamilton, 1967; Gould & Schliekelman, 2004; Huang *et al*., 2007; Galizi *et al*., 2014; Champer *et al*., 2016; Galizi *et al*., 2016). This approach in particular depends on the destruction of X-bearing sperm to produce males that only give rise to male progeny (Huang *et al*., 2007; Champer *et al*., 2016), and requires the ability to meiotically express an X-chromosome targeting element from the Y-chromosome (Beaghton *et al*., 2016; Galizi *et al*., 2016). The strategy described here could be used to dock such an X-chromosome targeting transgene (perhaps one based on CRISPR/Cas9 technology; Galizi *et al*., 2016; Zuo *et al*., 2017) on the Y-chromosome of many insects, including the malaria vector *Anopheles gambiae*, to facilitate the engineering of the promising X-chromosome shredding genetic control strategy that can be used to combat malaria and other vectored pathogens (Gould & Schliekelman, 2004; Champer *et al*., 2016; Hall *et al*., 2016; Papathanos *et al*., 2014).

Experimental procedures

Construct design and assembly

To generate vector ABy, the base vector used to generate vectors AByA–AByJ, several components were cloned into the *piggyBac* plasmid pBac[3xP3-DsRed] (Li *et al*., 2017b) using Gibson assembly/enzymatic assembly (EA) cloning (Gibson *et al*., 2009). First, a Gypsy insulator fragment amplified with primers ABy.1 and ABy.2 from *Drosophila* genomic DNA, the 3xP3 promoter amplified with primers ABy.3 and ABy.4 from plasmid pBac[3x-P3EGFP afm] (Horn & Wimmer, 2000) and a *Drosophila* codon optimized tdTomato marker amplified with primers ABy.5 and ABy.6 from a gene-synthesized vector (Genscript, Piscataway, NJ, USA) were cloned into a *Bst*BI/*Not*I-digested pBac[3xP3DsRed] backbone using EA cloning. The resulting plasmid was digested with *Avr*II, and the following components were cloned in via EA cloning: an *attP* sequence from plasmid M{3xP3RFP attP} (Bischof *et al*., 2007) amplified with primers ABy.7 and ABy.8, and a CTCF insulator fragment amplified with primers ABy.9 and ABy.10 from *Drosophila* genomic DNA. All primer sequences are listed in Supplementary Table 1. To generate the final vectors containing homology arms specific to each putative Y-chromosome docking site, vector ABy was first digested with *Pme*I and each 5′ homology arm (amplified with primers specific to the Y docking site from *Drosophila* genomic DNA) was individually cloned in using EA cloning. Each resulting intermediate plasmid was then digested with *Eco*RI, and each corresponding 3′ homology arm (amplified with primers specific to the Y docking site from *Drosophila* genomic DNA) was cloned in using EA cloning. The 10 Y-chromosome docking-site-specific construct names and the primers used to amplify the 5′ and 3′ homology arms for each are listed in Supplementary Table 1. (The slight variation in the length of the homology arms was not expected, and not observed, to affect integration efficiency.) Vectors AByF and AByG are available from Addgene (Cambridge, MA, USA; nos. 111084 and 111083 respectively). To generate DNA DSBs for vector incorporation, sgRNAs targeting 10 distinct regions of the Y-chromosome were *in vitro* transcribed with the MEGAscript™ T7 Transcription Kit (ThermoFisher Scientific, Waltham, MA, USA; cat. no. AM1334) using a self-annealing set of primers, with a unique forward primer for each target site and a universal reverse primer. Genomic locations of each sgRNA target sequence, the target sequences themselves and primers used for *in vitro* transcription are listed in Supplementary Table 1. The sgRNA target sites with no predicted

off-target effects were selected using flyCRISPR Target Finder (Gratz *et al*., 2014).

Fly culture and strains

Fly husbandry and crosses were performed under standard conditions at 25 °C. Rainbow Transgenics (Camarillo, CA, USA) carried out all of the fly injections. Each construct was premixed with Cas9 protein (PNA Bio Inc., Newbury Park, CA, USA; cat. no. CP01-20) and *in vitro* transcribed sgRNAs at the following concentrations: 400 ng/μL cyclic donor plasmid, 40 ng/μL sgRNA and 300 ng/μL Cas9 protein. Constructs were injected into a *vasa*-Cas9 transgenic line marked with 3xP3-GFP [Bloomington Drosophila Stock Center (BSC), Bloomington, IN, USA; no. 51324, w[1118]; PBac{y[+mDint2]=vas-Cas9}VK00027]. Only transgenic males were recovered, and these were singly outcrossed to w- (w[1118]) virgin females to verify paternal transmission. Male progeny without the *vasa*-Cas9 transgene were selected and further crossed to w- to establish a stock. The AByF transgenic line is available from the Bloomington Stock Center (BDSC_78567).

Molecular characterization of Y-chromosome lines

To confirm correct insertion of transgenes on the Y-chromosome, PCRs were carried out on genomic DNA from transgenic males (Fig. 1). Briefly, genomic DNA was extracted from individual flies with the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA; cat. no. 69504) following the manufacturer's protocol. PCR was carried out using standard procedures to amplify the junction of the transgenes and the surrounding genomic region (as well as the unmodified target genomic region as a negative control and a region of the X-chromosome as a positive control) using primers listed in Supplementary Table 1. The PCR program utilized was as follows: 98 °C for 30 s; 35 cycles of 98 °C for 10 s, 56 °C for 20 s, and 72 °C for 30 s; then 72 °C for 10 min. PCR products were purified with the MinElute® PCR Purification Kit (Qiagen, cat. no. 28004) according to the manufacturer's protocol and sequenced with Sanger sequencing (Source BioScience, Nottingham, UK) utilizing the same primers as used for the PCRs. Sequences were analysed with DNASTAR software.

Generation and characterization of XXY females and XO males

To determine whether a marked Y-chromosome could be utilized to identify individuals that result from meiotic nondisjunction events, a single male from the AByF transgenic line (which showed stronger fluorescent marker expression levels than line AByG transgenic males) was crossed to virgins from the following X-chromosome balancer stocks from the BSC: BSC nos. 579, 723, 727, 939, 944, 946, 959, 976, 3347, 6002, 6007, 6019, 6219, 7200, and 27887. Each cross was set in triplicate. Progeny were screened to identify females with fluorescent red eyes (putative XXY individuals) and males without fluorescent red eyes (putative XO individuals). Frequency of putative XXY and XO individuals was calculated by dividing the total number of each individual type found for each type of cross by the total number of females or males respectively (Fig. 2C). Each XO individual was outcrossed to w-(w[1118]) virgins to test for fertility. To molecularly confirm the presence of the transgenic Y-chromosome in putative XXY females and the absence of any Y-chromosome in putative XO males, genomic DNA was extracted as earlier, and PCRs were performed as earlier with primers utilized for the AByF 3′ genomic DNA–transgene junction, the AByF 5′ genomic DNA–transgene junction, the AByF wild-type locus and the X-chromosome positive control locus (Supplementary Table 1).

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Disclosure statement

The authors declare no conflict of interest.

Author contributions

O.S.A and A.B. conceived and designed experiments. A.B. performed molecular and genetic experiments. All authors contributed to the writing, analysed the data and approved the final manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

TableS1. Primer sequences.