Mutations in the N-terminus of the Mod(mdg4) BTB domain reveal an unexpected role of Mod(mdg4) in chromosome segregation in female meiosis

Gwyneth D. E. Walker and Bruce D. McKee

Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville, Tennessee 37996

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ABSTRACT

Proper segregation of homologous chromosomes during anaphase I of meiosis is essential for ensuring that aneuploid gametes are not produced. Two proteins, MNM and SNM, assist in homolog conjunction in Drosophila males. In order to examine the role of disulfide bonding between BTB domains of Mod(mdg4), mutant Drosophila stocks were created using a co-CRISPR method to target white+ and resulting stocks were phenotypically analyzed. In this study, we show that disruption of a two amino-acid sequence at C10 of the Mod(mdg4)56.3 protein results in multiple phenotypic consequences, including nonlethal male and female nondisjunction and abnormal staining of synaptonemal complexes in germaria. These meiotic disruptions suggest that one isoform of MNM protein plays a role in female meiosis.

INTRODUCTION

Meiosis is a specialized process through which many eukaryotic cells gain genetic variation through the recombination of homologous chromosomes. During meiosis, DNA is replicated in a single round, followed by two rounds of chromosome segregation. It is during this first round of segregation that homologous chromosomes synapse and recombine, occurring during prophase I. During this stage, the homologous chromosomes must be aligned and held together properly to ensure that aneuploid gametes are not produced through missegregation of homologs during anaphase I (Page and Hawley 2003; McKee 2004). In many eukaryotes, these homologs are linked via synaptonemal complexes until meiotic recombination occurs, during which chiasmata are generated (Roeder 1997; Page and Hawley 2003, 2004). The formation of

chiasmata is essential for linking homologs until their segregation during anaphase I (Hawley 1988; Carpenter 1994).

In some species, meiosis occurs without recombination or chiasmatic linkage mechanisms. This is true for Drosophila males, in which homologs are paired through midprophase I. Despite their lack of synaptonemal complexes and chiasmata, the homologs must remain connected through another mechanism until reaching anaphase I, and these stable connections are now understood to be the result of the two proteins Modifier of Mdg4 in Meiosis (MNM) and Stromalin in Meiosis (SNM) (Thomas et al. 2005, M. Soltani-Bejnood et al. 2007). During prometaphase I and metaphase I, MNM and SNM colocalize to the pairing region of the bivalent chromosomes before disappearing at the start of anaphase I when bivalents are segregated (M. Soltani-Bejnood et al. 2007). Despite the discovery of MNM and SNM, many of the specifics of the molecular mechanisms for homolog conjunction remain undiscovered.

A further analysis into the protein MNM suggested that the BTB domain of the N-terminal common region of MNM was required for proper X-Y segregation in male Drosophila (M. Soltani-Bejnood et al. 2007). The mod(mdg4) locus gives rise to 31 unique chromosomal proteins, including MNM, which include a common 402-amino-acid N terminus but varying C termini resulting from alternatively spliced exons within the variable region (M. Soltani-Bejnood et al. 2007). The MNM protein includes a unique 100 amino acid C terminus and is expressed only in primary spermatocytes. Within this common region is an N-terminal BTB/POZ domain, which assist in protein homodimerization and multimerization. This BTB domain closely resembles the Drosophila GAGA factor, a transcription regulator with a C-terminal C2H2 zinc-finger motif in addition to an N-terminal BTB domain (M. Soltani-Bejnood et al. 2007). Similar to the multimeric complexes that the GAGA factor BTB-BTB interactions hold together,

Mod(mdg4) proteins, specifically MNM, encode specificity for interaction partners of the conjunction complex through formation of nuclear foci resulting from coalescence of multiple Mod(mdg4)-containing complexes (Gerasimova and Corces 1998; Gerasimova et al. 2000; Gause et al. 2001; Ghosh et al. 2001; Thomas et al. 2005).

MNM contains a zinc-finger sequence that is different from Mod(mdg4)67.2 and may be a DNA binding domain; however, it is also possible that the zinc-finger interacts with other proteins, or itself. The importance of these interactions for typical meiotic function is not well studied. Within the amino acid sequence for Mod(mdg4)56.3 is a cysteine at position 10. Due to dimer-dimer interactions, two monomeric cysteines are left adjacent to each other between two MNM dimers. For this interaction to occur, the MNM dimers must form a parallel structure to allow the cysteines to be adjacent. This pair of cysteines can then form a disulfide bond, providing stability for the MNM protein. This leaves the question of whether the disulfide bond and resulting stability of the MNM protein plays a role in its meiotic function within the conjunction complex.

A BTB domain similar to that of MNM is highly conserved in other Drosophila proteins, with a large subclass of these domains containing a leucine at position 9. Many of these BTB domains have a cysteine at position 8 instead of position 10, including a BTB zinc-finger protein that interacts strongly with MNM (Weber *et al.* 2020). Because the Mod(mdg4) BTB domain can interact with other BTB domains and form stable noncovalent bonds, it is assumed that a second BTB protein could interact with Mod(mdg4) and form structures stabilized by disulfide bonds. If this is the case, a multimeric protein complex could be formed to wrap and bundle 4 chromatids, thus playing a vital role in the conjunction complex and meiotic process as a whole. In order to investigate the plausibility of this occurrence, the cysteine at position 10 of the MNM protein

was mutagenized in the native copy of the gene and meiotic segregation in resulting mutant Drosophila strains was analyzed.

In this paper we examine the role of disulfide bonding within the MNM Mod(mdg4) conjunction complex protein in Drosophila male and female meiosis and show that, while the intended cysteine to serine mutation does not result in meiotic disruption, the unintended six base pair deletion at the mutation site of one of the mutant Drosophila cultures resulted in both male and female nonlethal meiotic nondisjunction. This suggests that one of the isoforms of the MNM protein plays a role in Drosophila female meiosis.

MATERIALS AND METHODS

Generation of mutant Drosophila: First, to ensure that the guide RNAs and donor DNA matched the target sequence, a 520bp region centered on the target cysteine codon in the mod(mdg4) gene was amplified from genomic DNA of the injection stock y^1 w^+ M{vas-Cas-9} Zh-2A stock (Bloomington Drosophila Stock Center #66554) using PCR primers Mod520-F and Mod520-R (Table 1). PCR products were confirmed with agarose gel electrophoresis, and then combined and purified using the Qiagen PCR purification kit. DNA was eluted and subcloned into the pAGM1311 vector (provided by S. Marillonett (Weber et al. 2011) via Addgene) using the New England BioLabs Golden Gate Assembly kit. Once cultured, plasmid samples were prepared according to the Qiagen Spin Miniprep kit and sequences were obtained by Sanger Sequencing using the sequencing primers 1311-F and 1311-R (Table 1), which are complementary to sequences immediately upstream and downstream of the cloning site in

pAGM1311. The sequences of four clones were essentially identical and well-matched to the genome reference sequence except for a 35bp insertion in the upstream intron.

A transcription unit was assembled in the pAGM8079 (C8) vector (provided by S. Marillonnet (Weber et al. 2011) via Addgene) consisting of the two guide RNA sequences flanked by the promoter and terminator from the Drosophila U63 gene. This was designed to express the guide RNAs constitutively and at high levels. The transcription unit also contained the subcloned product of a different PCR reaction using forward and reverse primers that included the two guide RNA sequences. The template for this PCR product was the clone 9121:gScmut-tRNA(rice) which contains a guide RNA scaffold sequence followed by the sequence of a tRNA from rice (Dang et al. 2015). The forward and reverse primers (Mod-cys-F and Mod-cys-R), which were both 58 bases, start with golden gate cloning sequences, then the 20 base variable sequence of gRNA1 (F primer) or the reverse complement of gRNA2 (R primer) followed by 20 bases of homology to the template (Table 1). The PCR product of this transcription unit was cloned into the pAGM1311 vector to create the clone 1311:TGCA-N20gRNA1-gRNA scaffold-tRNA(rice)-N20gRNA2-GTTT. This clone was assembled along with three other 1311 clones plus the end-linker clone pICH50914 (B9, provided by S. Marillonnet (Weber et al. 2011) via Addgene). The three other clones were i) 1311:CCAT-U63P-tRNA(DmeI)-TGCA, which contains a strong promoter from the Drosophila U63 gene followed by a Drosophila tRNA gene, ii) 1311:GTTT-gRNAscaffold-tRNA(rice)-pol3T-TGTG, which contains a second copy of the gRNA scaffold, a second rice tRNA gene, and the transcription terminator sequence from the U63 gene, and iii) Dummy30:CAGA-CCAT, a linker to connect the CCAT junction in clone i to the CAGA site in vector C8. The end-linker B9 creates a TGTG joint in the vector to match the TGTG joint in clone iii. The transcription unit is

composed of U63P-tRNA-N20gRNA1-gRNAscaffold-tRNA-N20gRNA2-gRNAscaffold-tRNA-terminator. The purpose of the tRNA genes is to allow synthesis of multiple guide RNAs in a single transcription unit (Port et al. 2016). The tRNAs provide cleavage sites for RNaseP, which cuts out each guide RNA (N20 + scaffold) from the transcript. These clones were confirmed with Sanger Sequencing, and a midi-prep was performed according to the Qiagen Plasmid Midi Kit.

In order to increase chances of recovering progeny that contained the missense mutation introduced into the G0 parents, a co-CRISPR approach was used (Levi et al. 2020, Ge DT et al. 2016). Two guide RNAs were selected using the CRISPR Optimal Target Finder website, one to target mod(mdg4) and one to target the white+ gene (Table 1). The mod(mdg4) guide RNA was optimally positioned for targeting C10 as its predicted cut site was immediately upstream of the TGC cysteine codon. There were two predicted off-target sites (sites that match the 12 3' bases of the guide RNA), one on chromosome arm 2R and the other on chromosome arm 3R. The 2R site was of only minor concern because the crossing scheme was expected to remove 2nd chromosomes exposed to the CRISPR procedure. The potential for a second-site mutation on chromosome arm 3R was a more serious concern but the chosen guide RNA was nevertheless judged to be superior to the alternative guide RNAs with no off-site targets, the two nearest of which would have targeted Cas9 to sites 25bp upstream or downstream of the HDR target site.

A single-stranded ultramer DNA oligo was then designed to serve as the donor DNA sequence for the mod(mdg4) edit and ordered from Integrated DNA Technologies. The ultramer was 195 bases, centered on the targeted cysteine codon (C10), and included 94 base and 97 base left and right homology arms that matched the corresponding sequences of the target fly strain as determined by the procedure described above. Base substitutions were introduced at three sites, a T to A substitution at the first position of the codon for C10 to mutate it to serine (TGC-AGC)

and two substitutions at the adjacent upstream codon for L9 to recode it from TTG to CTC. Together, these substitutions introduced a novel PvuII restriction site for molecular screening and also served to substantially alter the seed sequence to reduce the likelihood of Cas9 recutting of successful edits. We could not alter the PAM site (TGG) because it comprises a tryptophan codon with no degenerate sites. An injection mix containing the donor ssODN and the guide RNA transcription unit plasmid, both at 100ng/uL, was prepared and sent to Rainbow Transgenic Flies for microinjection into Drosophila embryos from the w^+ vas-Cas9 stock that expresses Cas9 from the promoter of the germline-specific vasa gene.

Drosophila culture methods and stock generation: Injected larvae were cultured in vials on cornmeal-molasses-yeast-agar medium. As the G0 generation hatched, virgin females and males were collected. G0 virgin females were crossed singly to *w; sna[Sco]/CyO; MKRS, Sb/TM6B, Tb* males. G0 males were crossed singly to *w; sna[Sco]/CyO; MKRS, Sb/TM6B, Tb* virgin females. Once F1 progeny began hatching, cultures were screened for white or red eyes, and vials of all white-eyed progeny were kept. From these vials, F1 Tb or Sb males were selected and crossed singly to 2-3 *w; sna[Sco]/CyO; MKRS, Sb/TM6B, Tb* virgin females. F2 males and virgin females were then crossed in groups to create mutant stocks. All such stocks were initially homozygous for the X-linked white (w) mutation that results in white eyes and heterozygous for either of the 3rd chromosome balancers MKRS, Sb or TM6B, Tb and for the specific mod(mdg4) mutation. However, the balancer was retained only in some stocks since some of the mutations were viable and fertile. Stocks that proved to contain a wild-type mod(mdg4) gene were discarded.

Molecular identification of mutations in MNM: Once stable stocks were established, progeny were examined and 3-5 heterozygous (Tb or Sb) flies were selected for molecular

analysis. Genomic DNA was prepared, and PCR was performed with the modmdg4500-F and modmdg4500-R primers (Table 1). The PCR products were purified and digested with the PvuII enzyme and run on a 1% agarose gel. Stocks that resulted in the desired bands at 246, 309, and 520/555 base pairs were kept. This pattern reflects heterozygosity for a successfully edited allele that contained the PvuII restriction site and for an unedited allele on the balancer without the restriction site. To confirm that the desired mutation was introduced, single male homozygous (Tb+Sb+) progeny from the mutant stocks established were collected. Genomic DNA was prepared, and PCR amplification was again performed with the modmdg4500-F and modmdg4500-R primers. Purified PCR products were run on a 1% agarose gel against PCR products digested with PvuII to compare cut vs uncut samples. Samples that contained bands at 246bp and 309bp but lacked a band at 520/555bp were Sanger Sequenced to confirm the presence of the desired mutation.

2nd Chromosome nondisjunction test: Male homozygous mutants (1c1, 3d2, 7a1) were crossed singly to female C(2)EN, b pr/O females. In these females, both 2nd chromosomes are attached to a single centromere and therefore always segregate together. At meiosis I, roughly equal numbers of 2-2 and nullo-2 (O) eggs are produced. When fertilized by regular haplo-2 sperm, the resulting trisomic (2-2/2) and haplo-2 (2/O) zygotes die early in development. However, if the males generate nondisjunctional diplo-2 or nullo-2 sperm, then viable progeny can result from fertilization of 2-2 eggs by nullo-2 sperm and nullo-2 eggs by diplo-2 sperm. When wild-type males are crossed to C(2)EN/O females, spontaneous nondisjunction yields an average of around 0.1 viable progeny per male. Mutants that cause high frequencies of nondisjunction in male meiosis (such as mm) generate up to 20-25 viable progeny per male

Thomas et al. 2005). A true NDJ frequency cannot be calculated because of the absence of regular progeny, but chromosome 2 NDJ is roughly proportional to progeny per male.

4th Chromosome nondisjunction test: Male homozygous mutants (1c1, 3d2, 7a1) were crossed singly to female C(4)RM, ci^D ey^R/O, an attached-4 chromosome marked with recessive mutations that result in interrupted cross-veins on the wings and reduced eye size. Regular 4th chromosome segregation generates haplo-4 gametes carrying the wild-type alleles of ci and ey, yielding C(4), ci ey/+ + and O/+ + progeny which have normal cross-veins and eyes. Paternal 4th chromosome NDJ or chromosome loss generates nullo-4 gametes. The resulting C(4), ci ey/O progeny express the cubitus interruptus and eyeless mutant phenotypes. Progeny from diplo-4 gametes express the wild-type phenotypes and cannot be distinguished from regular progeny. Chromosome 4 NDJ can be estimated by doubling the frequency of ci ey progeny.

Sex Chromosome nondisjunction(males) test: *w/BsYy+; sna[Sco]/CyO; MKRS, Sb/TM6B, Tb* males were crossed singly to homozygous female mutants (w/w; 1c1, 3d2, 7a1). Male *w/BsYy+; m/MKRS* progeny with the Sb phenotype and male *w/BsYy+; m/TM6* progeny with the Tb phenotype were recovered and crossed with virgin mutant females with the Sb or Tb phenotypes, respectively. *w/BsYy+; m/m (Sb^+or Tb^+)* males of the resulting F2 generation were then crossed singly to females from the C(1)RM, y^2 w^a su(w^a)/O stock. C(1)RM is an attached-X chromosome; these females generate attached-X and nullo-X eggs at roughly equal frequencies. Regular progeny are C(1)RM/$B^S Y y^+$ females with gray body color (y^+), narrow Bar eyes (B^S) and brownish (suppressed white-apricot) eyes and X, w/O males with grey body color, and white round (B^+) eyes. Nondisjunction during meiosis I generates XY and nullo-XY sperm, which yield X, w/$B^S Y y^+$ males with grey bodies and white, narrow Bar eyes and C(1)RM/O females with yellow bodies, black bristles (the y^2 phenotype) and round brownish eyes.

Nondisjunction at meiosis II generates XX sperm, which yield patriclinous X/X females with grey bodies and round white eyes, YY sperm that yield X/Y/Y progeny that cannot be distinguished from regular XY males, and nullo-XY sperm that yield X/O males with grey bodies and round white eyes. To estimate the total NDJ frequency, the patriclinous daughters are doubled (to account for the undetectable YY gametes) and added to the sum of all the other NDJ classes, which sum is then divided by total progeny. Since XO progeny from nullo-XY sperm can result from either meiosis I or meiosis II NDJ, the exact meiosis I and meiosis II NDJ frequencies cannot be determined. However, an estimate of their relative frequencies can be obtained from the ratio of 2x(XX females)/(XYY males), taking advantage of the fact that XX females and XYY males result only from NDJ at meiosis II or meiosis I, respectively.

Female sex chromosome nondisjunction test: To measure X chromosome nondisjunction, female homozygous mutants (1c1, 3d2, 7a1) that were also homozygous for X chromosomes marked with w were crossed to attached-XY y w^+ B/O males. In this cross, regular haplo-X eggs give rise to w^+ B females (X, w/attached-XY, y w^+ B) and w B^+ males (X, w/O). Nondisjunctional diplo-X eggs give rise to w B^+ females (X, w/X, w/O) as well as to lethal X/X/attached-XY progeny. Nondisjunctional nullo-X eggs give rise to y w^+ B males (attached-XY, y w^+ B/O) as well as to lethal O/O progeny. To estimate the nondisjunction frequency, the numbers of B male progeny and B+ female progeny were multiplied by 2 to account for the lethality of X/X/attached-XY and O/O progeny, then divided by the total number of progeny.

Ovary immunostaining: To aid in dissection and immunostaining, females were collected in vials containing medium and yeast paste. Several males were added to the vials. After 3 days, the ovaries were dissected. Ovaries were fixed in a formaldehyde/PBS solution and

blocked with a 1X PBST/BSA solution. Mouse monoclonal anti-C(3)G primary antibody was used at a 1:200 dilution. Alexa-Fluor 488 donkey anti-mouse IgG (H+L, Invitrogen) secondary antibody was used at a 1:1000 dilution. In order to examine formation of the synaptonemal complex in 1c1 mutants, ovaries were stained with DAPI. Slides were mounted using 2% n-propyl gallate, 80% glycerol in 1X PBS.

Microscopy: To image the dissected ovaries, the Zeiss AxioObserver microscope was used with a 40x and 100x lens. Images were taken in z-series, deconvolved and stacked via sum algorithm. For closer examination, images were taken with a Nikon Eclipse inverted microscope at a 100x objective. RGB images were created by combining 16-bit greyscale stacks, and greyscale images were created with 8-bit stacks.

Name	Sequence (5' to 3')
Modcys-Fb	CCTTGGTCTCTACATTGCAACGAGCAATTCAGCTTGTGCGTTTCAGAGCTATGCTGGAAA
White-Rb	CCTTGGTCTCTACAAAAACCCGCGAATTAATAGCTCCTGTGCACCAGCCGGGAATCGAAC
Modmdg4500-F	CCCTTGGTCTCAACATTACACAGTAGGTTGCGGTCA
Modmdg4500-R	CCCTTGGTCTCAACAACACGTAGAGCTACAGATCTG

Table 1. Primers and sequences used to construct the gRNA plasmid to create the genetically modified flies.

	1c1, 3d2, 7a1 Construct Sequences
Locus	Mod(mdg4)
Mod500 sequence including primers	TACACAGTAGGTTGCGGTCATTAAGCGAGATAAATGTAATTTACACACACGCACACACAGA CAGACGCGTGAAAATTTCCCAGTTTTTTTTGGCTGCAGTGCATTCTATTTTGTGTGTGCCT TTGCCATTTTTCGGCACTCGCTTCTGGCTGCTGTAAACAATGTTTTAGTTAGTTGCAGCAT TGAGTTTTAAACGCGCATTGCACTTTTTCGCCTGCAGCCAACAAACGCATAGATACAGAAA AGTATTGATTTTCGTCCAAGATGGCGGACGACGAGCAATTCAGCTTG/**TGC**TGGAA**C**AACT TCAACACGAATTTGTCGGCCGGCTTCCACGAGTCGCTATGCCGCGGCGACCTGGTGGACGT CTCGCTGGCCGCCGAGGGCCAAATAGTGAAGGCCCACCGATTGGTGTTATCCGTCTGCTCG CCCTTCTTCCGCAAGATGTTCACTCAGATGCCGTCGAACACCCACGCTATCGGTGAGTTTG TGCCCCTCAGCTCAGATCTGTAGCTCTACGTGTTGT
gRNA target	ACGAGCAATTCAGCTTGTGCTGG
Designed ssODN	cgcgcattgcacttttcgcctgcagCCAACAAACGCATAGATACAGAAAAGTATT GATTTTCGTCCAAGATGGCGGACGACGAGCAATTCAGC**C**T**CA**GCTGGAACAACTTCAACAC GAATTTGTCGGCCGGCTTCCACGAGTCGCTATGCCGCGGCGACCTGGTGGACGTCTCGCTG GCCGCCGAGGGCCAAAT

Table 2. Sequences for ssODN design. PAM site is underlined, introduced mutation sites are bolded, and gRNA double-stranded breaks are indicated with a slash (/).

RESULTS

Co-CRISPR editing resulted in three separate genotypic mutations: The original goal of using the co-CRISPR editing method was to distinguish between broods more easily from G0 flies with successful genomic editing of the desired site and those that did not have successful editing. This targeting was followed through the eye color of the F1 progeny of injected flies, where broods with all progeny containing white eyes were more likely to also contain the desired cysteine to serine mutation. Out of the parental crosses, 8 cultures of white-eyed F1 broods were created. From these white-eyed broods, 1-8 males, depending on availability in the F1 broods, were selected from each vial and crossed singly with *w; sna[Sco]/CyO; MKRS, Sb/TM6B, Tb* females. A total of 29 F2 generation broods were created from 8 different F1 white-eyed broods. After these broods hatched, F2 males and females were crossed in groups to create stocks. Once stocks were set up, molecular analysis of progeny was performed on 20 stocks. These 20 were

selected based on the availability of Tb or Sb flies in culture. Genomic DNA was prepared and run on an agarose gel, with each stock run as prepared genomic DNA, and genomic DNA digested with PvuII for comparison. Out of the 20 stocks run, 15 showed the desired bands at 230, 309, and 520/555bp while 4 showed no bands and 1 showed a single band at 520/555bp. 10 stocks were kept, 6 of which were created from different white-eyed F1 cultures (1b, 1c, 1f, 2g, 3a, 3c, 3d, 4b, 5a, 7a). These 10 stocks were selected based on phenotypic analysis of the F2 cultures for Tb/Tb+ and Sb/Sb+ markers in order to select predominantly homozygous (Tb+ or Sb+) cultures. The remaining 10 stocks were discarded. Multiple iterations of these 6 stocks had been created by crossing Tb x Tb F2 flies or Sb x Sb F2 flies, and this resulted in final stocks of 1b3Sb, 1c1Tb, 1f1Tb, 3a1Tb, 3a3Tb, 3d2Tb, 3d3Tb, 4b1Tb, 4b2Tb, 7a1Tb, 7a3Tb. Genomic DNA from single homozygous males from each of these 11 stocks was prepared and showed that males from stocks 1b3Sb, 1c1Tb, 1f1Tb, 4b1Tb, and 4b2Tb contained a six base pair deletion predicted to remove L9 and C10 without altering the reading frame, males from stocks 3a1Tb, 3a3Tb, 3d2Tb, and 3d3Tb contained both the cysteine to serine mutation and L9 mutation, and males from stocks 7a1Tb and 7a3Tb contained the C10 mutation but not the L9 mutation (Table 3). After sequencing, the 1c1, 3d2, and 7a1 broods were chosen to represent the three resulting mutations based on number of flies present and overall health of broods, and multiple stocks were made for phenotypic testing.

	Resulting Mutation Sequence
1c1	TACACAGTAGGTTGCGGTCATTAAGCGAGATAAATGTAATTTACACACACGCACACACAGACAG ACGCGTGAAAATTTCCCAGTTTTTTTTGGCTGCAGTGCATTCTATTTTGTGTGTGCCTTTGCCA TTTTTCAGCACTCGCTTCTGGCTGCTGTAAACAATGTTTTAGTTAGTTGCAGCATTGAGTTTTA AACGCGCATTGCACTTTTTCGCCTGCAGCCAACAAACGCATAGATACAGAAAAGTATTGATTTT CGTCCAAGATGGCGGACGACGAGCAATTCAGC------TGGAACAACTTCAACACGAAT
3d2	TACACAGTAGGTTGCGGTCATTAAGCGAGATAAATGTAATTTACACACACGCACACACAGACAG ACGCGTGAAAATTTCCCAGTTTTTTTTGGCTGCAGTGCATTCTATTTTGTGTGTGCCTTTGCCA TTTTTCAGCACTCGCTTCTGGCTGCTGTAAACAATGTTTTAGTTAGTTGCAGCATTGAGTTTTA AACGCGCATTGCACTTTTTCGCCTGCAGCCAACAAACGCATAGATACAGAAAAGTATTGATTTT CGTCCAAGATGGCGGACGACGAGCAATTCAGC**C**T**CA**GCTGGAACAACTTCAACACGAATTTGTC GGCCGGCTTCCACGAGTCGCTATGCCGCGGCGACCTGGTGGACGTCTCGCTGGCCGCCGAGGGC CAAATAGTGAAGGCCCACCGATTGGTGTTATCCGTCTGCTCGCCCTTCTTCCGCAAGATGTTCA CTCAG
7a1	TAAGCGAGATAAATGTAATTTACACACACGCACACACAGACAGACGCGTGAAAATTTCCCAGTT TTTTTTGGCTGCAGTGCATTCTATTTTGTGTGTGCCTTTGCCATTTTTCAGCACTCGCTTCTGG CTGCTGTAAACAATGTTTTAGTTAGTTGCAGCATTGAGTTTTAAACGCGCATTGCACTTTTTCG CCTGCAGCCAACAAACGCATAGATACAGAAAAGTATTGATTTTCGTCCAAGATGGCGGACGACG AGCAATTCAGCTT**CA**GCTGGAACAACTTCAACACGAATTTGTCGGCCGGCTTCCACGAGTCGCT ATGCCGCGGCGACCTGGTGGACGTCTCGCTGGCCGCCGAGGGCCAAATAGTGAAGGCCCACCGA TTGGTGTTATCCGTCTGCTCGCCCTTCTTCCGCAAGATGTTCACTCAGATGCCGTCGAACACCC ACGCTATCGGTGAGTTTGTGCCCCCTCAGCCTATGCCGTCGAACACCCACGCTATCGGTGAGTT TGTGCCCCCTCAGCCTC

Table 3. Sequences resulting from molecular analysis of mutant stocks. Mutations are bolded.

1c1 and 7a1 exhibit 2nd chromosome NDJ in male meiosis: Male Tb+ mutants from each of the three established stocks were crossed singly with C(2)EN virgin females in order to assess 2nd chromosome nondisjunction (NDJ). In these crosses, the presence of progeny indicated that NDJ was occurring. The large amount of progeny from 1c1 and 7a1 suggest that male meiosis NDJ is occurring, and consequently, the overall lack of progeny per male in the 3d2 crosses indicates that normal DJ is occurring. Furthermore, both the 1c1 and 7a1 progeny of these crosses has varying phenotypic traits, with some progeny having orange eyes and a light body color, while some retained the purple eyes and black body from C(2)EN mothers (Table 4). Progeny with orange eyes and light body carry two 2nd chromosomes from the father, while

progeny with purple eyes and black bodies carry the C(2)EN chromosome from the mother as a result of a nullo-2 sperm fertilizing a C(2) egg.

	Total Progeny	**Orange Eyes, Light Body**	**Purple Eyes, Black Body**	**W, Black Body**	**Total Males Tested**	**Progeny per Male**
1c1	225	39	183	3	23	9.78
3d2	2	1	1	0	25	0.08
7a1	37	14	23	0	14	2.64

Table 4. Phenotypic analysis of male mutant 2nd chromosome NDJ.

1c1 exhibits 4th chromosome NDJ in male meiosis: Another test for NDJ in male meiosis was to singly cross mutant homozygous males from the generated three stocks to C(4)RM, ci^{D} ey^{R} virgin females and assess progeny for eye size – ey^{R} is a recessive mutation that causes small eyes. It is important to note that the presence of progeny in these crosses does not represent NDJ, and progeny must be phenotypically scored to identify the occurrence of NDJ. 1c1 mutants produced larger numbers of progeny with the smaller eye mutation than 3d2 or 7a1 mutants, suggesting that there is 4th chromosome NDJ occurring in the 1c1 mutants (Table 5).

	Total Progeny	**Regular Eye Size**	**Smaller Eye Size**	**%NDJ**
1c1	160	141	19	23.75
3d2	244	242	2	1.64
7a1	68	68	0	0

Table 5. Phenotypic analysis of male mutant 4th chromosome NDJ.

1c1 and 7a1 mutations exhibit NDJ in female meiosis: The first determination of female fertility for all of the mutants was crossing homozygous female mutants with attached XY, y B/O

males and assessing vials for presence of progeny. All three mutant stocks, 1c1, 3d2, and 7a1 produced F1 generations in these crosses, signifying that all three female mutants were fertile. Secondly, these crosses assess for NDJ occurring during female meiosis. Female progeny with the Bar mutation and white+ wild-type eye are phenotypically representative of normal DJ, as are male progeny with Bar+ wild-type and white eye mutation. However, female progeny with B+/w eyes and male progeny with B/w+ eyes are phenotypically indicative of X chromosome NDJ. The percent of NDJ per mutation was evaluated by adding the number of female B+ progeny and number of male B progeny and then dividing by the total number of progeny (Table 6). 1c1 and 7a1 mutant stocks showed similar levels of sex chromosome NDJ in male meiosis, with 3d2 mutants showing very low percentages of NDJ comparatively (Table 7).

	Total Progeny	**w+,B female**	**w,B+ female**	**w+,B male**	**w,B+ male**	**%NDJ**
1c1	327	152	8	9	158	10.40
3d2	843	390	0	2	451	0.47
7a1	223	100	5	3	115	7.17

Table 6. Phenotypic analysis of female mutant X chromosome NDJ

	Total Progeny	**w+,B Female**	**w,B+ Female**	**w+,B Male**	**w,B+ Male**
1c1	5	2	0	0	3
3d2	38	15	0	1	22

Table 7. Phenotypic analysis of male mutant sex chromosome NDJ between w/BSYy+; m/m males and C(1)RM, y^2 w^a su(w^a)/O virgin females.

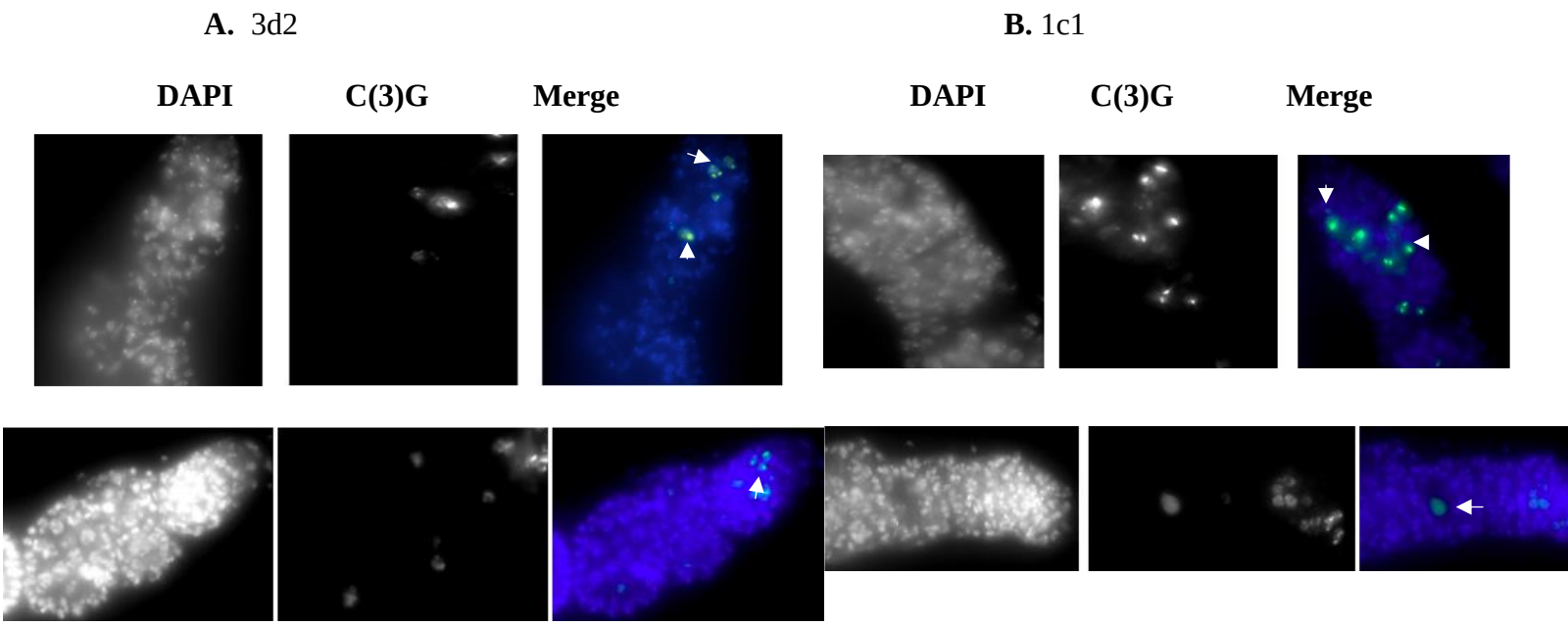


Figure 1. anti-C(3)G staining of nuclei in 3d2 control and 1c1 mutant.

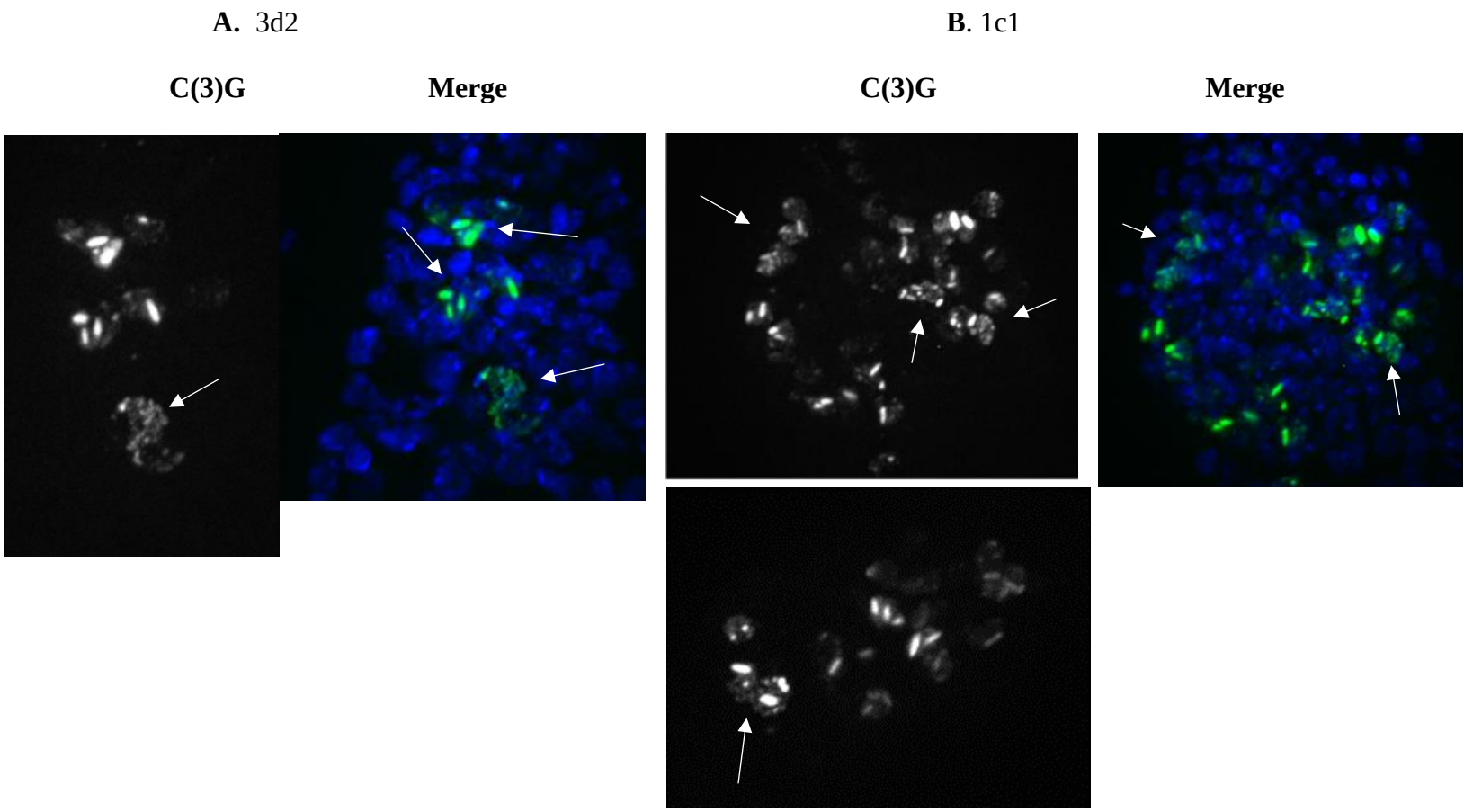


Figure 2. Confocal imaging of anti-C(3)G staining in 3d2 control and 1c1 mutant female ovariole nuclei.

C(3)G localizes to the germaria in 1c1: At the 100x level of magnification of the Zeiss microscopy images, the main similarity between the 3d2 control and the 1c1 mutant images is that C(3)G localizes to germaria in both (Figure 1). One apparent difference is the presence of thread-like staining along the chromosomes in region 2 of the germarium of the 1c1/1c1 mutant. In the confocal imaging at 100x, the lowest of the three nuclei in the 3d2 control sample shows thread-like staining of individual homolog pairs (see arrows). Similarly, in the 1c1 mutant confocal images at 100x, there are at least three nuclei with clear thread-like staining (see

arrows). The lower C(3)G 1c1 image also shows thread-like staining (see arrows). While full length thread-like staining of multiple homolog pairs is evident in the 3d2 merge image, full length staining is less clear in the mutant images; however, the appearance of thread-like staining in all five of these images suggests that both 3d2 and 1c1 do form synaptonemal complexes. Both 3d2 and 1c1 images show C(3)G localization in multiple stages and regions of the germarium. The most apparent difference between the 3d2 and 1c1 mutant images is the multitude of the staining in 1c1. While both 3d2 and 1c1 samples show bright cylindrical and spherical staining, compared to 3d2, 1c1 germaria show more stained structures, indicating that more cells are stained in the mutant and more SC are forming.

DISCUSSION

While the initial goal of this study was to identify the role of cysteine disulfide bonds within the mod(mdg4) locus of the Drosophila meiosis conjunction complex, it was determined that the mutants containing the desired mutations (3d2) showed no abnormal meiotic phenotypes through cross analysis or ovariole staining. However, mutants with a two amino-acid deletion at the mutation site (1c1) were found to have significant male and female NDJ, as well as atypical staining of SC formation in the germaria. As the MNM protein has thirty isoforms, it is plausible to conclude that at least one of these isoforms plays a role in female meiosis, as mutations in MNM do not affect female meiosis (Soltani-Bejnood et al. 2007).

The results of the NDJ crosses indicate that mod(mdg4) is required for chromosome segregation in both male and female meiosis. Specifically compared to 3d2, where no NDJ was found, and 7a1, where mild NDJ results were found, it is likely that the degree to which the

mod(mdg4) locus is mutated or deleted directly correlates to the level of successful meiotic function. The staining of the germaria confirms that C(3)G does localize to the nuclei of both 3d2 and 1c1, and shows no significant differences between the two ovarioles, suggesting that the 6bp deletion of 1c1 does not impair homolog synapsis. Therefore, the apparent NDJ must result from a defect in another step in chromosome segregation. One possibility is that, due to the prominence of staining in the 1c1 mutants, mod(mdg4) may be required to limit the number of cells per cyst that develop synaptonemal complexes.

The mutagenesis procedure used worked well using an alternative cloning method; however, there were two results posing potential issues. The first was the unexpected 35bp insertion in the upstream intron, and the second was the possibility for the second-site off-target mutation on chromosome arm 3R. The 35bp insertion was not a concern, as it was bypassed by sequencing genomic DNA only with the reverse modmdg4500 primer to avoid the the upstream region containing the insertion. Conversely, the second-site mutation is a remaining concern. Because the site lies within the six-banded gene (Flybase), which encodes a chromatin-binding protein, it is possible that the phenotypes of 1c1 and 7a1 mutants are due to this possible second-site mutation. However, the exact sequence that matches the target site is in an intron of six-banded, so that lowers the likelihood that the 3R mutation is the cause of the phenotypic expression. To further investigate this possibility, 1c1, 3d2, and 7a1 mutants can be crossed with a stock containing Df(3R)T16, which deletes the mod(mdg4) locus. So far, the results of these crosses for 1c1 support a mod(mdg4) mutant phenotype, not a second-site phenotype.

The use of the co-CRISPR method greatly improved accuracy and efficiency of phenotypic screening of mutant progeny. As seen in the restriction digest and gel electrophoresis results, co-CRISPR targeting allowed for a high success rate in culturing stocks with the desired

mutation. Out of the 20 stocks analyzed, 4 showed no bands, suggesting that the PCR reaction failed. Not considering these failed reactions, the co-CRISPR targeting was unsuccessful for 1 out of 16 samples, leaving 15 samples to be heterozygous for the mutant chromosome and resulting in a 94% successful targeting rate. This method saved time and resources from excessive culture setting, as broods could quickly be screened visually for eye phenotype.

This experiment was not free from error, the largest of which was neglect for collecting all results throughout the experiment. In the beginning, total numbers of parental, F1, and F2 crosses were not counted, as well as ratios of red, white, and mixed-eye color F1 broods. Furthermore, more careful phenotypic screening of set crosses could have been conducted, such as examining vials of female mutant X chromosome NDJ for Tb progeny to avoid contamination from Tb females. More careful scoring of all crosses could have been conducted to avoid contamination. Lastly, careful monitoring of G0 flies would have improved numbers of parental crosses set, as many G0 virgin females were lost due to breeding with brothers before they could be isolated for crosses with *w; sna[Sco]/CyO; MKRS, Sb/TM6B, Tb* males.

Further directions for this project would include completing crosses with the Df(3R)T16 stocks to examine results of mod(mdg4) locus deletion, specifically fertility, viability and NDJ. Mutants could also be crossed to phenotypically examine the role of mod(mdg4) in position effect variegation and as a chromatin insulator, specifically in the mutants created in this experiment.

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