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EXPLORING THE EFFECTS OF REPLICATION STRESS ON CHROMOSOME FRAGILE SITES IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Using Drosophila as a model, chromosome breakage and "fragile sites" were studied. Fragile sites are areas on a chromosome where breaks occur with increased frequency (Laird et al., 1987). In humans, common fragile sites are more susceptible to breaking due to late replication in these areas and other factors (Glover & Durkin, 2007). To test whether dicentric chromosome breakage is affected by late replication, aphidicolin, a DNA Polymerase-α inhibitor, was used to induce replication stress throughout the entire genome. Exposure to aphidicolin throughout spermatogenesis does not significantly affect the distribution or incidence of dicentric chromosome breakage in the male germline. This suggests that the replication stress induced by aphidicolin does not contribute to fragility of chromosomes under tension. These results provide insight into the distribution and characteristics of fragile sites within chromosomes, as well as the relationship between *Drosophila* fragile sites and the DNA replication process.

INTRODUCTION

DNA must be accurately copied and efficiently distributed during each cell division to maintain a healthy organism. During the cell cycle, DNA replication and chromosome segregation processes may be subjected to a variety of endogenous and exogenous stressors, interfering with their completion and jeopardizing the integrity of these processes (Gelot, Magdalou & Lopez, 2015). When strategies to counteract or alleviate the effects of these stressors fail, chromosome breakage may result. In response to a double stranded break, a cell may fuse the broken ends. This is problematic when multiple breaks occur, creating rearrangements within and between chromosomes (McClintock, 1950). These attempts at repair can cause additional gene mutation and ongoing genetic instability. The accumulation of mutations and copy number variants that result are often seen in the beginning stages of carcinogenesis (Glover & Durkin, 2007).

One source of chromosome breakage is stress that hampers normal DNA replication. For instance, conditions that delay or stall replication forks cause replication stress. A stalled fork could lead to a double stranded break when helicase continues to unwind the DNA into single strands, making these areas susceptible to breakage. The collapse of a stalled replication fork can result in a break (Zeman & Cimprich, 2014). Replication stress may occur naturally when low concentrations of necessary replication factors, including nucleotides, components of replication machinery, and histones, are encountered. Excessive replication origin firing in one area of the genome can lead to a depletion of these replication factors, causing replication stress in other areas of the genome. A deficiency of active origin sites within a stretch of the genome can also lead to stress, requiring continuous synthesis across a long stretch of DNA. Properties of the DNA can also contribute to replication stress. DNA lesions, crosslinking and the densely compacted structure of heterochromatin can all slow the progression of replication complexes through these areas. Replication stress can occur in any organism when problems arise during pre-replication, initiation, or elongation, resulting in a local delay in DNA synthesis (Gelot, Magdalou and Lopez, 2015). Replication stress can also be chemically induced. The drug aphidicolin binds DNA polymerase-α, inhibiting its ability to hydrolyze necessary ATP to incorporate dNTPs into a newly synthesized strand. At high concentrations of aphidicolin, all polymerase is completely inhibited, stopping cell cycle progression and eventually triggering apoptosis. With lower doses of aphidicolin, some polymerase remains uninhibited and free to synthesize DNA, but replication fork speed is reduced 2-10 fold, creating replication stress but allowing cell viability for investigation. Since aphidicolin delays S-phase, the cell cycle time is lengthened (Vesela et.al., 2017). Aphidicolin is a useful tool for studying replication stress and its effects on chromosome breakage.

Treatment of chromosomes with low doses of aphidicolin induces non-random breaks at specific loci known as fragile sites. These sites have been identified in several eukaryotes as common areas of breakage (Helmrich et. al., 2006, Song et. al., 2014, LaFave, 2011). In humans, two types of chromosome fragile sites have been identified. Rare fragile sites are found in less than 5% of individuals, can be inherited in a Mendelian manner, and are associated with genetic disorders including Fragile X syndrome. The increased frequency of breakage at these sites has been identified as a mechanism of mutation leading to genetic disorders. The second and largest class of fragile sites are common fragile sites (CFS). These sites are a normal component of genomes and are highly conserved between individuals. CFS are frequently involved in breakage and fusion during replication stress and have been identified as sites of frequent chromosome rearrangements and deletions in cancer cells (Glover & Durkin, 2007).

Using techniques to recover DNA sequences bound by the pre-replication complex, CFS are often found in areas of the genome far from origins of replication (Nordman & Orr-Weaver, 2012). This makes CFS replication reliant on polymerase working effectively over long distances (Gelot, Magdalou and Lopez, 2015). This process is slower than replicating short stretches of DNA from many origins, and when compared genome-wide these areas are 'late-replicating'. These areas may replicate so late that synthesis is not complete before cell division. This would normally halt the cell cycle to allow time to resolve these issues. Recombination or incorrect processing of these unreplicated strands could lead to the formation of ultra-fine DNA bridges between the two segregating sister chromosomes. In efforts to resolve these bridges, these areas may be targeted by nucleases resulting in breakage (Franchitto, 2013). In human cells, late replicating areas of the genome frequently coincide with common areas of breakage, indicating a possible connection between late replication and chromosome fragility (Laird et. al., 1987). CFSs are loci where low doses of aphidicolin reproducibly cause breakage (Glover et al., 1984). These experiments suggest a connection between the type of replication stress caused by treatment with aphidicolin and breakage at common fragile sites.

Chromosome breakage 'hotspots' have recently been identified in *Drosophila melanogaster*, a promising model to study fragile sites in an organism that can be genetically manipulated (Hill and Golic, 2015). To explore the relationship between the occurrence of fragile sites and replication stress, experiments were conducted to identify chromosome breakage patterns in *D. melanogaster* exposed to aphidicolin. These experiments examine the relationship between replication stress and chromosome fragility in the specific circumstance of chromosomes under tension in mitosis. This is an unexplored area and may lead to new insights regarding chromosome fragility and the role it plays in unchecked cell proliferation.

METHODS

Determining effective concentrations of aphidicolin:

To induce replication stress, *Drosophila* were grown on food containing low doses of aphidicolin. Experiments were conducted to determine the highest concentration of aphidicolin flies could be raised on without causing sterility. Aphidicolin was added to 2mL of food to produce final concentrations spanning 0μM to 100μM. Wildtype (*y w*) female virgins were crossed to wildtype males and allowed to lay eggs on the aphidicolin treated food. Progeny from this cross developed from egg to adult on the treated food. Adult males from this cross were mated with untreated females to determine the effects of different concentrations of aphidicolin on male fertility (Figure 1). Lower concentrations of aphidicolin (<50μM) were shown to have no significant effect on the fertility of *Drosophila*. However, food containing 100μM of aphidicolin has a significantly detrimental effect on fertility ($p<0.01$). These results prompted the use of higher levels of aphidicolin (100μM) in subsequent experiments. This concentration has a clear detrimental effect while giving enough viability and fertility for successful experiments and substantial sample sizes.

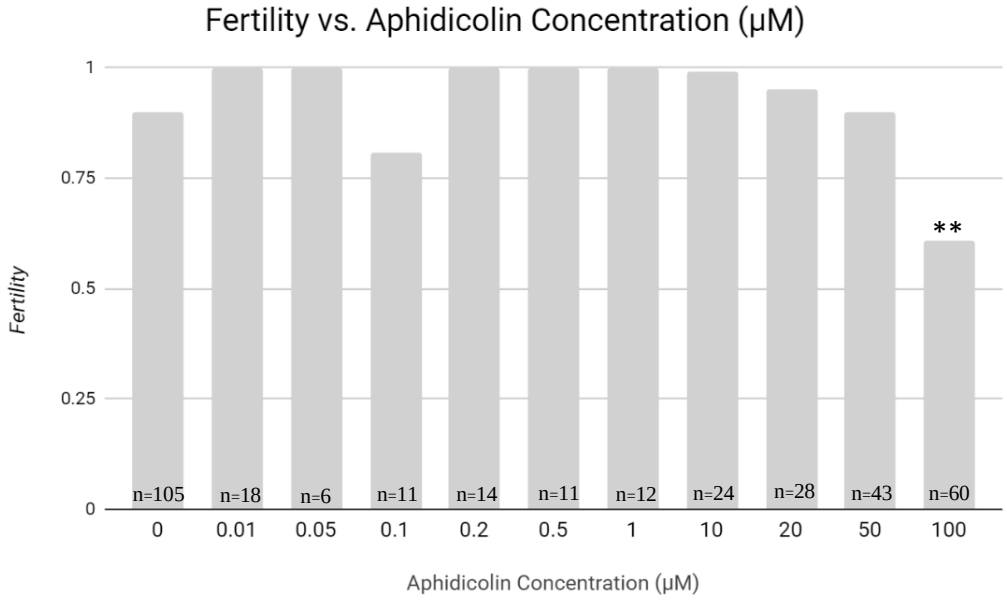

Figure 1: Fertility at increasing aphidicolin concentrations. Fertility is defined by number of progeny per male parent

A 100μM concentration of aphidicolin was achieved by diluting 2 μmol (66.7 μL) of aphidicolin in 500 μL water and adding this to liquid fly food to give a final volume of 2mL. Controls ate food prepared the same way, but without aphidicolin.

Chromosome fragility under tension:

Because breakage events are rare and typically lethal, a system to efficiently and effectively break chromosomes was used. Ring chromosomes, found and studied in *Drosophila*, humans and other organisms, were used to facilitate breakage. The site-specific recombinase, FLP, catalyzes recombination between the sister chromatids of this ring, creating a single chromosome with two centromeres (Figure 2). These dicentric chromosomes break during anaphase when each centromere is pulled to opposite poles of the cell.

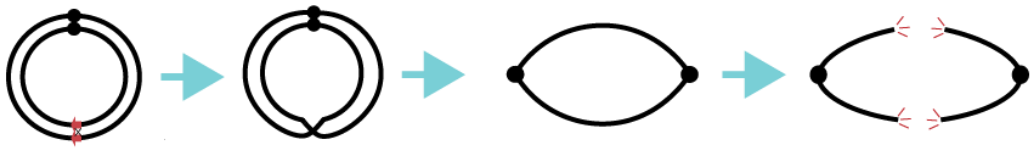

Figure 2: Dicentric chromosome formation and breakage

If this chromosome break does not result in lethality, the cell may heal the damage via de novo telomere addition to protect the DNA at the new ends from degradation. These broken, linearized chromosomes can be made in the male germline and recovered to generate stocks, allowing for further examinations (Hill & Golic, 2015). Theoretically, dicentric chromosomes have the potential to break at any point between centromeres. The double bridge made by sister chromatid exchange in a ring chromosome includes 2 copies of the entire *X* chromosome (Figure 3). If the arms break in similar sites, each cell may receive a complete *X* chromosome. After healing, such chromosomes may be recovered in viable sons.

Breaks that do not precisely correspond on both arms of the dicentric chromosome will result in duplications and deletions. A duplication of euchromatin may be recovered, but a deletion is likely to be lethal. On the other hand, breaks in heterochromatin are not limited in this way, since it carries no vital genes.

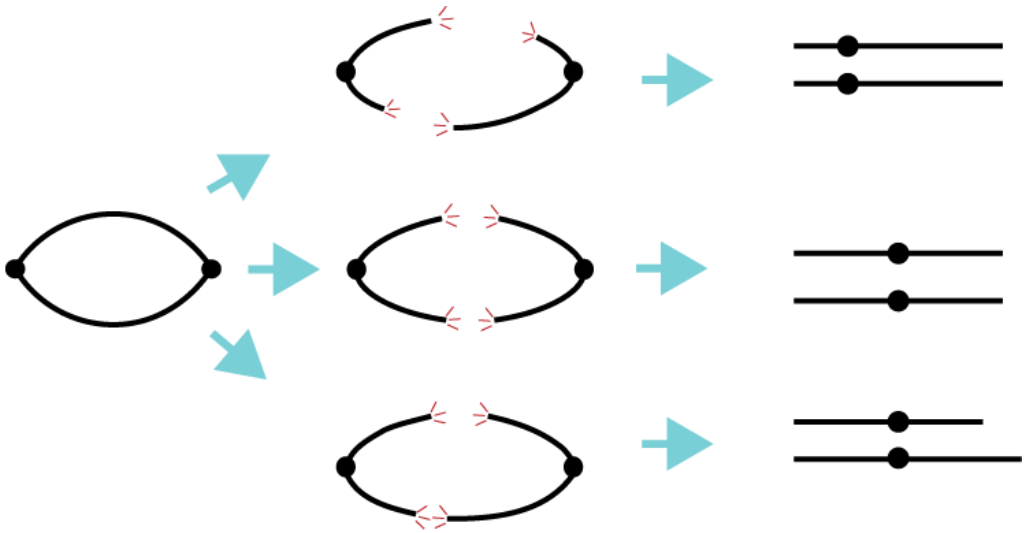

Figure 3: Breakage at various loci giving viable chromosomes and minor duplications and deletions

Fragile sites were identified by this system of inducing breakage of a ring chromosome. It was shown that the dicentrics do not break randomly, and hotspots of breakage are, by definition, regions of chromosome fragility. Microscopic analysis of the new chromosomal termini in mitotic chromosomes of neuroblasts and polytene chromosomes of salivary gland cells enables the identification of breakage sites. A cluster or 'hotspot' of breaks is indicative of a fragile site.

Structurally unique Ring-*X* chromosomes:

With established effective concentrations of aphidicolin and genetic methods to induce chromosome breakage, fragile sites can be examined under induced replication stress. Two structurally distinct Ring-*X* chromosomes, *R(1;Y) 11Ax2-8c* and *R(1)2-75B*, were used to study the role of replication stress in the sites of dicentric chromosome breakage. The two ring chromosomes used in these experiments contain the entire euchromatic *X* chromosome, with variable amounts of pericentric heterochromatin. Approximately half of *R(1;Y)11Ax2-8c* is heterochromatin, while *R(1)2-75B* has significantly less. This design allows for a more comprehensive study of the chromosomal structure in which breaks preferentially occur.

Genetic screen to recover opened Ring-*X* chromosomes:

Females carrying the Ring-*X* chromosome were crossed to males that express FLP and Hiphop in their germline with the transgenes *nosGal4*, *UASFLP*, and *UAShiphop*. FLP catalyzes the exchange that makes dicentric chromosomes and Hiphop promotes healing of the broken ends. This cross was allowed to lay eggs on aphidicolin treated food or control food. The offspring developed and males with these transgenes and the ring chromosome were crossed to females expressing *eyFLP*. The eyFLP gene expresses FLP recombinase in the eye throughout larval development. If the ring chromosome is present, recombination between FRTs forms dicentric chromosomes in the developing eye. The resulting breakage and cell death give males with unopened ring chromosomes an easily recognized phenotype: small, rough eyes. Linearized *X* chromosomes are not detrimentally affected by FLP because recombination between linear chromosomes does not result in dicentric formation. Therefore, males with opened rings and expressing FLP have wildtype eyes (Figure 4). Males carrying the opened Ring-*X* chromosome were singly mated to *C(1)DX* females to produce stocks of each individual chromosome breakage event.

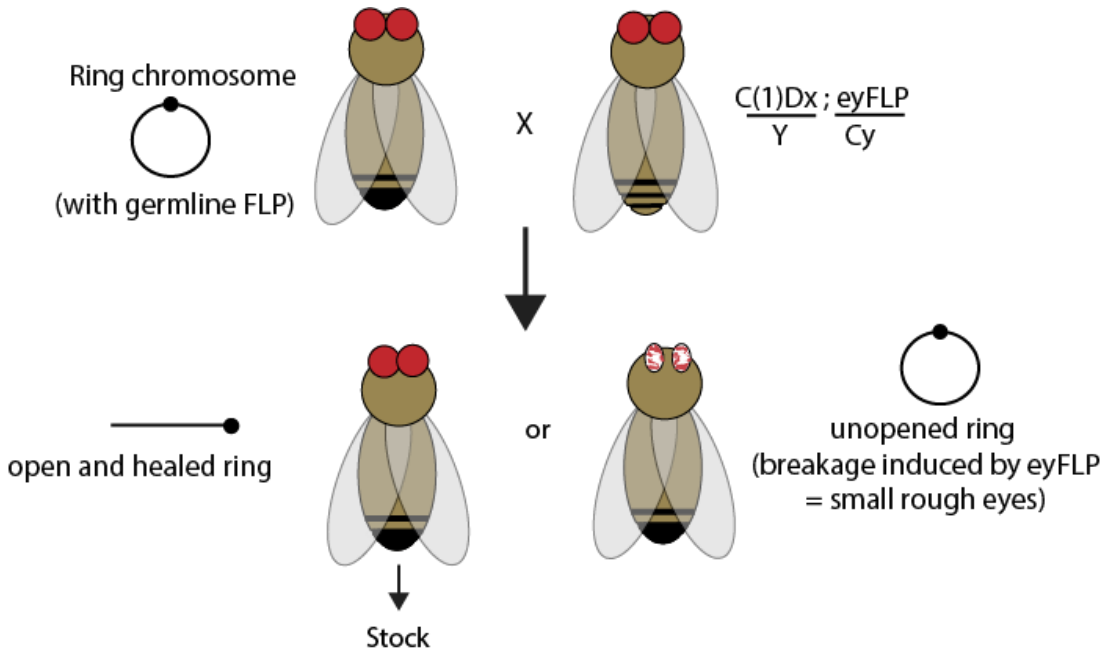

Figure 4: Genetic screen for unopened ring chromosomes using

As a control for comparing incidence and distribution of chromosome breakage, flies were raised on food without aphidicolin and tested as described. All crosses and conditions were held constant within these two experiments with the exception of aphidicolin treatment.

Determination of breakpoints:

After a linearized ring was recovered, cytological analysis was used to determine the breakpoint location of each new end. First, mitotic squashes were produced by dissecting and staining a larval brain with the fluorescent DNA binding dye DAPI. Analysis on a fluorescent microscope allows visualization of metaphase chromosomes. The karyotypes of these nuclei confirmed that the ring had become linear and allowed determination of whether the breaks were in heterochromatin or euchromatin.

Resolution of the euchromatic breakpoints can be specified further to a cytogenic band by polytene chromosome cytology. Salivary glands from third instar larvae were stained with Lacto Aceto-Orcein and imaged on a phase contrast microscope. This technique allows for resolution of the breakpoint to around 50kbp. This resolution is restricted by the resolution of polytene banding and light microscopy.

Heterochromatin does not replicate in polytene cells so it cannot be effectively visualized in polytene cytology. Thus, chromosomes broken within heterochromatin were further studied using a genetic recombination screen. The results from this screen indicate on which side of the centromere the breaks lie. R(1)2-75B has mutations in the X-linked genes *yellow* and *forked* (y^- f^-), so wildtype female (w^{1118}) flies marked with y^+ f^+ were crossed to males carrying the opened ring, R(1)2-75B. Breaks occurring on the side of the centromere closest to polytene band #1 will result in a linear *X* chromosome with the normal, wild-type orientation, with band #20 closest to

the centromere. Breakage on the opposite side yields an inverted linear *X* (Figure 5). When a normally oriented opened ring is crossed to w^{1118} flies, the incidence of recombinant offspring will be almost equivalent to non-recombinants because the yellow and forked genes are relatively far apart (~50 mu). However, crossovers between an inverted opened *X* and the w^{1118} chromosome do not give viable offspring because the resulting inversion loop will prevent recombinants. The recombination frequency was determined for each fly stock with an opened *Ring-X* chromosomes opened in heterochromatin. Recombination frequencies allowed a determination of which side of the centromere the breakpoint was on. A high recombination frequency indicates normal orientation, while a low frequency indicated a resulting inverted orientation.

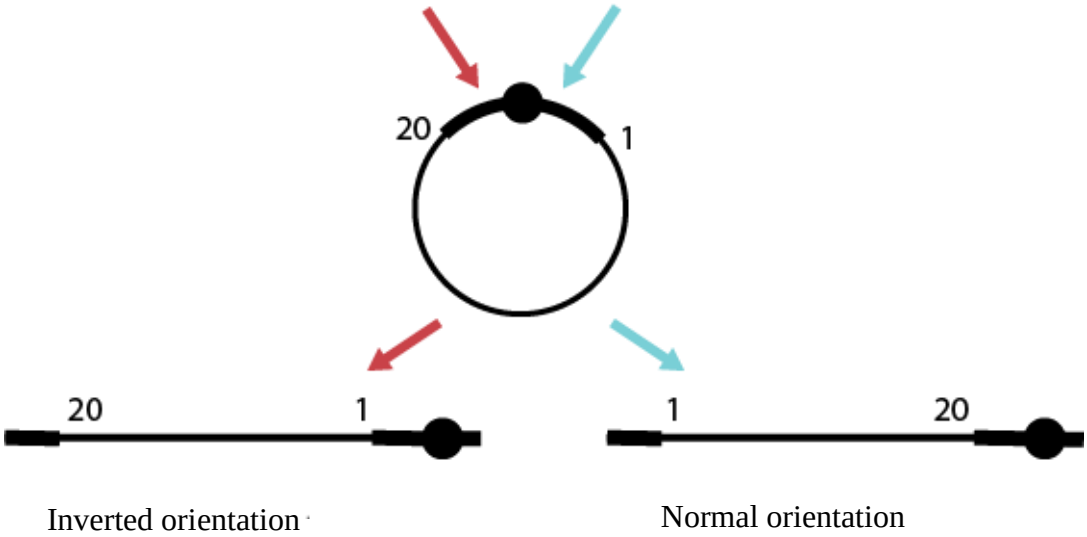

Figure 5: Breakage of a Ring-*X* chromosome produces a normally oriented or inverted linearized *X* depending on which side of the centromere it breaks

Effects of late replication on ring opening rates:

Previous work in the Golic lab identified R(1)2-75B as a Ring-*X* chromosome that is particularly hard to break. Linearized chromosomes are produced from R(1)2-75B at extremely low frequencies. However, due to the effects of aphidicolin on replication, more breaks may be produced when the entire genome is subjected to replication stress, increasing the rate of ring openings.

The number of males yielding opened rings was compared to the total of all males with the potential for breakage. This ratio gives the frequency of ring opening. Rates of breakage in chromosomes with and without exposure to aphidicolin are compared to investigate the effect of late replication on ring breakage rates.

Cytology:

To observe mitotic chromosomes, the brains of third instar larvae were dissected in 0.7% NaCl, swelled in 0.5% sodium citrate, and fixed in a 5.5:5.5:0.5 solution of HOAc: MeOH: H_2O. After fixation, the brain was squashed in 45% HOAc under a siliconized coverslip with bibulous paper and frozen on dry ice. The coverslip was flipped off with a razor blade and the slide was air dried. These squashes were stained with DAPI and viewed under UV fluorescence.

For polytene analysis of euchromatic breaks, third instar larvae were dissected in 45% acetic acid. Salivary glands were moved onto a coverslip with Lacto Aceto Orcein and a slide placed on top. The salivary glands were squashed by tapping the coverslip with a wooden stick and squashing with bibulous paper. These spreads were analyzed on a Zeiss phase-contrast microscope.

RESULTS

Rates of Ring Chromosome Opening:

To determine if replication stress has an effect on dicentric chromosome breakage, we compared rates of ring chromosome breakage in the presence and absence of aphidicolin. We chose the ring chromosome *R(1)2-75B* because it is notoriously difficult to open, so if replication stress does promote chromosome breakage, we expect to see more linearized chromosomes when flies are exposed to aphidicolin. Higher rates of chromosome opening would additionally allow for further characterization and observation of fragile sites.

Rates of chromosome breakage in flies carrying the *R(1)2* chromosome were determined (Figure 6).

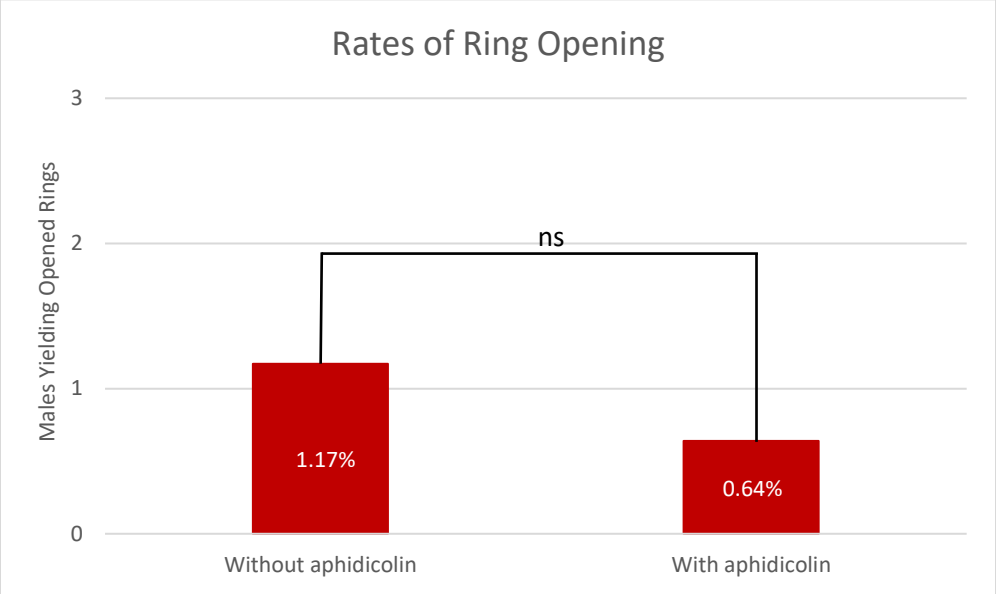

Figure 6: Rates of ring chromosome breakage with and without exposure to aphidicolin

Of the males that were screened for opened rings, only 0.64% and 1.17% were broken and recovered with and without aphidicolin, respectively. These ring opening rates are not significantly different from each other (p= 1.0, Fisher's exact test). Surprisingly, treatment with aphidicolin did not change ring breakage frequencies. Replication stress induced by aphidicolin does not affect the fragility of *Drosophila* chromosomes.

Distribution of chromosome breakage sites:

Linearized ring chromosomes with and without exposure to aphidicolin were compared to determine the effect of replication stress on breakpoint distribution. The analysis of two structurally distinct rings allowed for the comparison of breakpoint distribution between the chromosomes to determine if common sites of breakage are present.

R(1;Y)11Ax2-8c:

Previous work from the Golic laboratory determined that different chromosomes exhibit different arrays of breakage hotspots (Hill and Golic, 2015). In particular, this work identified one chromosome, *R(1;Y)11Ax2-8c*, with almost all breakage sites clustered in a single block of heterochromatin. We suspect that this region represents an extreme fragile site, perhaps owing to very late replication. Breakage at this specific fragile site may preclude less frequent breakage at the common fragile sites identified in other chromosomes. We theorized that if these flies are treated with a low dose of aphidicolin, the replication stress may make other regions fragile enough to break, potentially revealing novel fragile sites previously unstudied due to the high rate of breakage at the extreme fragile site.

Linearized *R(1;Y)11Ax2-8c* chromosomes were recovered and analyzed with mitotic microscopy technique described in Methods. Flies exposed to aphidicolin throughout development and the controls exhibited a strong preference for breakage within heterochromatin. The resulting linear chromosomes have heterochromatin at each end and can be recognized because sister chromatid cohesion is maintained in heterochromatin until the onset of anaphase, so in metaphase these breaks are easily distinguished from those in euchromatin because the sisters remain connected at the ends.

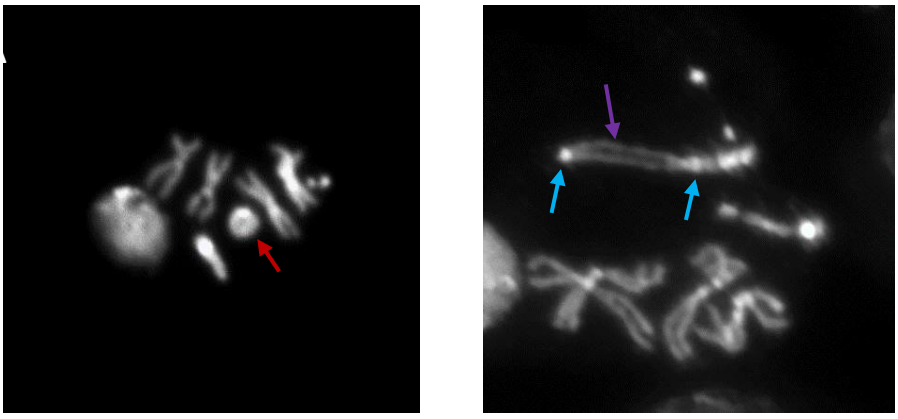

Figure 7: Mitotic squashes A: an unopened ring chromosome (red arrow) B: a R(1;Y) chromosome broken in heterochromatin. Heterochromatic regions represented by blue arrows. The purple arrow shows the euchromatic region.

Resolution from mitotic squashes was sufficient for identifying break sites in these crosses.

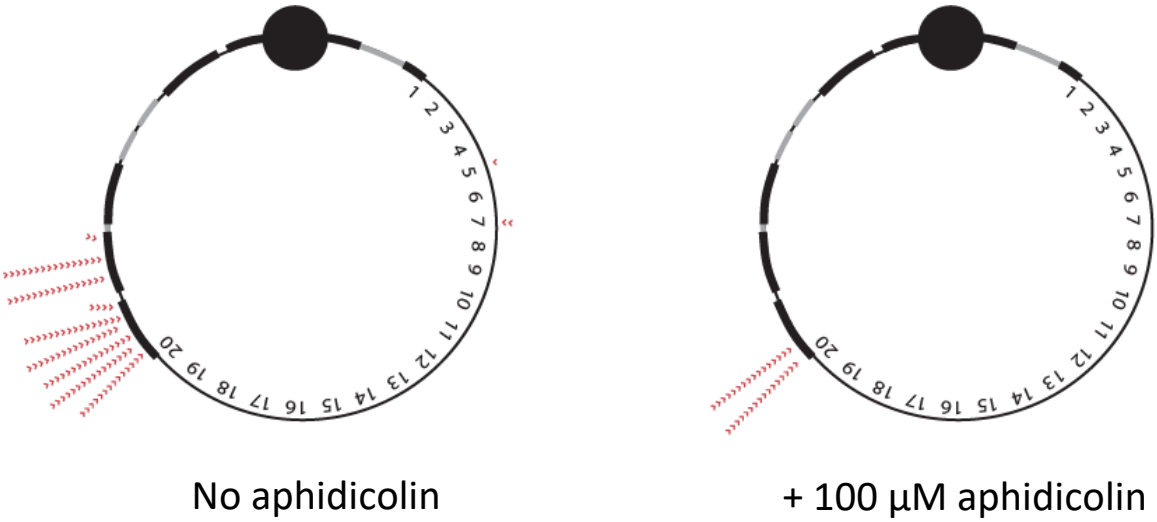

Figure 8: R(1:Y)11Ax2-8c breakpoints with and without exposure to aphidicolin. Arrows represent newly added telomeres on ends of breakpoints

The R(1;Y)11Ax2-8c chromosome is notable for its extreme fragile site in the heterochromatic block most distal from the centromere. 59 breakage events without exposure to aphidicolin were recovered by Kent Golic (unpublished). Breakpoint distribution was not affected by treatment with aphidicolin ($p<0.01$). 15 independent, aphidicolin-treated R(1;Y)11Ax2-8c openings were produced, all with breaks in the extreme fragile site. No novel breakpoints were observed after treatment with aphidicolin.

R(1)2-75B:

Breakage of a second Ring-X chromosome, R(1)2-75B, was also examined in the presence of aphidicolin. It has been established that this chromosome has a particularly low rate of breakage and healing (unpublished). Although aphidicolin did not have an effect on the rate of opening, it may affect the breakpoint distribution.

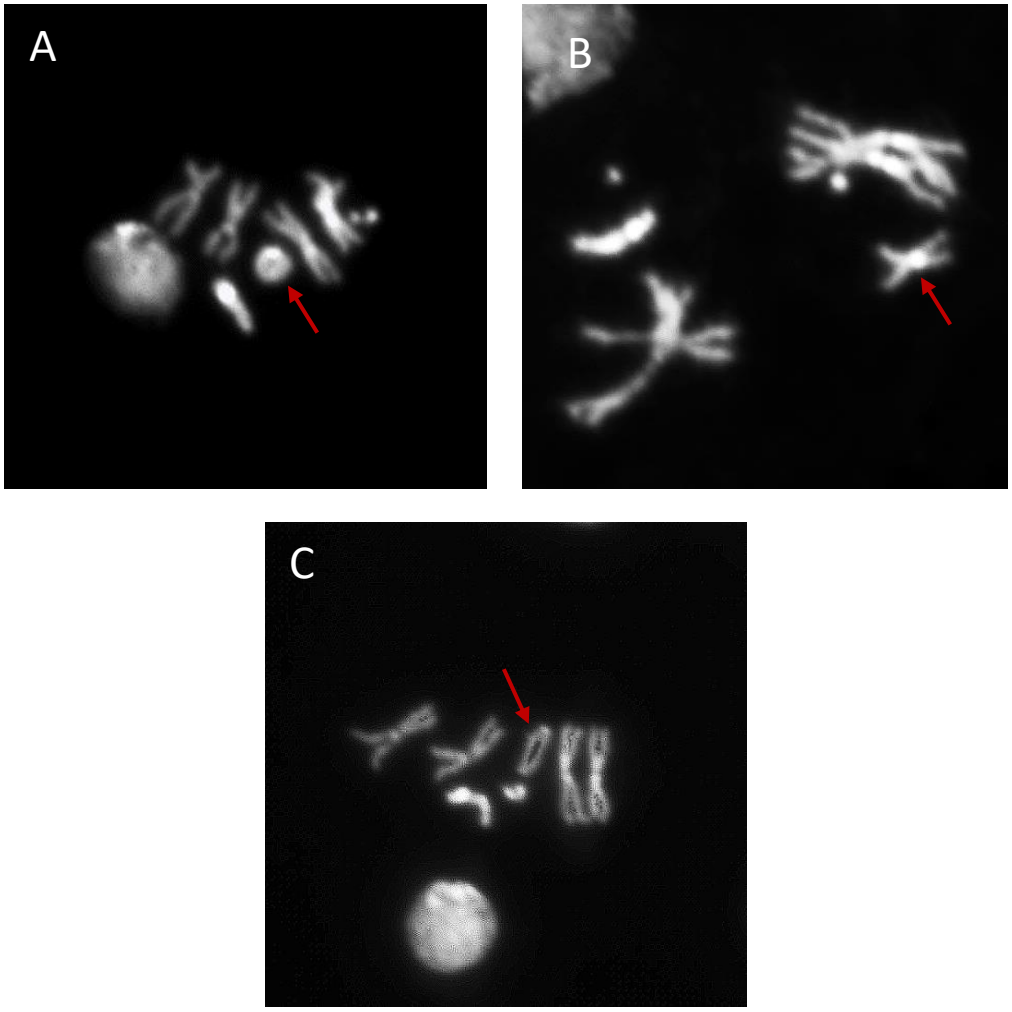

Figure 9: Mitotic spreads a R(1)2-75B chromosome A: an unbroken ring B: a ring broken in euchromatin and C: a ring broken in heterochromatin

When flies were treated with aphidicolin during gamete formation, one candidate male was recovered through our screen with a breakpoint in euchromatin. This chromosome was further investigated using polytene microscopy techniques. Euchromatin can be viewed in polytene spreads while heterochromatin aggregates to the non-replicating chromocenter. Analysis of this chromosome revealed breakpoints at polytene band #10B and #11C with a small duplication between these two breakpoints.

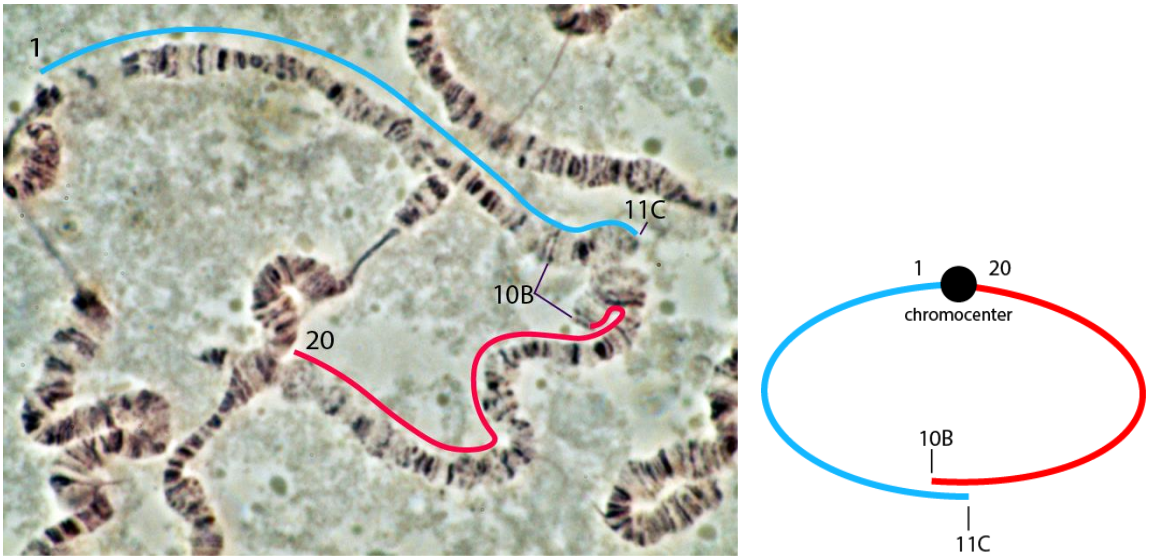

Figure 10: A: an image of a polytene chromosome with labeled breakpoints B: a diagram representing the broken X with a small duplication

The control (no aphidicolin) R(1)2-75B that I obtained all broke within heterochromatin and were further investigated using a recombination assay. Recombination frequencies provide insight into areas of the chromosome breakage with respect to the centromere (Table 1).

Chromosome	Recombination Frequency
D1	40.39%
D2	36.49%
D4	37.77%
D6	40.31%
D9	40.24%
D10	26.14%
D12	34.65%
D13	37.93%

Table 1: Recombination Frequencies of Chromosomes with Heterochromatic Breaks

All lines produced essentially normal frequencies of crossover events indicating that all of these chromosomes broke to give normally oriented linearized X chromosomes. These breaks are grouped within the heterochromatin on R(1)2-75B on the side closest cytology band 1.

The heterochromatic breaks recovered in this experiment are a subset of all the control openings found in Figure 11, the rest of the data for this figure was contributed by Hunter Hill. Using these refined breakpoint locations, a map depicting these sites of breakage was generated:

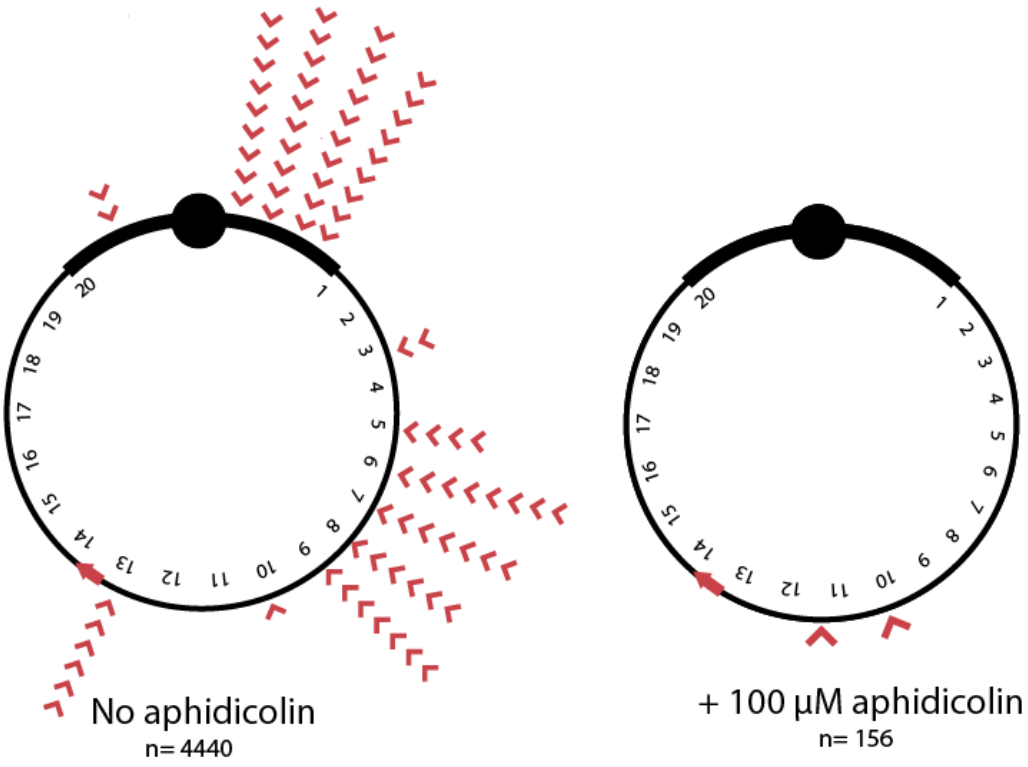

Figure 11: R(1)2-75B breakpoints with and without exposure to aphidicolin. Arrows represent newly added telomeres on ends of breakpoints

In the absence of aphidicolin, 21 breaks occurred in euchromatin and 20 in heterochromatin. When exposed to aphidicolin, 27 males that should have had broken rings were recovered. Only one chromosome was linearized, the rest retained a ring chromosome and somehow evaded the screen. When dicentric chromosomes are induced using *R(1)2-75B*, a high level of lethality is observed. This lethality, paired with the low availability of aphidicolin, severely limits the sample size of this experiment. Due to these limitations, there is not enough data to determine a significant correlation between breakpoints in *R(1)2-75B* chromosomes with and without treatment with aphidicolin.

DISCUSSION

The replication stress induced by aphidicolin did not have a substantial effect on the fragility of chromosomes. The Ring-X chromosome *R(1)2-75B* yields linearized chromosomes at a very low frequency (unpublished). It is difficult to open and recover using dicentric bridge creating techniques. Treatment with aphidicolin might have allowed for the visualization and classification of more fragile sites. However, comparing ring opening frequencies showed that the rate of chromosome breakage was not altered by treatment with aphidicolin. Even though the entire genome was subjected to replication stress, the incidence of chromosome breakage was not increased.

Additionally, treatment with aphidicolin did not change breakpoint distributions in ring chromosomes. Previous studies determined that the Ring-X chromosome *R(1;Y)11Ax2-8c* breaks almost exclusively in a block of heterochromatin that is normally found at the tip of the long arm of the Y chromosome (unpublished). Alteration of this pattern of breakage after exposure to aphidicolin would provide strong evidence for a connection between replication stress and fragile sites in this system. However, treatment with aphidicolin did not significantly change the breakpoint distribution, with nearly all identified fragile sites on this chromosome clustered in the previously identified single heterochromatic locus. The structurally distinct ring chromosome *R(1)2-75B* was also analyzed, but the effects of treatment with aphidicolin on the location of breakage in this Ring-X were inconclusive due to low sample size.

These analyses suggest that the fragile sites revealed by dicentric chromosome breakage are not the same as Common Fragile Sites in humans. Conservation of fragile site locations, as seen in humans, does not occur between the two *Drosophila* Ring-X chromosomes studied. *R(1;Y)11Ax2-8c* and *R(1)2-75B* exhibit different patterns of breakpoints. Fragility during chromosome stretching likely has an underlying mechanism that is unique from breakage at human common fragile sites. Replication stress may still play a role in some forms of chromosome breakage in *Drosophila*, but dicentric breakage is not affected by the late replication induced by aphidicolin, indicating a fundamental difference between these two phenomena.

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