Mutations in *mx*c Tumor-Suppressor Gene Induce Chromosome Instability in *Drosophila* Male Meiosis

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ABSTRACT. *Drosophila* Mxc protein is a component of the histone locus body (HLB), which is required for the expression of canonical *histone* genes, and severe *mx*c mutations generate tumors in larval hematopoietic tissues. A common characteristic of cancer cells is chromosomal instability (CIN), but whether *mx*c mutants exhibit this feature is unknown. Here, examination of post-meiotic spermatids created after male meiosis revealed that a fraction of the spermatids in hypomorphic *mx*c^{G46} mutants contained extra micronuclei or abnormally sized nuclei, corresponding to CIN. Moreover, we observed that the so-called lagging chromosomes retained between chromosomal masses separated toward spindle poles at telophase I. Time-lapse recordings show that micronuclei were generated from lagging chromosomes, and the abnormal chromosomes in *mx*c^{G46} mutants lacked centromeres. In normal spermatocyte nuclei, the HLB component FLASH colocalized with Mxc, whereas FLASH was dispersed in *mx*c^{G46} spermatocyte nuclei. Furthermore, we observed genetic interactions between Mxc and other HLB components in meiotic chromosome segregation, which suggests that inhibition of HLB formation is responsible for aberrant chromosome segregation in *mx*c^{G46}. Quantitative real-time PCR revealed that canonical *histone* mRNA levels were decreased in *mx*c^{G46}. Lastly, similar meiotic phenotypes appeared in the spermatids of *histone H4* mutants and in the spermatids in testes depleted for chromosome-construction factors. Considering these genetic data, we propose that abnormal chromosome segregation leading to CIN development results from a loss of chromosome integrity caused by diminished canonical histone levels in *mx*c mutants.

Key words: Chromosome instability, *Drosophila*, meiosis, tumor-suppressor gene

Introduction

Genomic instability is a characteristic of most cancer cells. Human cancer cells present at least two classes of instability: Microsatellite instability (MIN) and chromosomal instability (CIN) (Lengauer *et al.*, 1997). CIN is characterized by chromosome structural changes and number variations and is detected in several types of cancer cells. Conversely, MIN is the condition of genetic hypermutability that results from impaired DNA mismatch repair in cells retaining normal ploidy (Lengauer *et al.*, 1997; Negrini *et al.*, 2010). CIN can occur owing to diverse mitotic defects, such as sister-chromatid cohesion defects, merotelic centromere attachment, and defects of the spindle-assembly checkpoint (SAC) that monitors proper attachment between

the kinetochore and microtubules (Kops *et al.*, 2005; Gisselsson, 2008; Holland and Cleveland, 2009). Large-scale genome sequencing of several cancer cells from various species has revealed the most commonly mutated genes in cancer cells (Orr and Compton, 2013; Jones *et al.*, 2008). Several of these genes have been shown to encode proteins involved in cell cycle control and cell signaling pathways involved in cell growth and death have been identified (Cahill *et al.*, 1998; Orr and Compton, 2013; Roschke and Rozenblum, 2013). The human *Bub1* gene, a SAC gene, was first identified as mutated in human colorectal cancer cells showing CIN. Moreover, the mutations result in activity that makes cells with normal mitotic checkpoints defective (Cahill *et al.*, 1998). CIN is consistently associated with loss of a mitotic checkpoint function. SAC is a checkpoint that monitors proper attachment between chromosomes and microtubules (Holland and Cleveland, 2009; Kops *et al.*, 2005). The checkpoint is activated during prometaphase and, then, inactivated when all kinetochores on chromosomes are captured by microtubules. *Bub1* plays a role as an inhibitor that delays the metaphase to anaphase transition (Weaver and Cleveland, 2005). Unexpectedly, a

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Abbreviations: CIN, chromosomal instability; HLB, histone locus body; MIN, microsatellite instability; Pc-G, polycomb group; qRT-PCR, quantitative real-time PCR; SAC, spindle-assembly checkpoint.

few mutations in genes encoding proteins involved in chromosome segregation in mitosis have been identified in cancer cells (Negrini *et al.*, 2010). Other candidates that contribute to aneuploidy representing CIN in hereditary cancers are mutations in DNA repair genes such as BRAC1, RAD50, NBN, WRN, and BLM (Kennedy and D'Andrea, 2006; Ripperger *et al.*, 2009; Bachrati and Hickson, 2003; Negrini *et al.*, 2010). In sporadic cancers, however, mutations in DNA repair genes have been infrequently found in recent high-throughput cancer sequencing studies. Mutations in DNA damage checkpoint genes such as p53, ATM, and cyclin-dependent kinase inhibitors have been more frequently detected in the genome of cancer cells (Negrini *et al.*, 2010; Shiloh, 2003). In addition, deregulation of 12 oncogenic signaling pathways, such as RB, Wnt, and Hippo-mediated pathways, is closely interrelated with CIN in pancreatic cancers and glioblastomas (Orr and Compton, 2013).

Mutations or down-regulation of mitotic checkpoint genes have been found less frequently than expected in human cancer cells characterized by CIN. However, after apoptosis inhibition, *bub3*-depleted imaginal disc cells in *Drosophila* display neoplastic growth, CIN phenotype, and higher cell proliferative potential. A SAC gene, *bub3* plays a role as tumor suppressor in *Drosophila* imaginal discs in a condition of apoptosis inhibition (Silva *et al.*, 2013). Another mitotic checkpoint gene in *Drosophila*, *atm*, was identified as a genome instability-inducing gene. This gene is required for telomere maintenance in *Drosophila* cells, and its mutants exhibit CIN caused by aberrant telomere fusion (Oikemus *et al.*, 2004). Moreover, *polo* mutants also exhibit CIN in male meiosis as well as in mitosis (Sunkel and Glover, 1988). Polo is a protein kinase required in several stages of *Drosophila* cell division such as the recruitment of crucial components of the centrosome (Archambault and Glover, 2009; Llamazares *et al.*, 1991; Novak *et al.*, 2016). Aurora A is another kinase required for the centrosome cycle (Glover *et al.*, 1995; Inoue and Glover, 1998; Merigliano *et al.*, 2019). Centrosome dysfunction caused by *polo* or *aurA* mutations also results in the generation of malignant neoplastic cells in *Drosophila* (Castellanos *et al.*, 2008; Glover, 2005).

Lethal mutants of the tumor-suppressor gene *mxc* show the malignant blood-neoplasm phenotype in *Drosophila* larval hematopoietic organs (Gateff, 1994; Remillieux-Leschelle *et al.*, 2002; Shrestha and Gateff, 1982). *mxc* was initially reported as a member of Polycomb-group (Pc-G) gene family (Saget *et al.*, 1998; Santamaria and Randsholt, 1995). The Pc-G proteins assemble a protein complex required for the modification of nucleosomes composed of core histones. The hypomorphic *mxc* mutants showed a reduction in gonad size in both males and females, suggesting that the mutations affect germ cell proliferation (Docquier *et al.*, 1996). Furthermore, the mammalian nuclear protein NPAT, encoded by the ataxia telangiectasia-

mutated locus, was shown to be a functional ortholog of *Drosophila* Mxc (White *et al.*, 2011). NPAT expression is enhanced by E2F, a key transcription factor phosphorylated by cyclin E-CDK2 at the G1/S boundary, and this upregulates the transcription of replication-dependent *histone* genes (Gao *et al.*, 2003). Both Mxc and NPAT are components of the Histone Locus Body (HLB), a nuclear body associated with the *histone* gene cluster; HLB is required for the transcription and pre-mRNA processing of *histone* genes (Marzluff *et al.*, 2008; Nizami *et al.*, 2010; Duronio and Marzluff, 2017). In *Drosophila*, the HLB complex is formed in a hierarchical manner, and Mxc is recruited on the *histone* gene cluster as a component of the initial HLB; the complex is commonly required for the transcription of five canonical *histone* genes (Salzler *et al.*, 2013; Terzo *et al.*, 2015; White *et al.*, 2011; Yang *et al.*, 2014).

Here, we investigated the appearance of the CIN phenotype in post-meiotic cells of *mxc* mutant males. *Drosophila* male meiosis provides a highly suitable experimental system for investigating CIN because of these reasons: First, the primary spermatocytes undergoing meiosis I allow precise observation of chromosome construction because the cells are considerably larger than somatic cells, and, second, a post-meiotic cell, termed onion-stage spermatid, harbors a single nucleus and a single mitochondrial derivative; this characteristic cell enables precise examination of meiotic defects, if any, and the nucleus size in post-meiotic spermatids depends on their DNA content (Castrillon *et al.*, 1993; González *et al.*, 1989; White-Cooper, 2004; Inoue *et al.*, 2012; Ichihara *et al.*, 2007; Kitazawa *et al.*, 2012). If meiotic defects occur during chromosome segregation and cytokinesis, post-meiotic spermatids exhibit abnormally sized nuclei and contain multiple micronuclei, respectively (Inoue *et al.*, 2012; Tanabe *et al.*, 2017). Furthermore, spermatids can be observed in testes depleted of cell-division factors by inducing testis-specific expression of dsRNAs against mRNAs encoding specific factors by using a Gal4/UAS system (Duffy, 2002; Inoue *et al.*, 2012; White-Cooper, 2012).

By using time-lapse imaging, we demonstrated that the extra micronuclei and abnormally sized nuclei in the *mxc* mutants originated from lagging chromosomes. The formation of HLB was disrupted in *mxc*^{G46} spermatocyte nuclei. We also found genetic interactions between Mxc and other HLB components in meiotic chromosome segregation. Considering these data, we speculate that the failure of HLB formation results in abnormal chromosome segregation in *mxc*^{G46}. Quantitative real-time PCR (qRT-PCR) demonstrated that canonical *histone* mRNA levels were decreased in *mxc*^{G46}. Here, we propose a hypothesis regarding the role of CIN appearance in the post-meiotic cells of the *mxc* mutant. Our findings indicate that *mxc* is required for proper chromosome segregation during *Drosophila* male meiosis.

Methods

Drosophila stocks

Two hypomorphic alleles for *mx*c gene on the X chromosome, *mx*c^{G46} and *mx*c^{G43} (Santamaria and Randsholt, 1995) were obtained from Bloomington Stock Center. To investigate genetic interactions with *mx*c, we used following mutants; *Lsm10*⁰⁶⁶¹⁶ (Godfrey *et al.*, 2009), *snRNA:U7*^{EY11305} (Godfrey *et al.*, 2006), *coil*^{B220} (Liu *et al.*, 2009), *mute*^{EY22147} and *FLASH*^{L01602} (Rajendra *et al.*, 2011). *GFP-βTubulin*, *RFP-βTubulin*, and *mRFP-Histone H2Av* were used as a normal control for cytological imaging and time-lapse observations (Inoue *et al.*, 2004; Kitazawa *et al.*, 2014; Tanabe *et al.*, 2017). *Ub-GFP-cid* (Talbert *et al.*, 2018) was used to visualize centromeres. *Ub-GFP-Mxc* was a gift from Robert J. Duronio (Univ. North Carolina, Chapel Hill). For depletion of *mx*c, *UAS-mxcRNAi*^{HMS000444} was used (Bloomington Stock Center #BL32446). Expression of the dsRNA using *Actin-Gal4* as a driver makes it possible to reduce *mx*c mRNA levels in the larvae by 37.3%. For spermatocyte-specific dsRNA expression in depletion experiments, *UAS-dir2*; *bam-Gal4::VP16* stock was used. *His4*^{Scim} mutation was isolated as one of dominant chromosome inheritance modifier mutations which cause abnormal chromosome segregation in female meiosis (Dobie *et al.*, 2001). To generate hemizygotes for the mutation, we used *Df(2L)ED1462* (KYOTO Stock Center). To induce expression of *His4* mRNA carrying a poly (A) tail at the 3' end, we used *UAS-His4ORF-3xHA*^{F000777} stock from Fly ORF. To deplete DNA polymerase or chromosome construction factors by RNA interference, the following *UAS-RNAi* stocks were used: *UAS-DNApol-α180RNAi*^{HMJ23033}, *UAS-SMC2RNAi*^{GL00440}, *UAS-DNApol-ε255RNAi*^{HMC03330} and *UAS-CAP-D2RNAi*^{GL00523}. *UAS-His3.3B RNAi*^{8989R-2} has been reported to be able to deplete *His3.3B* mRNA efficiently (Umemori *et al.*, 2009). These stocks were obtained from Bloomington Stock Center.

Quantitative real-time PCR (qRT-PCR) analysis

For qRT-PCR analysis, total RNA was extracted from pharate adult flies using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis from the RNA was carried out using a PrimeScript II High Fidelity RT-PCR kit (Takara, Shiga, Japan) with random primers. qRT-PCR was performed using FastStart Essential DNA Green Master (Roche, Mannheim, Germany) and a LightCycler Nano (Roche, Basel, Switzerland). *RP49* was used as a normalization reference (Oka *et al.*, 2015). Relative mRNA levels were quantified using LightCycler Nano software version 1.0 (Roche, Basel, Switzerland). The primers used were as follows: *histone H1* (L: 5'-AGTTGCAACGTCCGCTTC-3', R: 5'-TTGTGCCAGCAGATCCAG-3'), *histone H2A* (L: 5'-GAAGGGAACTACGCAGAGC-3', R: 5'-AGCCAACTCGAGAACCCTCAG-3'), *histone H2B* (L: 5'-TTCGTCAAGGCGATGAG-3', R: 5'-CGAGCGCTTGTGTAGTGA-3'), *histone H3* (L: 5'-GAGACCGAGCTTCTAATCC-3', R: 5'-CTTCCTGCAGAGCCATAACC-3'), *histone H4* (L: 5'-GCGTCATCGCAA

AGTACTGC-3', R: 5'-CCAGATATGCGCTTCACACC-3'). Each sample was duplicated on the PCR plate, and the final results average three biological replicates. For the quantification, the ΔΔCt method was used to determine the differences between target gene expression relative to the reference *RP49* gene expression.

Preparation of post-meiotic spermatids

For evaluation of nuclei in post-meiotic spermatids, we used previously described protocols (Inoue *et al.*, 2004; Tanabe *et al.*, 2017) and viewed using phase contrast microscopy. A pair of testes from pharate adults or newly eclosed adult flies (0–1 day old) were dissected to isolated spermatocyte cysts in Testis buffer (183 mM KCl, 47 mM NaCl, 10 mM EDTA, pH 6.8) and covered with 18×18 mm-coverslip (Matsunami, Osaka, Japan) to flatten the cysts. For observation of nuclear organization in spermatids after meiosis II, cysts of spermatids collected from adult testes in Testis buffer supplemented with DAPI were mildly flattened under a cover slip, and phase contrast micrographs and fluorescence images were successively acquired (Inoue *et al.*, 2004). To observe fixed spermatid samples, we removed the coverslip after freezing the slides, and transferred them into 100% methanol for 5 min. Consequently, the samples were rehydrated in 1× PBS (137.0 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄·12H₂O, 1.8 mM KH₂PO₄) for 10 min and then stained with DAPI. Under a phase contrast microscope, we observed cysts of spermatids after the completion of meiosis II, at the stage called onion stage and a slightly after stage. We scored the spermatids as cells showing meiotic chromosome segregation phenotypes; cells containing dot-like micronuclei and those having abnormally sized nuclei: with diameter less than a half, larger than the double of normal nuclei, which are as large as Nebenkerns.

Live cell imaging of primary spermatocytes

To observe chromosome segregation in living primary spermatocytes, we performed time-lapse observation as described previously (Inoue *et al.*, 2004; Inoue *et al.*, 2012; Tanabe *et al.*, 2017). To trace chromosome segregation and dynamics of microtubules simultaneously in male meiosis, flies expressing *mRFP-Histone H2Av* and *GFP-βTubulin* were used. Testis cells dissected in BRB 80 Buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8) were laid out under mineral oil (Trinity Biotech, Bray, Ireland) filled in open chambers on a clean glass cover slip. Time-lapse imaging of primary spermatocytes was performed under a fluorescence microscope (IX81, Olympus, Tokyo, Japan) outfitted with excitation, emission filter wheels (Olympus, Tokyo, Japan). At each 60-s time interval, near-simultaneous GFP and/or RFP fluorescence images were captured with a CCD camera (Hamamatsu Photonics, Shizuoka, Japan). Image acquisition was controlled through the Metamorph (Molecular Device, Sunnyvale, CA, USA) software.

Immunofluorescence microscopy

Testis cells were fixed according to the method as described above. The slides were permeabilized in PBST (PBS containing 0.01% Triton-X) for 10 min, followed by blocking with 10% normal goat serum in PBS for 30 min at room temperature. Primary antibodies were used at the following concentrations: Polyclonal rabbit anti-FLASH, 1:2000 (a gift of Z. Dominski, The University of North Carolina, Chapel Hill) and polyclonal rabbit anti-GFP, 1:2000 (Thermo Fisher Scientific, Waltham, MA, USA). After incubating over night at 4°C, the slides were repeatedly washed in PBS and subsequently incubated with Goat Anti-Rabbit IgG (H+L) Alexa Fluor 488 or 594 (Thermo Fisher Scientific, Waltham, MA, USA) for 2 h at room temperature. The slides were then washed in PBS for 10 min. The samples were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Microtubules were visualized by expression of *GFP-βTubulin* (Inoue *et al.*, 2004) or *RFP-βTubulin* (Kitazawa *et al.*, 2014) and image acquisition was performed as described above.

Results

Extra micronuclei and abnormally sized nuclei appear in post-meiotic cells in hypomorphic mxc mutants and in testes depleted of mxc

Hemizygotes for the hypomorphic *mxc* mutations *mxc^{G43}* and *mxc^{G46}* showed sterility in both males and females, instead of a malignant phenotype as observed in lymph glands of a lethal allele of the gene *mxc^{mbn1}* (Remillieux-Leschelle, *et al.*, 2002). The *mxc^{G46}* hemizygous males possess somewhat smaller testes containing less spermatid-cysts and fewer cells at the apical region of testis compared with controls (Fig. 1A, 1B). The *mxc^{G43}* was a temperature-sensitive allele. Most of the hemizygotes (2.8% of eclosed sibling male progenies (n=64) from heterozygous females balanced by FM7 chromosome) died at the larval to pharate adult stages at 25°C, whereas more *mxc^{G43}* adult males (30.1% of sibling male progenies (n=108)) could be obtained at 19°C. The hemizygous males reared at 19°C possessed shorter, thinner, and non-coiled testes (Fig. 1C). Most of the mutant males had almost empty testes devoid of living cells or developing cysts at 25°C (Supplementary Fig. 1A, B), whereas certain amount of cells and cysts remained in testes of mutant males reared at 19°C.

Compared with the testes in wild-type males, fewer cells at the tip and fewer cysts could be seen in the *mxc^{G46}* and *mxc^{G43}* (at 19°C) testes. Thus, we carefully examined whether some phenotypes related to chromosome instability, a common characteristic of tumor cells, could be observed in the post-meiotic cells called onion-stage spermatids from the testes of the *mxc* hypomorphic mutants and from *mxc*-depleted testes. First, we confirmed that almost

all onion-stage spermatids in control males contained single nuclei of almost the same size; only 4 among 834 (0.5%) spermatids of the stage (from 12 control males) showed abnormalities in the number and size of nuclei (Fig. 1D). This indicates that both chromosome segregation and cytokinesis during male meiotic divisions occurred properly, because nuclear size in post-meiotic cells increases and decreases according to the amount of chromosomes that the nuclei carry (González *et al.*, 1989).

In contrast to the aforementioned observation, we detected, at low frequencies, spermatids containing micronuclei (arrow in Fig. 1E, 1E') or abnormally sized nuclei (either smaller or larger nuclei) (arrows in Fig. 1F, 1F') in hemizygotes for the mildest allele, *mxc^{G46}* (4.1% among 441 spermatids from 10 flies). These phenotypes typically suggest improper chromosome segregation during male meiotic divisions. In rare *mxc^{G43}* adults obtained at 25°C, we observed almost empty testes devoid of live spermatids (Supplementary Fig. 1A, B), which agrees with a published result (Landais *et al.*, 2014). We found that *mxc^{G43}* is a temperature-sensitive allele. In the mutant males obtained at 19°C, we scored onion-stage spermatids carrying micronuclei or smaller nuclei under this condition; the same meiotic phenotype appeared at a higher frequency (23.7% of onion-stage spermatids (n=779)) from *mxc^{G43}* males reared at 19°C (Supplementary Fig. 2B). However, the effects of this allele were too severe for further investigation to examine its meiotic phenotypes or genetic interaction with other gene mutations, even if the males were reared at 19°C. Notably, these meiotic phenotypes in both *mxc^{G43}* and *mxc^{G46}* mutants were rescued by ubiquitous expression of GFP-Mxc, which can rescue a lethal phenotype of amorphic *mxc* mutants. Fewer than 0.8% of onion-stage spermatids (n>200) from *mxc^{G43}* and *mxc^{G46}* males having the ubiquitous expression of GFP-Mxc reared at 25°C showed the abnormalities (Supplementary Fig. 2C). We also observed spermatids showing the meiotic phenotype in *mxc*-depleted testes (*bam>mxcRNAi*, Supplementary Fig. 2E) (2.6% among 441 spermatids from 17 flies). From these genetic data, we conclude that micronuclei and abnormally sized nuclei suggesting chromosome instability in meiotic divisions appeared as a consequence of a reduction in *mxc* function and expression. We have performed further observations of the meiotic phenotype using the *mxc^{G46}* mutant, rather than *mxc^{G43}*, because more live cells can be observed in its testes.

Micronuclei originated from lagging chromosomes were generated during male meiosis in mxc^{G46}

To identify aberrant meiotic abnormalities with which the production of micronuclei was induced, we examined chromosome distribution and microtubule organization in *mxc^{G46}* spermatocytes undergoing meiosis I. In control testes, all anaphase I cells (64 cells from 13 flies) contained

Fig. 1. Testes and post-meiotic cells, after completion of meiosis II, containing extra micronuclei and abnormally sized nuclei in *mxc^{G43}* and *mxc^{G46}* mutant males. (A–C) Phase-contrast (PC) micrographs of adult testes. Insets in A–C present magnified view of tip region of each testis. (A) Normal testis from the control strain (*w¹¹¹⁸*) contains testis cells at the apical region and developing spermatid cysts in abundance. (B) Testis from the *mxc^{G46}* mutant. The testis retains some cells and cysts. (C) Testis from the *mxc^{G43}* mutant reared at 19°C. The testis contains fewer cells and cysts. Both mutant males possess smaller testes containing fewer apical cells and developing cysts (B, C) in comparison with a control testis (A). Arrowheads indicate apical tip of testes. Bar: 100 μ m. (D–F) Phase-contrast micrographs of spermatids at the onion stage and a slightly later stage. (D'–F') Fluorescence images of the same cells stained with DAPI. DNA is colored in red (D–F) or white (D'–F'). (D–D'') Control spermatid. (E–E'') Spermatid from the *mxc^{G46}* mutant having an extra micronucleus (arrow in E, E', E''). Margin of the cell is encircled by a dotted line. (F–F'') Spermatid from the *mxc^{G46}* mutant having abnormally a smaller nucleus (left arrow in F–F'') and a large nucleus (right arrow in F–F''). A cell containing a smaller nucleus and a larger nucleus is encircled with the dotted line. Bar: 10 μ m.

equally separated chromosomal masses (Fig. 2A, 2B, and 2A', 2B'), whereas in *mxc^{G46}* testes, 11.5% of anaphase I to telophase I cells (78 cells from 17 flies) contained lagging chromosomes (arrow in Fig. 2C, 2C'), unequally separated chromosomal masses (arrow in Fig. 2D, 2D'), and micronuclei at the end of meiosis I (arrow in Fig. 2E, 2E'). These abnormal cells were regarded as a consequence of abnormal chromosome partitioning. Consistently, we found such abnormal cells in 9.5% of 201 anaphase II to telophase II cells in the mutant testes (Fig. 2F, 2F' for a control, arrow in Fig. 2G, 2G'). Next, to determine the process in male meiosis during which the lagging chromosomes and micronuclei were generated, we performed a time-lapse live-imaging analysis that allowed us to trace chromosomal behavior and microtubule dynamics during male meiosis I.

In a control cell (Fig. 3A), sets of homologous chromosomes aligned on the metaphase plate ($t=54'$), and the chromosomes segregated properly ($t=72'$). All meiosis I spermatocytes recorded in the time-lapse imaging underwent proper chromosome segregation ($n=7$). Conversely, we certainly observed abnormal behavior of chromosomes and microtubules during *mxc^{G46}* male meiosis after chromosome congression. At least 2 mutant cells among 9 that we continuously examined using time-lapse imaging displayed similar abnormal chromosome segregation (Fig. 3B). Chromosome alignment appeared to occur properly at metaphase ($t=43'$) but subsequently, lagging chromosomes were generated at early anaphase I ($t=65'$) and they ultimately formed a few chromatin fragments ($t=88'$). It was uncertain whether fragments were linked to each other through

Fig. 2. Aberrant chromosome segregation in meiosis I and II cells from *mxc^{G46}* mutants. (A–G) Fluorescence micrographs of fixed anaphase I cells prepared from adult male testes expressing GFP-tagged Tubulin. Control meiotic cell at anaphase I (A) and telophase I (B). (C) Anaphase I cell from the *mxc^{G46}* male containing lagging chromosomes (arrow) in the middle of separated chromosomes. (D) Early telophase I cell from the *mxc^{G46}* male. Notably less amount of chromosome masses (arrow) are partitioned to the right of the constricting cell, and an excess amount of chromosome mass is on the left. (E) A *mxc^{G46}* spermatocyte at the cytokinesis stage after telophase I. Micronucleus in the daughter cell to the right (arrow). (F) Control telophase II cell. (G) Telophase II cells containing unseparated chromatin masses (arrow in G, G'). (A'–G') DAPI staining. Bar: 10 μ m. Chromosomes and microtubules are colored in red and green, respectively.

under-condensed chromatin fibers. This formation of lagging chromosomes and the presence of unequally separated chromosomal masses represent a unique meiosis-related phenotype of *mxc^{G46}* males.

Lagging chromosomes lacking centromere marker CID are present in meiosis I cells of *mxc^{G46}* males

To ascertain whether the lagging chromosomes remaining at the middle of separating chromosomes in anaphase I

cells (Fig. 3B) lack centromeres, we examined distribution of the protein CID, a *Drosophila* homolog of CENP-A, in male meiotic cells (Fig. 4). All control cells from anaphase I to telophase I contained equally separated chromosomal masses, on which anti-GFP-CID immunostaining was detected as multiple dots (27 cells from 8 flies, Fig. 4A). All CID-fluorescence dots were localized on each chromosomal mass facing toward the poles. By contrast, 18.5% of the *mxc^{G46}* primary spermatocytes (27 cells from 8 flies) contained lagging chromosomes or unequally separated

Fig. 3. Time-lapse imaging of live cells undergoing meiosis I: control versus *mxc*^{G46}. (A) Normal cell undergoing meiosis I (control); $t=0'$: time at which cell poles are formed by microtubule asters. (B) Mutant (*mxc*^{G46}) cell undergoing meiosis I; $t=0'$: time at which three condensed chromosomes are visible in a nucleus. After chromosome congression occurred properly at metaphase I ($t=43'$), lagging chromosomes appeared (arrowheads, $t=65'$ and thereafter). At the end of meiosis I, a few chromatin fragments appearing decondensed were retained at the cell center ($t=88'$). Bar: 10 μ m. Chromosomes and microtubules are colored in magenta and green, respectively.

chromosomal masses. In these cells, no CID signal on the DNA that lags behind at the spindle equator was observed (arrow in Fig. 4B, 4B''), even though a cluster of the CID signals was localized at the end of every separated chromosomal mass closest to the spindle poles and facing outwards. In the hyper-exposed image presented in Fig. 4B'', we could not find any intermediate DNA fiber connecting the lagging-like chromosome to chromosome masses with kinetochores around the right spindle pole, although we cannot exclude the possibility that the lagging DNA was still connected with chromosomes having a centromere(s).

Intracellular localization of Mxc in premeiotic and meiotic spermatocytes

To elucidate the meiotic role, if any, of the Mxc protein, we examined the localization of GFP-Mxc, which can rescue the phenotypes of all *mxc* mutants, in primary spermatocytes before and during meiosis I. Consistent with a previous result showing that Mxc is localized in the nuclei of

early embryos as a single nuclear body called a histone locus body (HLB) associated with the histone gene cluster and their mRNAs (White *et al.*, 2011), Mxc was localized at a single site in interphase nuclei of spermatocytes during most of the premeiotic stages (Supplementary Fig. 3), as well as on condensing chromosomes at prophase I (Supplementary Fig. 4A). In the cyst containing 16 primary spermatocytes, the cells enter a developmental growth stage, which is subdivided into stages S1 to S6. The cells increase their volume, which is associated with expression and accumulation of proteins and mRNAs required for meiosis and spermatid development (Cenci *et al.*, 1994, Inoue *et al.*, 2012, Tanabe *et al.*, 2017). We classified premeiotic spermatocytes according to chromatin structure and cell morphology by phase contrast micrographs. In the S1 growth stage, corresponding to premeiotic S phase, Mxc had already formed single bodies on a chromatin (Supplementary Fig. 3A). We performed immunostaining of spermatocytes expressing GFP-Mxc with MPM2 antibody, which can recognize phosphorylation of HLB, required for its

Fig. 4. Absence of centromere marker CID on lagging chromosomes in anaphase I cells of *mxc^{G46}* males. (A–B) Anti-GFP immunostaining of fixed anaphase I cells prepared from adult male testes expressing GFP-tagged CID. Dotted line: outer boundary of each cell. (A) Control primary spermatocyte at anaphase I/telophase I. (B) Primary spermatocyte at anaphase I-like stage, from *mxc^{G46}* testes, containing a lagging chromosome (arrow) in the middle of separated chromosomes. The lagging chromosome(s) lacked CID, a centromeric protein. Similar abnormal meiotic features were scored in 18.5% of all cells at anaphase I to telophase I. (A'–B') DAPI staining, (A''–B'') anti-GFP immunostaining, and (A'''–B''') phase-contrast (PC) micrographs of the meiotic spermatocytes. We examined 27 spermatocytes from flies of each genotype. Bar: 10 μ m. Chromosomes and CID are colored in magenta and green in the merged images, respectively.

activation (White *et al.*, 2011). The Mxc-containing nuclear body was not overlapped with the MPM2 signals and appeared in cells that seemed to be at later S1 stage, suggesting that the Mxc-containing HLB has not been phosphorylated, unlike active HLB seen in embryonic nuclei. As the growth phase of spermatocytes progressed, cells in S2 stage can be recognized by the presence of mitochondrial aggregates observed under a phase contrast microscope (Supplementary Fig. 3B'). A larger Mxc focus and smaller additional focus or foci were observed at a higher frequency (40.6% $n=32$ cells) (Supplementary Fig. 3B). In a later S3 stage spermatocytes increase markedly in size, and the GFP-Mxc in most cells was localized on a single site (24/31). In the remaining cells, two Mxc foci existed on a set of homologous chromosomes (Supplementary Fig. 3C). In either case, no Mxc foci overlapped with MPM2 immunostaining signals. At the middle of the S4 growth stage, nucleoli increased in size and were overlapped with the larger MPM2 foci in the nuclei (Supplementary Fig. 3D', D''). The Mxc foci generated into single sites per nuclei became overlapped with anti-MPM2 immunostaining signals (arrowheads in Supplementary Fig. 3D'', 3D'''), indicating that the HLB was phosphorylated by kinases such as Cdk1 as observed in embryonic cells (White *et al.*, 2011). After the S5 stage, which is a mature stage just before meiotic division I, cells showed two MPM2 signals (Supplementary Fig. 3E, 3E'''). A larger focus corresponded to the nucleolus on a X-Y chromosome pair. The Mxc-containing HLB was overlapped with smaller MPM2 foci on one of the autosomes, although it is uncertain that the Mxc foci were formed on the histone gene cluster.

Next, we examined the GFP-Mxc signal in spermatocytes undergoing meiotic division I and II. We classified

cell division stages according to the meiotic microtubule structure visualized by RFP-Tubulin until prometaphase I, in which three major sets of homologous chromosomes are formed on a bipolar spindle (Supplementary Fig. 4A). A clear single Mxc focus can be seen on one of the condensing chromosome sets. At metaphase I, in which the chromosome sets congressed at the cell equator, the single Mxc foci almost entirely disappeared; Mxc appeared to be released from chromatin as the chromosomes condensed and dispersed through a nuclear space surrounded by a nuclear envelope (see an inset of Supplementary Fig. 4B'). Subsequently, the Mxc foci reappeared as multiple dots in the nuclear space between segregating chromosomes at late anaphase I (Supplementary Fig. 4C). Lastly, at the end of meiosis I, the fluorescence signal was observed in the nucleus, and single Mxc bodies can be seen in one of the sister nuclei at telophase I cells (Supplementary Fig. 4D and E). Similar Mxc localization was detected in secondary spermatocytes undergoing meiosis II (Supplementary Fig. 4F–H). Until metaphase II, Mxc localized both in the cytoplasm and on a single site of the chromatin, and, subsequently, the Mxc signal reappeared in the space between segregating chromosomes at telophase II.

Formation of HLB complex containing Mxc and FLASH is disrupted in mature pre-meiotic spermatocytes in *mxc^{G46}*

We next immunostained spermatocytes from control testes to determine the localization of Mxc and another HLB component, FLASH, in pre-meiotic spermatocytes (Fig. 5A, arrow). In control spermatocytes expressing GFP-Mxc, the GFP fluorescence was detected at a single site on one set of paired homologous chromosomes (Fig. 5A). Anti-

Fig. 5. Colocalization of Mxc and FLASH in pre-meiotic spermatocytes. (A–B) Immunostaining of pre-meiotic spermatocytes expressing GFP-Mxc with an antibody against FLASH, another HLB component. (A, B) Pre-meiotic spermatocytes; (A', B') DAPI staining. (A'', B'') GFP fluorescence of GFP-Mxc. (A'''–B''') Anti-FLASH immunostaining. The pre-meiotic spermatocyte at the mature stage contains a single focal spot of Mxc on a chromosomal mass composed of a set of homologous chromosomes (arrow). (B–B''') Primary spermatocytes containing two Mxc foci on the same set of homologous chromosomes (arrowheads). We examined >150 spermatocytes from flies of each genotype. (C–C'') FLASH was dispersed in all pre-meiotic spermatocytes from *mxc^{G46}* mutant; we examined 358 spermatocytes from *mxc^{G46}* flies. Bar: 10 μ m.

FLASH staining demonstrated that this immunoreactive signal was also localized on a single site on chromatin, which overlapped with the Mxc signal. It is consistent with a previous observation that FLASH and Mxc colocalize with each other at a single site on chromatin in *Drosophila* early embryos (White *et al.*, 2011). Moreover, we detected two foci of GFP-Mxc on a set of homologous chromosomal mass at an earlier growth stage as described above (Fig. 5B, arrowheads), both of which were overlapped with anti-FLASH immunostaining signals. These immunostaining data indicate that two separate HLBs containing both Mxc and FLASH are assembled and then gathered in normal pre-meiotic spermatocytes.

By contrast, in *mxc^{G46}* spermatocytes, almost no FLASH-containing nuclear bodies was detected in the nucleus (Fig. 5C–5C''), and the anti-FLASH immunostaining signal was instead dispersed through the nucleoplasm of pre-meiotic spermatocytes. The results suggest that the formation of the HLB was inhibited in *mxc^{G46}* spermatocytes.

Decline of all canonical Histone mRNA levels in *mxc^{G46}* testes and a phenocopy of *mxc^{G46}* by induced expression of abnormal histone H4 mRNA with poly(A) tails

Formation of HLB containing Mxc and FLASH was dis-

rupted in *mxc^{G46}* mutant spermatocytes, and, thus, we next investigated whether mRNA levels of canonical histone genes, which are HLB targets, are affected in mutant testes. We quantified the mRNA levels of five canonical *histone* genes in *mxc^{G46}* adult testes using qRT-PCR. Besides mRNA levels of H1, H2A, and H2B decreasing to ~60% of control levels, those of H3 and H4 decreased to 23% and 5%, respectively (Fig. 6A). Given that meiotic aberrations in *mxc^{G46}* were potentially a result of decreased canonical Histone levels, we tested whether Histone alterations caused similar meiotic aberrations: We generated males hemizygous for a *His4* mutation, *His4^{Scim}*, which was previously isolated as a dominant mutation that causes abnormal chromosome segregation in female meiosis (Dobie *et al.*, 2001). As expected, hemizygous males (*His4^{Scim}*/*Df(2L)ED1462*) also showed meiotic phenotypes that were observed in post-meiotic cells of *mxc^{G46}* males, albeit at a low frequency (arrow in Fig. 6C, 6C', 23/565 cells from six flies; 4.3%), but it was still ≥ 8.6 -fold higher than that in controls (4/785 cells from 12 flies; 0.5%). We also observed a similar meiotic phenotype in spermatids from spermatocytes depleted of mRNA encoding the non-canonical histone, His3.3B (Akhmanova *et al.*, 1995) (*bam*>*His3.3B*RNAi) at a frequency of 15.2% (58/381 cells from six flies, Supplementary Fig. 2D, 2F).

Abnormal canonical *histone* mRNAs carrying poly(A)

Fig. 6. Histone gene mRNA levels and abnormal nuclei containing extra micronuclei were observed in spermatids from hemizygotes for *His4* mutation and spermatids expressing *His4* mRNA with a polyadenylated tail. (A) Quantification of the mRNA levels of five canonical histone genes in mxc^{G46} . Total RNA was prepared from adult testes of control males (*w/Y*) and mxc^{G46} males. Relative mRNA levels of the genes were calculated and normalized to the control level, which was set to 1.0 (*w/Y*) (* $p > 0.05$, ** $p > 0.01$, *** $p < 0.001$, Student's *t*-test). qRT-PCR analysis to quantitate the amount of mRNA of each gene was repeated three times. Error bars represent s.d. (B–D) Micrographs of spermatids at onion stage and a slightly later stage. (B'–D') Fluorescence micrographs of the cells stained with DAPI. DNA is colored in red (B–C) or white (B'–C'). (B''–D'') Phase-contrast (PC) micrographs of the cells. (B–B'') Control spermatids. (C–C'') Spermatids from a hemizygous male for *His4^{Scim}/Df(2L)ED1462*. Arrows indicate micronuclei. (D–D'') Spermatids expressing *His4* mRNA with a polyadenylated tail. In many of the abnormal spermatid nuclei, chromatin was unevenly distributed in the nucleus (arrows). A spermatid possessing micronuclei (arrow in inset D, D') is encircled by a dotted line (inset in D). Bar: 10 μ m.

tails at the 3' end were previously reported to be produced in the *mxc* mutants (White *et al.*, 2011); these mRNAs are devoid of a poly(A) tail in normal cells. Thus, to examine whether the production of abnormal *histone* mRNAs with poly(A) tails is also involved in the meiotic phenotype in mxc^{G46} mutants, we induced ectopic expression of *histone H4* mRNA artificially possessing a 3' poly(A) tail in testes (*bam>HisH4-poly(A)*). Post-meiotic cells having micronuclei and abnormally sized nuclei were also detected in spermatids at a low frequency (arrow in inset of Fig. 6D, arrows in Supplementary Fig. 2K) (10/587 cells from six flies, 1.7%). Moreover, these post-meiotic cells contained nuclei exhibiting uneven chromatin distribution (arrows in Fig. 6D, arrows in Supplementary Fig. 2L, 89/587, 15.2%),

in contrast to the control spermatid nuclei (Fig. 6B). Furthermore, 23.1% (6/26 cells) of the abnormal spermatids derived from *mxc*-depleted testes (*bam>mxcRNAi^{HMS000444}*) also displayed a similar chromatin phenotype (arrows in Supplementary Fig. 2E), although it remains uncertain whether this nuclear phenotype is related to the meiotic phenotype derived from abnormal chromosome segregation. We conclude that a partial reduction of HLB function in mxc^{G46} mutants resulted in a decline in canonical Histone expression in testes, and that production of *histone* mRNA with poly(A) tails occurring as a consequence of *Mxc*-containing HLB disruption might also contribute to the meiotic chromosome segregation defects.

Table I. GENETIC INTERACTIONS BETWEEN *MXC* AND GENES ENCODING HLB COMPONENTS IN CHROMOSOME SEGREGATION DURING MEIOTIC DIVISIONS.

Genotype	Post-meiotic spermatids with abnormal nuclei (%) ^a	Spermatids examined (cells (# of flies)) ^b	Anaphase I cells showing abnormal chromosome segregation (%) ^c	Anaphase I cells examined (cells (# of flies)) ^d
control (w/Y)	0.5	785 (10)	0.0	77 (11)
<i>mx^{G46}/Y</i>	4.1	441 (10)	11.5	78 (17)
<i>coil/+</i>	0.0	789 (15)	0.0	30 (5)
<i>FLASH/+</i>	0.0	547 (6)	0.0	32 (5)
<i>Lsm10/+</i>	0.5	1,174 (25)	0.0	30 (5)
<i>mute/+</i>	0.1	707 (13)	0.0	30 (5)
<i>snRNA:U7/+</i>	0.4	772 (10)	0.0	35 (5)
<i>mx^{G46}/Y;coil/+</i>	14.6	584 (7)	24.4	45 (7)
<i>mx^{G46}/Y;FLASH/+</i>	17.4	1,503 (21)	28.3	46 (8)
<i>mx^{G46}/Y;Lsm10/+</i>	15.4	624 (12)	20.0	30 (10)
<i>mx^{G46}/Y;mute/+</i>	13.0	768 (11)	35.3	51 (6)
<i>mx^{G46}/Y;snRNA:U7/+</i>	16.6	597 (6)	17.4	46 (9)

^a) Frequencies of post-meiotic spermatids at onion stage containing abnormally sized nuclei or extra micronuclei featuring the specified genotypes.

^b) >441 post-meiotic cells from >6 male flies of each genotype were scored.

^c) Frequencies of primary spermatocytes undergoing anaphase I, which contains lagging chromosomes or unequally separated chromosomal masses featuring the specified genotypes.

^d) >30 anaphase I cells (>3 cysts) from at least 5 male flies of each genotype were examined.

Genetic interactions between *mx^{G46}* and genes encoding other HLB components in chromosome segregation during male meiosis

Next, we investigated whether inhibition of HLB formation in *mx^{G46}* mutant testes was involved in the appearance of lagging chromosomes and the production of micronuclei. We examined genetic interactions between *mx^{G46}* and genes encoding these HLB components, namely Coilin, FLASH, Lsm10, Mute, and U7 snRNA. For this purpose, the hypomorphic mutation *mx^{G46}* was quite useful because of its less severe phenotype. Through genetic crosses, we generated *mx^{G46}* flies carrying a recessive mutation for each HLB component in heterozygous state, because homozygous mutations of these components are lethal at the embryonic stage. We then examined the nuclear organization of post-meiotic spermatids to ascertain whether there was a significant increase in spermatids containing excess micronuclei or abnormally sized nuclei (Supplementary Fig. 5A–L, Table I). As mentioned in the first paragraph of the Results, males hemizygous for *mx^{G46}* displayed the meiotic phenotype at a low frequency (4.1% of the spermatids). We recorded very few abnormal cells among the post-meiotic cells heterozygous for a mutation in each of the five HLB components (Coilin, FLASH, Lsm10, Mute, U7 snRNA) ($n > 540$ cells of each genotype, Supplementary Fig. 5C–5G). As compared with the frequencies in *mx^{G46}* mutants and males heterozygous for HLB mutations, a higher frequency of the meiotic phenotype was observed in *mx^{G46}* mutants carrying a mutation in each of the five genes of the HLB components (Table I, Supplementary Fig. 5B–5L). The frequencies in mutants ranging from 13.0% (*mx^{G46}/Y;mute^{EY22147}/+*) to 17.4% (*mx^{G46}/Y;FLASH^{LL01602}/+*) were ≥ 3.0 -fold higher than the frequency

of 4.1% in *mx^{G46}/Y* (Supplementary Fig. 5H–5L). Moreover, we examined whether the number of anaphase I spermatocytes at containing lagging chromosomes increased after the introduction of a recessive mutation in *mx^{G46}* (Supplementary Fig. 6B–6L). We did not record any abnormal anaphase I cells in control testes ($n = 77$, Supplementary Fig. 6A), and detected lagging chromosomes or unequally separated chromosomal masses in *mx^{G46}* anaphase I cells ($n = 78$) at a low frequency (11.5%) (Supplementary Fig. 6B). However, relative to the frequency of such anaphase I cells in *mx^{G46}*, we measured a higher frequency of cells presenting the abnormal meiotic features in *mx^{G46}* males heterozygous for each of the HLB mutations (Supplementary Fig. 6H–L, Table I). The frequencies in mutants ranging from 17.4% (*mx^{G46}/Y;FLASH^{LL01602}/+*) to 35.3% (*mx^{G46}/Y;mute^{EY22147}/+*) were ≥ 1.5 - to 3.0-fold higher than the frequency in *mx^{G46}/Y*. We confirmed that the meiotic phenotype was absent in the heterozygotes for HLB mutations (Supplementary Fig. 5B–5L, Supplementary Fig. 6B–6L). From these genetic data, we concluded that a reduction in HLB gene dosage enhanced the meiotic phenotype of *mx^{G46}*, and thus we speculate that a failure of HLB formation is involved in aberrant chromosome segregation in meiotic divisions of *mx^{G46}* mutants.

Micronucleus phenotype in *mx^{G46}* mutants can be observed by downregulation of chromosome construction factor genes but not DNA-replication genes

As mentioned above, a decline in the level of canonical histone mRNAs as well as production of abnormal histone mRNA with poly(A) tails are involved in chromosome segregation defects in *mx^{G46}* mutants. Moreover, a previous

study indicated that the hypomorphic *mx*c mutation induces DNA-replication stress in germline stem cells, affecting the maintenance of the cells (Landais, *et al.*, 2014). Thus, we examined whether DNA-replication defects induced by depletion of DNA polymerase genes in premeiotic spermatocytes influence chromosome segregation during male meiosis. We first examined whether ectopic expression of dsRNA against mRNA encoding *pol* α or *pol* ϵ can reduce the level of the corresponding mRNA in testes of *bam>DNApol- α 180RNAi* (*bam>pol- α RNAi*) and *bam>DNApol- ϵ 255RNAi* (*bam>pol- ϵ RNAi*) flies, and confirmed that the mRNA levels in the two sets of flies were reduced by 6.7% and 47.8% of controls, respectively (Supplementary Fig. 7A). We found that almost all spermatids in testes expressing dsRNA against *pol* α (99.8% of spermatid nuclei examined (n=405 cells from three flies), Supplementary Fig. 2G) or *pol* ϵ (99.6% (n=472 cells from five flies), Supplementary Fig. 2H) contained normal nuclei indistinguishable from those in control spermatids. We thus conclude that DNA replication stress induced by downregulation of DNA replication genes is not involved in the meiotic phenotype. Furthermore, we examined whether a decline in chromosome components other than histones results in the meiotic phenotype. We depleted the chromosome-construction factors CAP-D2 and SMC2 by ectopic expression of the corresponding dsRNA, and confirmed that the mRNA levels were reduced by 35.2% and 54.1% of controls, respectively (Supplementary Fig. 7B). In contrast to depletion of *DNA polymerase* mRNAs, we observed abnormal spermatids containing micronuclei or abnormally sized nuclei in testes depleted of CAP-D2 (25/550 cells (4.6%), Supplementary Fig. 2I) from seven *bam>CAP-D2RNAi* flies) or SMC2 (17/405 cells (4.2%) from six *bam>SMC2RNAi* flies, Supplementary Fig. 2J). These frequencies were low but still higher than those in the controls (1/1,362 cells (0.07%) from 15 *bam-Gal4/+* flies). Thus, the micronucleus phenotype as observed in *mx*c^{G46} can be reproduced in cells with downregulation of the chromosome-construction factors.

Discussion

Partial loss of *mx*c function gives rise to post-meiotic cells with CIN

We demonstrated here that the *mx*c hypomorphic mutants *mx*c^{G46} and *mx*c^{G43} and male flies with a testis-specific depletion of these genes contain abnormal post-meiotic cells harboring micronuclei or abnormally sized nuclei. The meiotic phenotypes are reminiscent of CIN, characteristic of cancer cells (Lengauer *et al.*, 1997; Negrini *et al.*, 2010). The *mx*c^{G46} mutation is a nonsense allele, encoding a truncated protein lacking residues after K¹⁶⁴³ of the 1,873-residue long protein in full length. Our finding that the

assembly of FLASH-containing HLB is disrupted in the mutant is consistent with reports that the 195 C-terminal amino acids, lacking in *mx*c^{G46}, are essential for recruiting FLASH to the HLB (Terzo *et al.*, 2015; White *et al.*, 2011). The C-terminal region is dispensable for survival of individuals, although it is indispensable for proper meiotic divisions. *mx*c^{G43}, a semi-lethal allele, encodes a protein lacking residues after K¹⁴⁸². The mutant allele encoding a protein featuring a longer C-terminal truncation showed a more severe germline cell phenotype. A consensus amino acid sequence for the AT-hook domain that binds the minor groove of DNA at AT-rich stretches exists between K¹⁴⁸² and K¹⁶⁴³ (Reeves and Nissen, 1990; White *et al.*, 2011; Terzo *et al.*, 2015). As a previous study demonstrated that the AT-hook domain is not necessary to concentrate exogenous Mxc in the HLB, it is unlikely that this motif is responsible for the differences in the meiotic phenotypes between the two alleles. Furthermore, it is possible that unknown domains existing between K¹⁴⁸² and K¹⁶⁴³ facilitate HLB assembly and are responsible for the differences. Moreover, we showed that *mx*c^{G43} is a temperature-sensitive allele. The C-terminal region between K¹⁴⁸² and the C-terminal end may play an indirect role in the conformation of the protein through interaction with other regions.

Although we observed distinctive cellular localization of Mxc in premeiotic spermatocytes, we did not find any cells possessing smaller or larger nuclei, or any anucleate cells, in the testes of *mx*c mutants. Moreover, in >50 primary spermatocyte cysts from over six flies of each genotype, we did not detect abnormal cysts containing fewer primary spermatocytes. It is possible the mutation predominantly affect meiosis, and abnormal mitoses of spermatogonia may occur at a lower frequency. Alternatively, the absence of spermatogonial defects might be ascribed to the lack of convenient and sensitive methods that allow us to detect aneuploidy in these cells. In summary, the CIN phenotype that appeared in post-meiotic cells may result from a partial loss of function of the tumor suppressor gene *mx*c.

Generation of lagging chromosomes lacking centromeres is involved in CIN in *mx*c mutants

The appearance of abnormal cells carrying micronuclei and/or abnormally sized nuclei among post-meiotic cells in *mx*c^{G46} suggests that chromosome segregation occurred improperly during meiotic divisions. Our time-lapse imaging revealed that chromosome masses were retained in the cell equator of the mutant cells. The chromosome masses are equivalent to the lagging chromosomes or chromatids in mitosis (Bakhoun *et al.*, 2014). Previous findings indicate that chromosome breakage occasionally occurs in chromosomes carrying poorly condensed regions owing to a tangling of chromatin fibers (Girdham and Glover, 1991). Other studies have also reported that lagging chromosomes arise from the formation of chromatin and chromosome

bridges owing to chromosome-construction defects (Gerlich *et al.*, 2006). Furthermore, *histone H1* depletion leads to the formation of abnormally extended, tangled, and twisted chromosomes (Maresca *et al.*, 2005). Our results and previous studies suggest that it is more likely that the centromere-lacking lagging chromosomes arose because of the chromosome breakage resulting from the reduced expression of canonical Histones. Once the lagging chromosomes are produced, they transform into extra micronuclei after nuclear membrane formation around the chromosome fragments. During cytokinesis, if a lagging chromosome is transported by chance to the daughter cell that should not have originally received the chromosome, CIN develops. At the same time, we observed abnormally sized post-meiotic cells and micronuclei less frequently compared with meiotic cells showing abnormal chromosome segregation. A lagging chromosome retained at the cell equator may be properly partitioned by chance during cytokinesis. If the chromosome fragment is accidentally incorporated into a daughter cell, in which the fragment should be originally transmitted, the CIN phenotype would not appear in the post-meiotic cells. Alternatively, deficiency or excess of part of a chromosome may be insufficient to be recognized as altered nuclear size.

Loss of HLB influences chromosome assembly through downregulation of histone gene expression

Here, in meiotic cells of *mx^c^{G46}* males, we found that an HLB component, FLASH, did not form the nuclear body. FLASH plays an essential role in both transcription of canonical *histone* genes at S phase and 3'-end processing of replication-dependent *histone* pre-mRNAs (Salzler *et al.*, 2013; Terzo *et al.*, 2015; White *et al.*, 2011; Yang *et al.*, 2009). Accordingly, the reduction in the levels of all canonical *histone* mRNAs in *mx^c^{G46}* is consistent with the inhibition of FLASH-containing HLB. We further verified that a reduced expression of five canonical *histone* genes causes similar meiotic phenotypes. We observed similar meiotic phenotypes in hemizygotes for *histone H4* mutation, while notable meiotic phenotypes in any of the adult testes expressing dsRNA against each of the canonical histone mRNAs (data not shown). These mRNAs cannot be readily depleted sufficiently by overexpressing their dsRNAs by using the Gal4/UAS system—the *Drosophila* genome contains >100 sets of *histone* genes in a chromosomal region known as the *histone* gene cluster (Domier *et al.*, 1986). We also identified clear genetic interactions between *mx^c* and other HLB genes in the appearance of the meiotic phenotypes. Considering these observations and the genetic results collectively, we speculate that a reduction of chromatin components owing to reduced HLB activity caused by the *mx^c* mutation ultimately led to the formation of poorly condensed chromosomes, resulting in broken chromosome fragments lacking centromeres at these fragile regions.

The *mx^c^{G46}* mutation was previously reported to affect the DNA-replication process by generating DNA damage, termed DNA-replication stress (Landais *et al.*, 2014). They reported that mRNA of *histone H3* but not of other Histones was increased in *mx^c^{G46}*, and the unbalanced accumulation of canonical Histones was suggested lead to the DNA-replication stress. However, we failed to confirm these previous results. We neither detected DNA-damage foci in *mx^c^{G46}* spermatocytes by anti- γ -His2Av immunostaining (data not shown) nor observed the meiotic phenotypes in post-meiotic cells derived from meiotic cells depleted for DNA-replication enzymes. Nevertheless, we cannot exclude a possibility that another DNA polymerase may have compensated for the DNA replication defects that occurred due to the depletion of DNA polymerase ϵ (Kesti *et al.*, 1999). Given these results, we consider it more likely that chromosome-construction defects, rather than DNA damage derived from DNA-replication stress, are involved in the CIN found in meiotic divisions in *mx^c^{G46}* mutant males.

Conclusions

We observed meiotic phenotypes corresponding to CIN—a common feature of malignant tumor cells—at a low frequency in *mx^c* hypomorphic mutants; however, this characteristic could not be readily examined in a more severe *mx^c* mutant exhibiting a malignant blood-neoplasm phenotype. If defects in chromatin structures that influence microtubule-kinetochore interaction occur, cell-cycle progression in somatic cells is invariably arrested at the G2/M checkpoint before the M phase (Weaver and Cleveland, 2005). Conversely, progression of male meiotic divisions in *Drosophila* is not notably impeded because the spindle checkpoint is not as strict as that in mitotic cells (Rebollo and González, 2000). Therefore, we successfully detected abnormal post-meiotic cells derived from aberrant chromosome segregation during meiotic divisions. Although most tumor cells originate from somatic cells, once initial pre-cancer cells have lost proper mitotic-checkpoint regulation, cell-cycle control becomes less strict. Cells harboring certain aberrant chromosome structures might also enter M phase. Previously, CIN was reported to be associated with the loss of function of a mitotic checkpoint in human cancer cells (Cahill *et al.*, 1998; Kops *et al.*, 2005). There are some reports of chromosome aneuploidy contributing to tumorigenesis (Gisselsson, 2008; Holland and Cleveland, 2009). As a recent study also demonstrated, once micronuclei are produced from unsegregated chromosome fragments or whole chromosomes, they contribute to CIN at the next cell division (He *et al.*, 2019). Considering these results, we hypothesize that a subtle aberration in chromosome construction can induce CIN in cooperation with the perturbation of M-phase-checkpoint regulation. The *mx^c*

mutants can serve as a useful model for investigating how CIN occurs at the onset of carcinogenesis.

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Conflicts of interest

The authors declare no conflicts of interest associated with this study.

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