No Detectable Effect of the DNA Methyltransferase DNMT2 on Drosophila Meiotic Recombination

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ABSTRACT Epigenetics is known to be involved in recombination initiation, but the effects of specific epigenetic marks like DNA methylation on recombination are relatively unknown. Studies in Arabidopsis and the fungus Ascomobolus immersus suggest that DNA methylation may suppress recombination rates and/or alter its distribution across the genome; however, these patterns appear complex, and more direct inquiries are needed. Unlike other organisms, Drosophila only have one known DNA methyltransferase, DNMT2, which is expressed in the ovaries and historically has been thought to be responsible for limited genomic DNA methylation. To test for a role of DNMT2 on the frequency and distribution of recombination, I compared recombination rates between Dnmt2 −/− and Dnmt2 +/+ Drosophila melanogaster individuals in two euchromatic regions and one heterochromatic region across the genome. I failed to detect an altered pattern of recombination rate in the absence of DNMT2 in all regions surveyed, and conclude that other epigenetic effects are regulating recombination initiation in Drosophila.

Epigenetics has long been predicted to play a role in the initiation of meiotic recombination. Observations of variation in recombination rate within and between individuals, sexes, populations, and across the genome (such as euchromatin vs. heterochromatin) suggest a role beyond DNA sequence in determining locations of recombination events (Lichten and Goldman 1995; Barthes et al. 2011). An association between open chromatin formation and double strand breaks, the first step in the initiation of recombination, has been identified in yeast, dog, and several plants (Berkowitz et al. 2009; Pan et al. 2011; Auton et al. 2013; Choi et al. 2013; Hellsten et al. 2013), and the histone H3K4 methyltransferase PRDM9 influences the distribution of recombination sites in human and mouse (Buard et al. 2009; Baudat et al. 2010; Berg et al. 2011; Grey et al. 2011; Brick et al. 2012; Acquaviva et al. 2013; Sommermeyer et al. 2013). However, the possible roles of specific epigenetic marks apart from H3K4me3 are less understood. For example, the relation between recombination initiation and the best-characterized epigenetic factor, DNA methylation, appears complex and remains relatively unexplored.

DNA methylation describes the transferring of a methyl group (CH3) to the 5th position of a cytosine residue, typically at CpG sites and repeat elements (Robertson and Wolffe 2000). The reaction is catalyzed by a family of conserved proteins known as DNA methyltransferases: DNMT1, the maintenance methyltransferase, ensures proper inheritance of methylation patterning after replication in somatic cells; DNMT3s (3A, 3B, 3L), the de novo methyltransferases, establish DNA methylation patterns during embryogenesis; and DNMT2, an enigmatic methyltransferase with conserved catalytic motifs, has a historically disputed function (Yoder et al. 1997; Dong et al. 2001; Schaefer and Lyko 2010; Barthes et al. 2011; Krauss and Reuter 2011). These genes function within an ancient regulatory mechanism shared by animals, plants, and fungi, serving in diverse roles often related to repression of gene expression (Feng et al. 2010; Zemach et al. 2010; Zemach and Zilberman 2010; Jurkowski and Jeltsch 2011; Nanty et al. 2011).

A link between DNA methylation and recombination was first hypothesized by Rossignol and Faugeron (1994) and Yoder et al. (1997) in which DNA methylation promotes genome integrity through the suppression of recombination between dispersed repetitive sequence. More direct evidence of a functional link between DNA methylation and recombination is somewhat limited, but a study in the fungus Ascomobolus immersus showed crossover formation was reduced several hundred fold in an in vivo methylated hotspot compared

KEYWORDS DNA methylation
DNMT2
recombination
Drosophila
epigenetics
with an unmethylated hotspot (Maloisel and Rossignol 1998). More recently, several reports in Arabidopsis paint a more nuanced pattern. For example, in the absence of MET1 (the DNMT1 homolog), researchers independently observed a pattern of increased recombination in euchromatin and decreased recombination in (typically hyper-methylated, transposon rich) heterochromatin (Melamed-Bessudo and Levy 2012; Miroouze et al. 2012; Yelina et al. 2012). Each study found that the total number of crossover events was not different between met1 mutants and the wild type, indicating that the loss of DNA methylation affects the distribution of crossovers but not their overall number. When wild-type Arabidopsis transcription start and termination sites are examined specifically, DNA methylation is decreased in recombination hotspots relative to transcription start or termination sites in which recombination was absent (Choi et al. 2013), supporting data from A. immersus. In contrast, some indirect evidence in humans pointed to a positive association between recombination rate and DNA methylation (Sigurdsson et al. 2009).

In this study, I explore the complex and perhaps contradictory role of DNA methylation in the determination of recombination events in the model system Drosophila melanogaster. D. melanogaster possesses the DNA methyltransferase DNMT2 and a methyl binding domain protein, MBD2/3, which typically binds to methylated DNA and recruits chromatin remodelin complexes (Tweedie et al. 1999; Roder et al. 2000; Ballesta et al. 2001; Marhold et al. 2004a). Transcripts of Dmnt2 were particularly enriched during early stages of embryonic development; expression in adult flies was limited to female ovaries, and there was no activity in male testes (Lyko et al. 2000), consistent with a role in recombination, which is female-specific in Drosophila. Furthermore, like MBD2 knockout mice, Drosophila null mutants of MBD2/3 were viable and fertile but revealed chromosome segregation defects (Lyko et al. 2006).

DNA methylation has experienced a controversial history in Drosophila, but has been reportedly detected at low levels, ranging from 0.21 to 1% across the Drosophila genus (Tweedie et al. 1999; Gowher et al. 2000; Lyko et al. 2003a; Marhold et al. 2004b; Salzberg et al. 2004; D’Avila et al. 2010; Takayama et al. 2014). One of its functions in Drosophila was thought to be retrotransposon silencing and stabilization of repeats, similar to its role in vertebrates and plants (Salzberg et al. 2004; Phalke et al. 2009; Krauss and Reuter 2011). In a new study, researchers reveal a wholly unique DNA methylation pattern in D. melanogaster, finding DNA methylation to be very localized, strand asymmetrical, dynamic, concentrated in CA- and CT-rich five base pair motifs, and most likely involved in gene expression (Takayama et al. 2014). The role of DNM2 in this recent study is nonintuitive; in its absence, DNA methylation remains, although with altered patterns (Takayama et al. 2014).

Therefore, to determine whether there is a detectable effect of DNA methylation and/or the DNA methyltransferase Dmnt2 on the distribution and frequency of recombination rate in Drosophila, I assayed recombination at one heterochromatic region spanning the centromere of chromosome 3 and two euchromatic regions on the X chromosome in Dmnt2+/− and control (Dmnt2+/+) D. melanogaster. I did not detect any change in recombination rate or distribution in the absence of Dmnt2 and conclude that other epigenetic factors are determining sites of recombination events in Drosophila.

**MATERIALS AND METHODS**

**Stocks and crossing scheme**

For all crosses, virgin flies were collected, separated by sex, and aged for 7 d. The crossing scheme (Figure 1) consisted of the following:

(A) crossing a D. melanogaster Dnmt2−/− element excision line Dmnt299 (Schafer et al. 2010) to wild-type D. melanogaster Zim29 to generate variability to score recombination events. (B) F2 females were crossed to a D. melanogaster chromosome 2L deficiency line over a balancer, Df(2L)BSC826/SM6a (#27900; Bloomington Stock Center, Bloomington, IN). F2 females were collected, and females carrying the SM6a balancer were identified by the curly wing phenotype and discarded. (C, D) The remaining F2 females (bearing 0−1 functional copies of Dmnt2) were crossed to wild-type males (Zim29) in single pair crosses, allowed to lay eggs, and subsequently genotyped after larvae appeared. Undesired genotypes (see section Scoring recombination for genotyping methods) were discarded, and F3 progeny were collected from remaining vials. Recombination was assayed in these individuals.

**Reverse transcription**

To ensure that individuals of Dmnt299/Df(2L)BSC826 genotype were Dmnt2−/−, reverse-transcription polymerase chain reaction (PCR) was completed. For each line (Dmnt299, Df(2L)BSC826/SM6a, Zim29, Dmnt299/Df(2L)BSC826), I prepped RNA from approximately 25 pooled flies of varying life stages using the QIAGEN RNaseasy kit, QIAGEN QIAshredder kit, and QIAGEN DNase kit. For each line, I used genomic DNA, H2O, and a negative control reverse transcription product as controls. For the reverse transcription reaction, the following recipe was used: 2 μL of 10× PCR buffer (15 mM MgCl2), 1.5 μL of 50mM MgCl2, 0.8 μL of 25 mM dNTPs, 1 μL of 50 μM experimental R primer, 1 μL of 50 μM control intron R primer, 0.5 μL of 40U/μL RNase inhibitor, 0.1 μL of 200U/μL MMLV reverse transcriptase, 9.1 μL of diethylpyrocarbonate H2O, and 4 μL of RNA mix + H2O to get to 500 ng of RNA. The reverse transcription program consisted of: 15 min at 42° followed by 5 min at 99°. Then, a PCR was completed with the following recipe: 2.5 μL of 10× PCR buffer (15 mM MgCl2), 1.5 μL of 2mM dNTPs, 1.25 μL, 10 μM experimental F primer, 1.25 μL of 10mM experimental R primer, 0.3 μL of DNA polymerase, 16.2 μL of H2O, and 2 μL of reverse transcription reaction product. The PCR program consisted of an initial denaturing step of 95° (60 sec), three touch-down cycles of 94° (30 sec) − 56° (30 sec) − 72° (45 sec) each, three touch-down cycles of 94° (30 sec) − 53° (30 sec) − 72° (45 sec) each, followed by 33 main cycles of 94° (30 sec) − 50° (30 sec) − 72° (45 sec) each. I used the trade embargo (TREM) gene as a control with the following primers: forward: CAG TAA GTG TGA ATC CGT GC TGT GGT GC; reverse: GCA TGC TCA TA AT GTG CTT GA TGG GAT GC. The primers used for flanking the Dmnt2 intron were forward: GGT CT TG AACT ATT GTA TG GGCA TGG GC; reverse: TA AT GTG CGC CATA A CCG CAT TG GC.

**Scoring recombination**

Flies were collected in 96-well plates and frozen at −20°. DNA was extracted following the protocol of Gloor and Engels (1992), using 49.5 μL of squish buffer [10 mM Tris-HCl (pH 8.2), 1 mM ethylendiaminetetraacetic acid, 25mM NaCl] + 0.5 μL of proteinase-K. A zirconium bead was placed in each well, and plates were then shaken using a QIAGEN TissueLyser II for 45 sec. The DNA preps were then incubated at 37° for 30 min and 95° for 2 min in a thermal cycler. The PCR recipe consisted of 0.5 μL of forward primer +M13 tag (CAC GAC GT GT TGA ATC CGT GC TGT GGT GC added to 5’ end of forward primer), 0.5 μL of reverse primer, 0.4 μL of 700IRD or 800IRD-labeled M13 tag, 1.3 μL of MgCl2, 10× buffer, 1 μL of 2 mM dNTPs, and 0.2 μL of Taq polymerase in a 10-μL reaction volume. The same PCR program was used as described previously. Products were visualized on a polyacrylamide gel using a LICOR 4300.
To identify crosses with desired genotypes, F2 females were genotyped at three loci on the X chromosome (Table 1 and Figure 1, C and D) to ensure heterozygosity \(Df(2L)BSC826/Zim29\) across the region of interest, and at one locus on chromosome 2L (Table 1 and Figure 1, C and D) to identify if the genotype was \(Dnmt2^{299}/Df(2L)BSC826\) (null, experimental) or \(Df(2L)BSC826/Zim29\) (null, control). Vials from parents of the desired genotypes were kept, and the F3 progeny were collected; all other vials and their progeny were discarded.

Recombination was scored in F3 progeny by genotyping at the Chromosome 2L marker, the centromere of Chromosome 3 (Table 1 and Figure 1, C and D). For each locus, eight combinations of genotypes were crossed to wild-type males \((Df(2L)BSC826/Zim29)\) and control females \((D. melanogaster Dnmt2^{299}/Df(2L)BSC826)\) across the region of interest at two markers spanning relatively high recombination (2.68 Mb, 10 cM). This delineation of recombination events in euchromatin in the absence of Dnmt2 was used to account for possible large-scale changes in the distribution of recombination events. (B) F1 females were crossed to a D. melanogaster chromosome 2L deficiency line (blue, with deficiency denoted by an open circle) over a balancer (green), \(Df(2L)BSC826/SM6a\) \((#27900\) Bloomington Stock Center, Bloomington, IN). F2 females were collected, and females carrying the SM6a balancer were identified by the curly wing phenotype and discarded. (C) F2 females \((Dnmt2^{299}/Df(2L)BSC826)\) were crossed to wild-type males \((Zim29)\), and experimental recombination was surveyed in the F3 progeny. (D) F2 females \((Df(2L)BSC826/Zim29)\) were crossed to wild-type males \((Zim29)\) and control recombination was surveyed in the F3 progeny. Markers used to assess genotype of F2 females and to assay recombination in F3 progeny are denoted in (C) and (D) as asterisks. More information about the markers is included in Table 1.

Figure 1 Crossing scheme. The crossing scheme follows the genotype of Chromosomes 2, 3, and X and consists of: (A) crossing a D. melanogaster Dnmt2 p-element excision line \(Dnmt2^{299}\) (gray, with p-element excision denoted in red) \((\text{Schaefer et al. 2010})\) to wild-type D. melanogaster Zim29 (yellow) to generate variability to score recombination events. (B) F1 females were crossed to a D. melanogaster chromosome 2L deficiency line (blue, with deficiency denoted by an open circle) over a balancer (green), \(Df(2L)BSC826/SM6a\) \((#27900\) Bloomington Stock Center, Bloomington, IN). F2 females were collected, and females carrying the SM6a balancer were identified by the curly wing phenotype and discarded. (C) F2 females \((Dnmt2^{299}/Df(2L)BSC826)\) were crossed to wild-type males \((Zim29)\), and experimental recombination was surveyed in the F3 progeny. (D) F2 females \((Df(2L)BSC826/Zim29)\) were crossed to wild-type males \((Zim29)\) and control recombination was surveyed in the F3 progeny. Markers used to assess genotype of F2 females and to assay recombination in F3 progeny are denoted in (C) and (D) as asterisks. More information about the markers is included in Table 1.

Statistics
Recombination fractions between experimental and control individuals were compared using an unpaired t-test (GraphPad Software, Inc. La Jolla, CA). A power analysis was completed using the “pwr” package in R [Statistic: (Cohen 1988) R package: Stephane Champely].

RESULTS
Recombination across a euchromatic region
To identify an effect of the DNA methyltransferase gene Dnmt2 on meiotic recombination, I created a variable deficiency line \((Dnmt2\) null fly stock (confirmed with reverse transcription) and identified recombinants in two adjacent euchromatin regions on the X chromosome. Region 1 spans assembly positions 494,471 to 3,240,200 bp \((\text{Adams et al. 2000})\) and represents a region of relatively low recombination \((2.75\) Mb, approximately \(5\) cM), whereas Region 2 spans assembly positions 3,240,200 to 5,922,673 bp and represents a region of relatively high recombination \((2.68\) Mb, \(10\) cM). This delineation was used to account for possible large-scale changes in the distribution of recombination events in euchromatin in the absence of Dnmt2.

In total, 1,536 \(Dnmt2^{+/−}\) (control) individuals and 1,177 \(Dnmt2^{−/−}\) individuals were scored. In Region 1, I identified no significant difference between \(Dnmt2^{−/−}\) and control individuals \((P = 0.86);\) Control: 3.02% recombinant, 1.07 cM/Mb; Experimental: 3.16% recombinant, 1.11 cM/Mb; Figure 2). Results were similar for Region 2 \((P = 0.84);\) Control: 10.58% recombinant, 3.57 cM/Mb; Experimental: 8.98% recombinant, 3.07 cM/Mb; Figure 2). A power analysis showed with these sample sizes, I could detect an effect size of \(d = 0.1\) (power = 0.8, significance level 0.05).
Recombination across a heterochromatic region

Upon finding no significant difference in euchromatic recombination rates, I sampled recombination rates for a subset of the aforementioned individuals across the heterochromatic centromere of Chromosome 3. This was done to account for possible changes in the distribution of recombination rate across a heterochromatic region in the absence of Dnmt2, as seen in Arabidopsis (Melamed-Bessudo and Levy 2012; Miroouze et al. 2012). I scored 200 Dnmt2 +/- individuals and 152 Dnmt2 -/- individuals at markers at assembly positions 3L: 16,327,010 and 3R: 4,531,276, and once again found no significant difference in recombination rates (P = 0.79; Control: 20.5% recombinant, 1.61 cM/Mb; Experimental: 18.4% recombinant, 1.44 cM/Mb; Figure 2). With this diminished sample size, the power to detect small differences in recombination rate is naturally decreased; (power = 0.8, significance level 0.05; N= 200, d = 0.28; N = 152, d = 0.32), although the results are consistent with the increased sample size results from the euchromatic region. I therefore conclude that the DNA methyltransferase DNMT2 has no detectable effect on recombination in D. melanogaster.

DISCUSSION

Although DNA methylation influences the recombination landscape in fungus A. immersus and Arabidopsis, I detected no effect of knocking out the DNA methyltransferase Dnmt2 on recombination in Drosophila. The crossover analyses were limited to two genomic regions, but these regions captured both low- and high-recombination areas of euchromatin, and the centromere of chromosome 3, a highly repetitive heterochromatic region. It remains possible that DNA methylation exhibits intergenerational epigenetic inheritance, as the Dnmt2 -/- females were created using a heterozygous (Dnmt2 +/-) mother (Figure 1), but this seems unlikely. Organisms typically undergo one or more rounds of epigenetic reprogramming, in which epigenetic signatures are erased and reprogrammed in the germline and in the early embryo. The failure of this process results in epigenetic inheritance, and although there is evidence of this occurring, especially in plants, it is not thought to be a widespread phenomenon (Heard and Martienssen 2014). As such, DNMT2 appears to have no major effect on rates of recombination in specific regions of the genome, and DNA methylation more generally also may not affect recombination in Drosophila.

The presence of DNA methylation in Drosophila and other Dnmt2-only systems is a long-debated issue (Schafer and Lyko 2010; Raddatz et al. 2013). Although the protein DNMT2 shows strong sequence and structural conservation to established methyltransferases, the enzymatic activity was found to be much weaker (Okano et al. 1998; Dong et al. 2001). Various studies reporting DNA methylation in Drosophila (Tweedie et al. 1999; Gowher et al. 2000; Lyko et al. 2000; Lyko 2001; Lyko et al. 2003a,b; Marhold et al. 2004a,b; Salzberg et al. 2004; Phalke et al. 2009; Krauss and Reuter 2011) may have been confounded by contaminations from other organisms, detection limits, low antibody specificity, and/or false positives (Zemach et al. 2010; Raddatz et al. 2013). More advanced bisulfite sequencing with thorough controls even questioned the existence of DNA methylation in Drosophila (Raddatz et al. 2013).

Alternatively, a new study that enriched methylated DNA through size, the power to detect small differences in recombination rate is naturally decreased; (power = 0.8, significance level 0.05; N= 200, d = 0.28; N = 152, d = 0.32), although the results are consistent with the increased sample size results from the euchromatic region. I therefore conclude that the DNA methyltransferase DNMT2 has no detectable effect on recombination in D. melanogaster.

Table 1 Genetic markers used to score recombination

| Marker Name | Primer Name          | Physical Distance/Genetic Distance Between Markers | Sequence (F/R) |
|-------------|----------------------|---------------------------------------------------|---------------|
| Marker X1   | DMELX_494471F        | Marker X1-Marker X2: 2.75 Mb/5 cM                 | CGAGCGGCTGTCTATTGCGTC |
|             | DMELX_494621R        |                                                   | TCATTTCATTAGTGTTGCACATGAG |
| Marker X2   | DMELX_3240050F       | Marker X2-Marker X3: 2.68 Mb/10 cM                | GAAACAGTGTATTGCGCAACATGAG |
|             | DMELX_32400200R      |                                                   | CTTGCGCAATTGTCACATGAGTAC |
| Marker X3   | DMELX_5922532F       | Marker X1-X3: 5.43 Mb/15 cM                       | GAGTCGTTGCGATCGGATAGACCT |
|             | DMELX_5922673R       |                                                   | CCGCTCATTGACATGACCGGCT |
| Marker Chr2 | DMEL2L_12024260F     | NA                                                | CGTCAATTCATTGAACATCTTG |
| Marker 3L*  | DMEL3L_16327010F     | Marker 3L-Marker 3R: 12.75 Mb/4 cM                | GATTCACTCGTGAGCTAGATGAGC |
| Marker 3R*  | DMEL3R_4531346F      |                                                   | CGCCCTTGAGAAAAGTGCAACGT |
| Marker 3R*  | DMEL3R_4531276R      |                                                   | CACCCCTGAGAAAAGTGCAACGT |

NA, not available.

a Used in conjunction with the restriction enzyme BsoBI. See the section Materials and Methods.
immunoprecipitation before bisulfite conversion has revealed a unique and dynamic genomic methylation pattern, suggesting that previous studies (Zemach et al. 2010; Raddatz et al. 2013) lacked sufficient coverage to detect methylation (Takayama et al. 2014). This most recent study presents strong evidence for an unidentified de novo methyltransferase in *Drosophila*, showing that genomic methylation persists in the absence of DNMT2 (although with altered patterns of DNA methylation) (Takayama et al. 2014).

The finding that there is no effect of the gene *Dnm2* on meiotic recombination supports recent data and points to other epigenetic mechanisms directing recombination in *Drosophila*. Indeed, there is some evidence that histone modifications in *Drosophila* may mimic the role of DNA methylation in transcriptional processes in other invertebrates (Cedar and Bergman 2009; Chodavarapu et al. 2010; Nanty et al. 2011; Hunt et al. 2013). Additionally, although DNA methylation was reportedly involved in specific transposons in *Drosophila* (Phalke et al. 2009), the small RNA Piwi-piRNA pathway is known to be the main genome defense system against repetitive elements in the germline (Aravin et al. 2007; Brenneneck et al. 2007; Blumenstiel 2011).

Clearly, *Dnm2* and *Drosophila* DNA methylation research has experienced a tumultuous 20 years, although new research appears to challenge the role of DNA methylation in transpositional processes in other invertebrates (Cedar and Bergman 2009; Chodavarapu et al. 2010; Nanty et al. 2011; Hunt et al. 2013). Additionally, although DNA methylation was reportedly involved in specific transposons in *Drosophila* (Phalke et al. 2009), the small RNA Piwi-piRNA pathway is known to be the main genome defense system against repetitive elements in the germline (Aravin et al. 2007; Brenneneck et al. 2007; Blumenstiel 2011).

**ACKNOWLEDGMENTS**

I thank Bret Lesavoy, Leigh Raskauskas, and particularly Brenda Manzano-Winkler for genotyping help and Ting Lu for the Zim29 *D. melanogaster* stock. Mohamed Noor provided helpful comments on the manuscript and provided guidance throughout the experiment. This work was supported by a National Science Foundation Doctoral Dissertation Improvement Grant awarded to C.S.S.H.

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Communicating editor: M. C. Zetka